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1

Monoclonal Antibodies Specific for Leukocyte Adhesion Molecules

Selective Protocols of Immunization and Screening Assays for Generation of Blocking, Activating and Activation Reporter Antibodies

Carlos Cabañas and Francisco Sánchez-Madrid

1. Introduction

The specificity, homogeneity, and ability to be produced in unlimited amounts have made monoclonal antibodies (MAbs) an extremely useful tool for the study of a great variety of molecules involved in cellular adhesion phenomena. In many cases, the detailed biochemical and functional characterization of members of the integrin, selectin, immunoglobulin, and cadherin families of adhesion receptors, and their specific cellular and tissue distribution have only been made possible through the development and use of specific MAbs to these molecules.

Very often, the binding of a MAb to a membrane receptor involved in cell adhesion affects the function of the molecule, and results in inhibition or enhancement of the ability of the cell to adhere to the specific ligand. These functional effects of MAbs usually reflect a direct or physical involvement of the epitope recognized in ligand interaction; in other cases, however, the functional effects exerted by MAbs can only be explained through the induction of conformational changes in the adhesion receptor. Those MAbs that reduce the ability of an adhesion molecule to interact with specific ligands are usually referred to as "blocking" or "inhibitory" antibodies. Conversely, those MAbs that are able to enhance the interaction of an adhesion receptor with its ligand are generally termed "activating" or "stimulatory" antibodies. A third group of

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MAbs comprise those antibodies that recognize the functional state of adhesion molecules and that react with specific epitopes whose expression correlates with the functional activity of the adhesive receptor; these antibodies are usually termed "activation reporters," and since many of them seem to recognize the specific conformation of the adhesion molecule after its interaction with ligand, they are also termed antibodies specific for "ligand-induced-binding sites" or simply "LIBS-type MAbs" ***(1–7)***.

In our laboratories, we have generated over the last 10 years a large number of MAbs against cell membrane molecules with distinct functional properties. The use of many of these MAbs has allowed us to identify novel molecules that are implicated in specific cellular adhesion phenomena, as well as to discover novel functional activities of already known adhesion molecules; in addition, we have isolated and elucidated the biochemical and functional characteristics of many leukocyte adhesive proteins. Here, some basic and optimized protocols for selective immunization of mice and for screening assays useful in the generation of MAbs against functional epitopes of leukocyte adhesion molecules are described.

2. Materials

1. Balb/c female mice can be obtained from Iffa Credo (Lyon, France). Outbred animals from 6–8 wk to 4 mo are used.
2. The mouse myeloma P3X63Ag8.653 and Sp2 cell lines were purchased from the American Tissue Culture Collection (ATCC).
3. CNBr-activated CL-4B Sepharose was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden.
4. Polyethylene glycol, hypoxanthine, aminopterin, thymidine (HAT), and HT selective media for hybridomas, EDTA, ethanolamine, Triton X-100, NaCl, $MgCl_2$, $MnCl_2$, PMSF, and octyl glucoside were all purchased from Sigma (St. Louis, MO).
5. RPMI-1640 medium and fetal calf serum were purchased from Flow Laboratories (Irvine, Scotland, UK).
6. Flat-bottomed, 96-well culture plates were purchased from Costar (Cambridge, MA)
7. The β1-specific stimulatory MAb TS2/16 was a generous gift of T. A. Springer (The Blood Transfusion Center, Boston, MA) ***(8)***.

3. Methods

3.1. Immunization of Mice with Intact Live Cells

Intact live cells expressing detectable levels of the adhesion molecule of interest on their surface can be efficiently used as immunogen for generation of MAbs. In addition, the immunization with live cells is a simple method for the generation of MAbs against previously uncharacterized or novel adhesion

receptors whose expression on the surface of the immunizing cells is suspected ***(9,10)***. Immunization with live cells is also highly recommended when a MAb against a cell-surface antigen that is expressed specifically on a particular cell type or lineage is desired. In this case, the reactivity of the MAbs obtained is screened against a panel of cell lines of different origin, and those MAbs that specifically react with the cell type used for immunization but not with other cell types, can be easily identified.

1. Prime animals ip on d –48 and –33 with 5–20 × 10^6 cells resuspended in 500 µL of an isotonic buffer, such as phosphate-buffered saline, pH 7.4 (PBS) (without adjuvant) using a 25-gage needle.
2. Three days prior to the fusion (d –3), give the animals a final boost by injecting 5–10 × 10^6 cells resuspended in 300 µL of PBS in one of the veins of the tail.
3. Surgically remove spleens from the immunized mice on d 0, and carry out fusion of spleen cells with P3X63Ag8.653 or Sp2 mouse myeloma cells at a 4:1 ratio using polyethylene glycol as fusing agent according to standard techniques ***(11)***.
4. Clone the growing hybridomas by limiting dilution or semisolid agar according to standard protocols (the reader is referred to one of the recent excellent books covering the different strategies for generation of MAbs) ***(12–14)***.

3.2. Immunization and Screening Methods for Generation of "LIBS-type" MAbs

The generation of MAbs specific for activation epitopes of adhesion molecules has facilitated studies on the function of these receptors ***(1,5,15)***. These activation-reporter MAbs recognize epitopes whose expression is not constitutive, but correlate with the functional activity of a given adhesion molecule. Since this type of MAb has the ability to discriminate between different states of activation of a given adhesion molecule, it can be used as a probe to monitor the functional state of these molecules.

When generation of MAbs to different activation-reporter epitopes for a particular adhesion molecule is sought, immunization of mice with the purified adhesion molecule is the best alternative. Ideally, the method employed for purification of the adhesion molecule should yield it in an activated conformation, so that activation-specific epitopes are exposed on the molecule and can be recognized by the mouse immune system. Using this strategy, we have recently generated a group of MAbs (HUTS) specific for LIBS-type or activation-reporter epitopes of β1 integrins, which have already revealed their usefulness in the study of integrin activation ***(7,15)***. The approaches employed for purification of human β1 integrins, for subsequent immunization of animals, and for the screening and selection of these HUTS MAbs are described here in detail to illustrate general strategies for generation of LIBS-specific antibodies. This protocol can easily be adapted for generation of LIBS anti-

bodies specific for other members of the integrin family or other families of cellular adhesion receptors.

1. Purification of human β1 integrins can be performed by immunoaffinity chromatography. To obtain purified β1 integrins in an activated state, prepare a chromatography column by coupling a stimulatory β1-specific MAb (such as TS2/16 or 8A2, *[2,8]*) at 2 mg/mL to 3 mL of CNBr-activated CL-4B Sepharose, following the manufacturer's instructions. Stimulatory MAbs are able to activate adhesion molecules by inducing the conformation of the molecules that favors their interaction with ligand (high-affinity conformations). Most importantly, the divalent cation Mn^{2+} (200 μ*M*), which is known to induce activation of most members of the β1, β2, and β3 integrin subfamilies, should always be present throughout the immunoaffinity purification and subsequent immunization of mice in order to preserve β1 integrins in the active conformation.
2. Triton X-100 homogenates of surgical specimens from different human tissues can be used as the starting source material for purification of β1 integrins. The tissues are diced, sieved, and lysed in 300 mL of lysis buffer for 2 h *(7)*.
3. The cell lysate is centrifuged at 3000 × *g* for 30 min at 4°C, then ultracentrifuged at 100,000 × *g* for 1 h at 4°C, and finally precleared by passing it through a 2-mL column of glycine-Sepharose CL-4B (pre-equilibrated in lysis buffer) and loaded onto the 3-mL column of MAb TS2/16 covalently coupled to Sepharose (pre-equilibrated in lysis buffer) at a flow rate of 0.5 mL/min.
4. The column is sequentially washed with 15 mL of lysis buffer and 15 mL of washing buffer *(7)* and bound β1 integrins are eluted with an ethanolamine buffer, pH 12.0, at a flow rate of 0.5 mL/min *(7)*. Fractions containing β1 integrins can be identified by SDS-7% PAGE followed by silver staining.
5. Immunization of Balb/c mice is performed by injecting ip 5–10 μg of purified β1 integrins in PBS containing 200 μ*M* Mn^{2+} at d –48, –33, –18, and iv on d –3.
6. Spleen cells from immunized mice are fused on d 0 with Sp2 mouse myeloma cells at a 4:1 ratio according to standard techniques, and distributed in 96-well culture plates.
7. After 2 wk, hybridoma culture supernatants are harvested and screened by testing their reactivity against human cells (T-lymphoblasts) expressing β1 integrins. The reactivity of each hybridoma supernatant is determined by flow cytometry under conditions of: (a) integrin inactivation induced by the total absence of divalent cations (divalent cation chelator EDTA is added to the hybridoma culture supernatants at a final concentration of 3 m*M*), and (b) high integrin activation induced by the presence of 500 μ*M* Mn^{2+}.
8. The hybridomas showing differential reactivity under the two conditions of integrin activation described in the previous step are selected and cloned by limiting dilution, according to standard techniques.
9. Immunoprecipitation, flow cytometry, and cell adhesion analyses with the MAbs selected have to be carried out to confirm that the antibodies are indeed specific for "activation-reporter" epitopes of β1 integrins.

3.3. Screening of MAbs Based on Their Effects on Cell Attachment to Specific Ligands Immobilized on a Solid Phase

Under appropriate conditions, most cell types are able to attach and adhere to a plastic surface that has been coated with a protein ligand specific for a particular adhesion receptor expressed on the surface of the cells. This type of adhesion assay allows a simple and rapid screening of MAbs that are specific for a given molecule, and display either blocking or activating functional properties. For instance, selection of either blocking or activating MAbs specific for the leukocyte integrin LFA-1 can be rapidly accomplished by measuring the inhibitory or stimulatory effects on the basal level of attachment of LFA-1-expressing cells to plastic wells coated with the LFA-1-specific ligands ICAM-1, ICAM-2, or ICAM-3.

1. Coat the plastic surface (usually the wells of a flat-bottomed 96-well plate) with specific protein ligands by incubating it overnight at 4°C (or for 2–3 h at 37°C) with an appropriate dilution of the adhesive ligand dissolved in a neutral or slightly alkaline buffer.
2. Saturate any remaining free plastic sites with 2% bovine serum albumin (BSA) dissolved in PBS. (We have found that in many cases, boiling the BSA solution before saturating the plastic plates results in lower nonspecific background levels of cell attachment.)
3. Wash the wells three times with PBS and one with RPMI medium, and the cells expressing the adhesion receptor specific for the immobilized ligand are added.
4. Add an aliquot (10–50 µL) of the appropriate hybridoma culture supernatant, and finally add the cells to each well resuspended in a volume of 50–100 µL of RPMI or an isotonic/neutral buffer (the actual number of cells added to each well usually ranges from 5×10^4 to 3×10^5 depending on the size of the cells).
5. Allow the cells to settle onto the bottom of the wells for 10 min at 4°C and then transfer the plates to a 37°C/5% CO_2 incubator for 30–60 min.
6. Using a multichanel pipet, wash the wells very gently 3–5 times with 200 µL of warm RPMI medium (or PBS buffer).
7. Quantitation of the percentage of cells that remain attached can be calculated by a variety of methods. In our experience, staining the attached cells with a solution of crystal violet represents an inexpensive and reliable method for quantitation that provides rapid and consistent results. The wells are first washed twice with PBS, and the cells are subsequently fixed with 3.5% formaldehyde in PBS (10 min at room temperature) and finally dyed with a crystal violet solution (0.5% w/v in 20% methanol) for 10 min at room temperature. Then, absorbance at 540 nm is measured in an ELISA detector (Pasteur Laboratories, Paris, France), and optical density is a linear function of the number of cells. A calibration curve (optical density vs number of cells) should be constructed for each cell type used in the assays (*see* **Note 1**). To calculate the percentage of cell attachment, basal cell adherence to a nonspecific protein,

such as BSA (cell binding to BSA-coated wells is constant enough for each cell type and must always be <5%), is always substracted from the attachment values (on a specific adhesive ligand) obtained in the presence of the respective MAbs. The final results can be expressed as percent of control (control: cell attachment to the specific ligand in the absence of MAb is considered 100% of adhesion). Assays should be performed in triplicate. Total cellular input is calculated by spinning wells with the original number of cells added to each well, and then fixing, staining, and measuring optical density.

3.4. Screening of MAbs Based on Its Effect on Homotypic Cell Aggregation Assays

The effect of MAbs on homotypic cell aggregation, i.e., the formation of clusters of cells of the same type or lineage, represents a simple method for selection of MAbs specific to leukocyte adhesion molecules and/or their ligands. Many immortalized leukocytic cell lines (as well as purified populations of normal lymphocytes) that grow in suspension are able to form homotypic cell aggregates either spontaneously or when induced by a variety of stimuli. These include monocytic (U937, HL60), erythroleukemic (K562), B-lymphocytic (JY, Ramos), and T-lymphoid (JM, Jurkat) cell lines (*see* **Note 2**).

1. Add 1×10^5 cells resuspended in 50 µL of RPMI medium to the wells of a flat-bottomed 96–well, tissue-culture microtiter plates containing 20–50 µL of the MAb-producing hybridoma culture supernatants to be tested.
2. Transfer the plates to a 37°C/5% CO_2 incubator and assess visually the effect of the different MAbs on the ability of cells to form homotypic aggregates at different time-points ranging from as little as 15 to 24 min or even 48 h (*see* **Note 3**).

This type of assay can be used to screen either adhesion-blocking or adhesion-activating MAbs. In the first case, homotypic aggregation is induced by treating the cells with agents that induce activation (i.e., an enhancement of the affinity or the avidity) of either the adhesion receptor or the counter receptor responsible for intercellular aggregation (*see* **Note 4**). This activation can be induced by chemical agents that activate cells (such as phorbol esters or calcium ionophores), by changes in the extracellular conditions (for instance, altering the divalent cation concentrations), or by addition of an activating MAb to the cell culture. The inhibitory or blocking effects of the hybridoma supernatants on the induced formation of intercellular aggregates can then be easily assessed by visual inspection of the wells at different time-points (*see* **Note 5**).

MAbs of the second type, adhesion-activating, are selected based simply on their ability to induce or accelerate the formation of intercellular homotypic aggregates in unstimulated cultures of the selected target cells. We consider an aggregation induction assay to be positive when more than 50% of the cells are aggregated.

4. Notes

1. Other methods can be used to quantify the cells adhered to ligand-coated plates, such as fluorescence analysis, but they require more expensive equipment. In this assay, cells are loaded in complete medium (RPMI 1640 medium supplemented with 10% fetal calf serum) with the fluorescent dye BCECF-AM (Molecular Probes, The Netherlands), and added in RPMI medium containing 0.4 BSA to 96-well dishes (Costar) (6×10^4 cells/well) previously coated with the protein ligands. After incubation for 20 min at 37°C, unbound cells are removed by three washes with RPMI medium, and adhered cells quantified using a fluorescence analyzer (CytoFluor 2300, Millipore Co.).
2. Despite the simplicity of the homotypic aggregation asssay, this type of screening method has been used succesfully in our laboratories, and in those of other investigators, as the initial assay to select functional MAbs against adhesion molecules. However, it is worth keeping in mind that in some cases the stimulation or inhibition of homotypic aggregation caused by a number of MAbs is not a result of their specific effects on a particular adhesion molecule, but is rather owing to "nonspecific" effects of antibodies, such as crossbridging.
3. The most important parameters to be taken into consideration when assessing the effects of MAbs on the formation of cellular homotypic aggregates are modifications in the number, size, and kinetics of formation of cell clusters. For instance, sometimes, depending on the affinity and/or the concentration of antibody, a blocking MAb will only be able to delay the formation or reduce the size of the homotypic cellular clusters rather than completely inhibiting their formation.
4. The formation of homotypic cell aggregates not only requires the expression of both a particular adhesion receptor and its specific ligand (or counterreceptor) on the surface of the cells, but also depends on the state of activation of these molecules. The state of activation of a particular adhesion molecule reflects its ability to interact with ligand molecules and this status can be assessed at the biochemical (affinity) or cellular (avidity) level. Most importantly, the affinity and/or avidity of many adhesion molecules is not constant, and can be rapidly regulated by many intracellular and extracellular factors, including blocking or activating MAbs.
5. For quantitative measurement of cell aggregation, a modification of the method previously described ***(16,17)*** is used. The number of free cells is counted by using a special mask, consisting of squares (0.5 mm) under the plate. Within each well, at least five randomly chosen areas are counted, after which the mean and the total number of free cells by well is calculated.

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2

Epitope Mapping

Horace M. DeLisser

1. Introduction

The region of an antigen that interacts with an antibody is defined as an epitope. For protein antigens, epitopes may involve a single length of the polypeptide chain (sequential or linear epitopes) or may be composed of several widely separated, discrete amino acid sequences that come together in the folded native portion (conformational or discontinuous epitopes) ***(1)***. Complete definition of the structure of an epitope can be achieved by X-ray crystallography of antigen–antibody cocrystals, but to date only a limited number of protein epitopes (all of the discontinuous type) have been defined by this method ***(1,2)***. These studies, however, have suggested that the epitopes of native protein consist of 15–22 residues with a smaller subset of 5–6 residues contributing most of the binding energy. It is important to note that these critical residues may not be arranged in a linear sequence ***(1)***.

An important tool for analyzing the structure–function relationships of protein antigens involves localizing the epitopes of functionally active monoclonal antibodies (MAbs) against the protein. This approach has helped to further our understanding of PECAM-1, a cell adhesion molecule of the immunoglobulin gene (Ig) superfamily that has been implicated in leukocyte transendothelial migration, integrin activation in leukocytes, and cell–cell adhesion (reviewed in ***3***). Localization of the binding epitopes of a number of active MAbs against human PECAM-1 has allowed us to define several functional regions within the molecule's extracellular domain ***(4)***. The epitopes of antibodies that inhibited PECAM-1-mediated leukocyte transendothelial migration were located in the N-terminal Ig-like domains. The binding regions for antibodies that activate integrin-function in leukocytes were found throughout the extracellular domain, but those that had the strongest activating effect mapped to the

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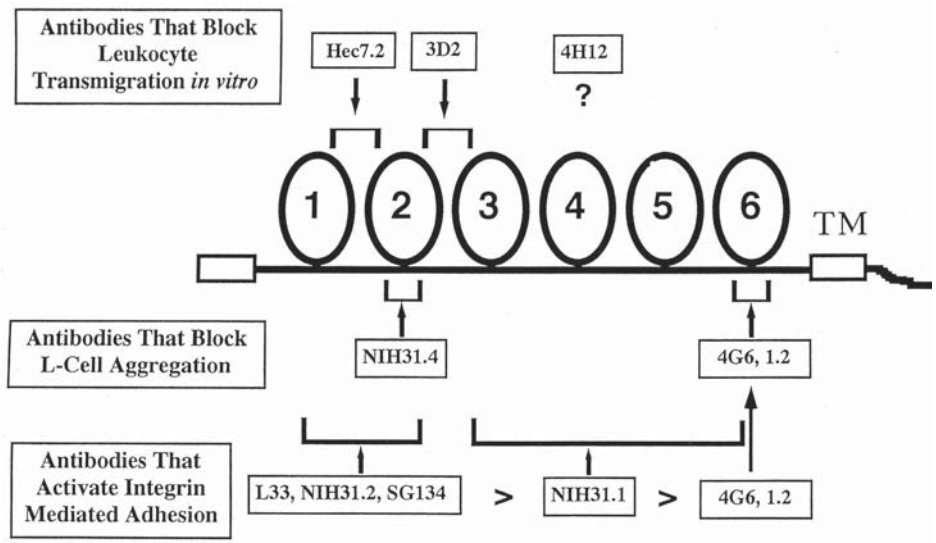


Fig. 1. The location of functional epitopes on PECAM-1. The binding regions of functional anti-human PECAM-1 MAbs are shown on a schematic representation of the PECAM-1 molecule. The first open box represents the signal sequences. Each of the six extracellular Ig-like domains is shown as an oval. The transmembrane (TM) is represented by the second open box. Three functional groups of antibodies were identified: (1) antibodies that blocked leukocyte transendothelial migration mapped to complex epitopes in the N-terminal domains of the molecule; (2) antibodies that inhibited PECAM-1-dependent heterophilic aggregation bound to regions in Ig-like domains 2 or 6; and (3) antibodies that activated integrin-mediated adhesion bound to all regions of the extracellular domain, but antibodies with the strongest activity mapped to the most N-terminal regions of the molecule (from ref. ***4***, used with permission).

N-terminus of the molecule. Also, antibodies that blocked PECAM-1-dependent heterophilic aggregation bound to either the second or sixth Ig-like domain **(Fig. 1)**. These findings have been essentially confirmed by comparable studies by Liao and associates, who also found that anti-human PECAM-1 MAbs that block migration through the extracellular matrix mapped to Ig-like domain 6 ***(5)***.

Several approaches have been used to define the epitopes of MAbs. These include:

1. Competitive antibody binding ***(6–8)***;
2. Immunological screening of recombinant expression libraries of random cDNA fragments ***(9–11)***;

3. Antibody binding to chemically synthesized overlapping peptides ***(12–14)*** or to fragments generated by proteolytic cleavage ***(15,16)***; and
4. Binding to recombinant proteins. Strategies involving recombinant proteins have used panels of sequential or overlapping deletion mutants ***(4,5,17)***, chimeric constructs composed of different species of the same molecule ***(4,18–21)***, bacterially expressed fusion proteins ***(14,22,23)***, and proteins generated by site-directed mutagenesis ***(4,21,24)***.

MAb epitope mapping generally occurs as a two-stage process. In the first stage, strategies are employed to localize the epitope to known functional or structural domains and/or to identify a contiguous region of <50 residues that contains the epitope. This is followed by fine epitope mapping in which critical sequences (≤10 amino acids) and/or residues are identified. Typically, a complete analysis will require two or more separate strategies ***(4,21,24,25)***. However, regardless of the approach used, it must be kept in mind that the loss of a binding epitope is not necessarily conclusive. This is particularly true for peptide or recombinant protein reagents, where associated changes in protein conformation rather than direct alterations in the epitope may alter antibody binding. Consequently, the preferred strategies are those that preserve the native structure and that allow for either the retention or actual gain of antibody binding.

The actual approach chosen for a given antigen and its antibodies depends on a number of factors, including facilities and expertise available, individual characteristics of the protein antigen, and the availability of the cDNA. If the molecule's cDNA is known, antibody binding can be studied in mammalian cells expressing mutant proteins (*see* **Note 1**). We and others have used this approach to map the epitopes of a number of MAbs to cell adhesion molecules ***(4)***. Analysis of constructs, particularly those in which the perturbation of the structure is minimal, expressed and analyzed in a cellular context is likely to represent more accurately an antibody's epitope.

A simple, "low-tech" approach can be employed in which recombinant proteins with targeted PCR-generated mutations are transiently expressed in COS cells. Deletion mutants and chimeric species constructs are engineered for surface expression and subsequently analyzed by immunofluorescence staining (*see* **Note 2**). Protocols for the transfection of COS cells grown on coverslips using calcium phosphate–DNA coprecipitation and immunofluorescence staining of COS cell transfectants are described below.

2. Materials

2.1. Preparation of COS Cells on Coverslips

1. Cell culture: COS-7 cells from the American Type Tissue Culture Collection (Rockville MD); DMEM with 10% FBS and gentamycin (100 µg/mL); trypsin/EDTA.

2. Preparation of coverslips: 70% alcohol; six-well culture plates; 11 × 22 mm glass coverslips (Thomas Scientific, Swedesboro NJ).
3. Tweezers for handling the coverslips.

2.2. Transfection of COS Cells on Coverslips by Calcium Phosphate–DNA Coprecipitation

1. Hanks buffer without calcium or magnesium (HBS); doubly distilled water (ddH_2O); PBS (pH = 7.4); 2.5 *M* $CaCl_2$.
2. Calf thymus DNA (18 mg for each six-well culture plate); DNA of interest (8 µg for each six-well culture plate).
3. Equipment: Inverted phase-contrast microscope; pipet-aid.
4. Sterile tubes and glassware: 15 mL conical tubes; 1.5 mL Eppendorf tubes; 20-mm Petri dish; 1-mL pipet; Pasteur pipet.

2.3. Fixing COS Cells on Coverslips in Six-Well Plates

1. 3% Paraformaldehyde in HEPES buffer (*see* **Note 3**).
2. 0.1 *M* glycine (stored at –20°C), PBS (pH = 7.4).
3. PBS with 0.02 % azide.

2.4. Immunofluorescence Staining of COS Cells on Coverslips

1. PBS (pH = 7.4); PBS with 4% fetal calf serum; TNC/NaCl (10 m*M* Tris-acetate, 0.5 m*M* $CaCl_2$, 0.5% NP-40, 0.15 *M* NaCl).
2. Staining jars for coverslips (Thomas Scientific, Swedesboro NJ).
3. Humidified Petri dish: Made by placing a filter paper into the bottom of a 100-mm Petri dish and saturating it with water. Two thin (1–2 mm) rods are then positioned closely, parallel to each other on the paper to provide support for the coverslips.
4. Antibodies: Antibodies of interest (diluted to 30–50 µg/mL if purified); appropriate fluorescently labeled secondary anitbodies.
5. Miscellaneous: Microscope glass slides; mounting medium (*see* **Note 4**); clear nail polish; tweezers for handling the coverslips.

3. Methods

3.1. Preparation of COS Cells on Coverslips

1. Culture COS cells in T-25 culture flasks at 37°C in a CO_2 incubator.
2. Rinse coverslips with 70% alcohol for 5 min, and then air-dry in sterile six-well culture plate (2 coverslips/well). Pipet 500 µL of fibronectin (10 µg/mL in PBS) onto each coverslip, and allow to sit for at least 1 h at room temperature. Suction off fibronectin.
3. For each confluent T-25 flask, remove cells with trypsin/EDTA, resuspend in 20 mL of media, and add 2 mL of the cell suspension to each well of the six-well plate. Culture for 24–36 h until wells are 80–90 % confluent (*see* **Note 5**).

3.2. Transfection of COS Cells on Coverslips by Calcium Phosphate-DNA Coprecipitation

1. One to 2 h before transfection, suction off the media from the six-well plate, and add 2 mL of fresh media to each well.
2. Immediately before transfection, confirm the precipitation reaction. Combine 500 µL of 2X HBS, 450 µL ddH_20, and 50 µL of 2.5 *M* $CaCl_2$ in 20-mm Petri dish, and allow to sit for 10 min. Confirm the presence of the precipitate by inverted phase-contrast microscope (*see* **Note 5**).
3. Aliquot 500 µL of 2X HBS into a 15-mL tube.
4. In a 1.5-mL Eppendorf tube, combine 8 µg of the DNA of interest (*see* **Notes 5**) and 18 µg of calf thymus DNA with sufficient amount of sterilized ddH_2O to achieve a final volume of 450 µL. Add 50 µL of 2.5 *M* $CaCl_2$ into the DNA solution, pipeting vigorously to ensure complete mixing. The above is sufficient for one six-well plate.
5. Using a Pasteur pipet, carefully add the DNA/$CaCl_2$ solution, a drop at a time, to the 2X HBS while simultaneously bubbling air through a 1-mL pipet from a pipet-aid into the HBS. After the addition of DNA/$CaCl_2$ solution is complete, allow the mixture to sit for 20 min at room temperature.
6. Pipet the entire mixture once, add 150 µL of the solution to each well of the six-well plate and incubate for 4–6 h at 37°C in a CO_2 incubator. (If the cells are to be evaluated by fluorescence activated cell sorting [FACS] analysis, Western blotting, or immunoprecipitation, then the entire mixture should be added to a 100-mm plate of subconfluent cells.)
7. After washing the wells three times with PBS, add 2 mL of complete media to each well and return to culture incubator.
8. After 36 h the coverslips will be ready to be fixed for immunofluorescence staining.

3.3. Fixing COS Cells on Coverslips in Six-well Plates

1. Wash wells twice with PBS, add 2.0 mL of 3% paraformaldehyde to each well, and incubate at room temperature for 20 min.
2. Suction off the paraformaldehyde, add 2.0 mL of 0.1 *M* glycine in PBS to each well, and incubate at room temperature for 15 min.
3. Wash each well twice with PBS for 5 min. Proceed to immunofluorescence staining, or store coverslips in PBS with 0.02% azide at 4°C.

3.4. Immunofluorescence Staining of COS Cells on Coverslips

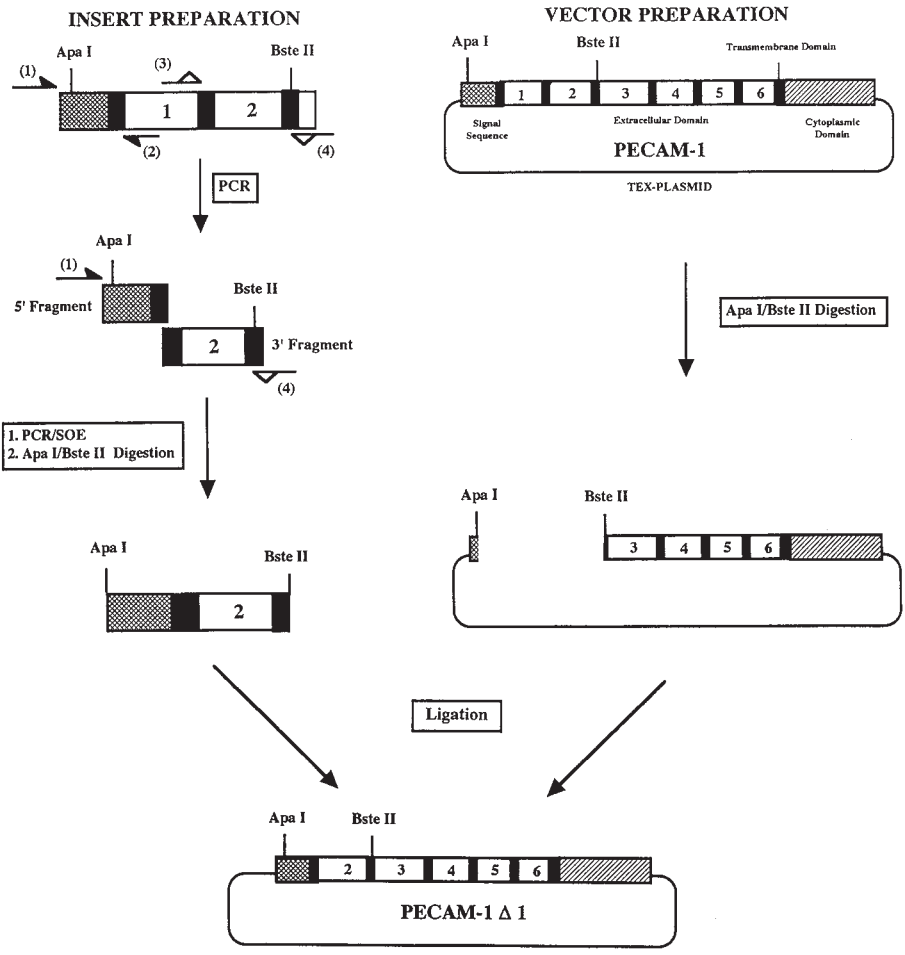
1. Incubate coverslips in TNC/NaCl for 1 min at room temperature.
2. Rinse coverslips with PBS, and transfer to staining jars with PBS/4% FBS. Incubate for 5 min at room temperature.
3. Transfer coverslips to the humidified Petri dish placing them cell side up on the rods. Cover the entire surface of coverslip with 50–100 µL of the antibody, replace the cover of the Petri dish, and incubate at room temperature for 1 h (*see* **Note 6**).

4. Dip each coverslip once in 250 mL of PBS, and transfer to staining jars with PBS/4% FBS. Incubate for 30 min at room temperature.
5. Transfer coverslips once again to the humidified Petri dish, placing them cell side up on the rods. Cover the entire surface of coverslip with 50–100 µL of the appropriate fluorescently labeled secondary antibody. Replace the cover of the Petri dish and incubate in the dark at room temperature for 30 min.
6. Mounting coverslips on glass slides: Place 10 µL of mounting medium onto the slide. Dip the coverslip once in 250 mL of PBS and once in 250 mL of water. Gently touch the edge of the coverslip against a paper towel to remove excess water. Immediately place the coverslip, cell side down, on the slide placing it over the mounting medium. Three to four coverslips can be easily positioned on the slide.
7. Once the coverslips have dried, paint the edges of the coverslips with clear nail polish to fix them on the slide. After the nail polish has hardened, the coverslips are ready to be viewed with immunofluorescence microscopy. Slides should be stored in the dark at 4°C when not being viewed.

4. Notes

1. Key to this and other recombinant strategies is the generation of mutants with well-defined deletions or substitutions, particularly when convenient restriction sites are not available. In our epitope mapping studies of the platelet endothelial cell adhesion-1 (PECAM-1/CD31) ***(4)*** we have made extensive use of a PCR-based strategy known as "Sequence Overlap Extension" (SOE) ***(26)***. This technique has allowed us to exploit available restriction sites to generate a variety of PECAM-1 deletion and human/mouse PECAM-1 chimeric mutants. In this approach, PCR is used to create two fragments of DNA that contain overlapping sequences. These two fragments are then used in a second PCR reaction to create an insert that can be cloned back into the original vector. **Figure 2** illustrates the use of this technique to generate a mutant missing the first extracellular immunoglobulin-like domain of human PECAM-1 (PECAM-1Δ1) ***(27)***.
2. There are two potential limitations to the use of COS cells in epitope mapping. First the mutation may result in a construct that will not express in COS cells. At times, one cDNA clone will be expressed, but another will not. Consequently, multiple cDNA clones should be tested, and mutant constructs should be

Fig. 2. *(opposite page)* Design of a mutant of human PECAM-1 missing the first immunoglobulin-like domain. Vector preparation. Shown is the full-length human PECAM-1 in the pESP-SVTEXP (TEX) expression vector digested with *Apa*I and *Bste*II restriction endonucleases. Depicted are the signal sequence and extracellular, transmembrane, and cytoplasmic domains. In the extracellular domain, the open and filled boxes represent the immunoglobulin-like homology domains and the interconnecting regions, respectively. Insert preparation. With full-length PECAM-1 as a template, primers 1 and 2 (filled half-arrows) were used to generate a 5' fragment (from the ApaI site to bp 246,



located immediately 5' to the sequence for domain 1, whereas primers 3 and 4 (open half arrows) were used to generate a 3' fragment (containing the sequences immediately following domain 1 and extending to the *Bste*II site). Primer 2 was complementary to the sequence immediately 5' to domain 1 and contained added base pairs that overlapped the region immediately following domain 1. Primer 3 was complementary to the sequence immediately following domain 1 and contained base pairs that overlapped the sequence immediately 5' to domain 1. The resulting 5'- and 3'-fragments therefore had overlapping sequences respectively at their 3'- and 5'-ends. The 5'- and 3'-fragments were then joined together by the PCR/SOE reaction using the two outside primers (primers 1 and 4). This mutated cDNA lacking the coding sequence for the first Ig-like domain of PECAM-1 was subsequently cut with *Apa*I and *Bste*II and then ligated into the previously digested TEX/PECAM vector (adapted from ref. ***27*** with permission.

sequenced to confirm the presence and integrity of the targeted mutations. Also, if antibody binding is weak, positive staining may be difficult to distinguish from background staining. Antibody binding of mutant proteins expressed in COS cells can also be evaluated by means of FACS analysis, Western blotting, or immunoprecipitation. These strategies, however, do have their own limitations. Since relatively few cells may express the protein, FACS analysis and immunoprecipitation may not be sufficiently sensitive to detect changes in antibody binding, and Western blotting requires that the antibody recognize denatured protein.

3. Preparation of 3% paraformaldehyde in HBS with 20 m*M* HEPES: A stock solution of 6% paraformaldehyde can be prepared by adding 6.0 g of paraformaldehyde to 100 mL of H_2O, followed by 3 drops of 1 *N* NaOH and gently heating at 60°C until the paraformaldehyde goes into solution. A stock solution of 40 m*M* HEPES in 2X HBS can be made by combining 50 mL of 10X HBS, 10 mL of 1*M* HEPES, and 190 mL water (adjusting pH to 7.2). Equal volumes of these stock reagents are added together to make the 3% paraformaldehyde solution. Stock solutions should be stored at –20°C.
4. Preparation of mounting medium (phenylene diamine): Add 1.2 g of polyvinyl alcohol to 3 g of glycerol in a 50-mL tube. Mix thoroughly, but gently with a glass rod. Add 3 mL of H_2O, mix well, and allow to stand at room temperature for at least 4 h. Add 5 mL of 0.1 *M* Tris HCl (pH = 8.5), and incubate in a 50°C water bath for 10 min. Then, quickly but thoroughly stir in an additional 1 mL of 0.1 *M* Tris HCl. Centrifuge at 2000*g* for 15 min. Prepare 50–100 µL aliquots and store at –70°C.
5. Transfection of near-confluent cultures (80–90%), generation of a fine precipitate (in contrast to one that is clumped), and use of DNA that is uncontaminated by large amounts of protein (OD 260/280 ratio ~ 1.7) all improve the efficiency of transfection.
6. The efficiency of transfection must be assessed for each transfection and each construct. Therefore, it is important to include in the staining an antibody that should react with the mutant construct (e.g., a polyclonal antibody or MAb whose epitope is distant from the engineered mutations).

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3

Sequencing of Antibodies

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1. Introduction

Several antireceptor monoclonal antibodies (MAbs) have been described to compete with ligands for receptor binding. The possibility that structures derived from the hypervariable or complementarity determining regions (CDR) of such antibodies display similarity with those of the ligand binding site of receptors has been documented, thereby allowing the understanding of the structural basis of receptor–ligand interaction. Thus, the determination of the structure of these CDR regions can allow the identification of sequences responsible for the activity of the antibodies.

As an example, we studied amino-acids sequences within CDR of a murine MAb: AC7. AC7 is an IgM, directed against the GpIIbIIIa receptor present on platelet and involved in platelet aggregation. After activation by agonists, the platelet glycoprotein GpIIbIIIa can bind to its ligand, fibrinogen, and promote platelet aggregation. Fibrinogen binding to GpIIbIIIa is mediated in part by an Arg-Gly-Asp- (RGD) like sequence. The RGD binding domain of GpIIbIIIa has been localized in a fragment of the GpIIIa subunit that includes the sequences between amino acids 109 and 171. AC7 has been produced against a synthetic peptide derived from the GPIIIa subunit (residues 109–128) and has been described to inhibit fibrinogen binding to its receptor and platelet aggregation in a dose-dependent fashion. In order to characterize the structural features of AC7 responsible for its ability to inhibit platelet GpIIbIIIa funtions, we sequenced the heavy- and light-chain variable region of AC7 cDNA, derived from mRNA of AC7 hybridoma cells by reverse transcription polymerase chain reaction (RT-PCR) procedure ***(1)***.

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2. Materials

2.1. RNA Extraction

1. Lysis buffer: Nonidet P40 13% and solution containing 10 m*M* Tris-HCl, pH 7.5, 0.15 *M* NaCl, 1.5 m*M* $MgCl_2$, pH 7.5.
2. Phenol.
3. Phenol/chloroform/isoamyl alcohol (50/48/2).
4. RNase inhibitor (Boehringer, Mannheim, Germany).
5. Apparatus: microcentrifuge.

2.2. RNA Reverse Transcription

1. Primer sequences are designed to maximize homologies with published sequences ***(2)***. 3' oligonucleotides primers correspond to conserved sequences of light- and heavy-chain variable regions of murin immonoglobulins.
   a. 3' Oligonucleotide primer corresponding to light chain: 3' CK1 (459/488) 5'-ACTGTTCAGGACGCCATTTTGTCGTTCACT-3'.
   b. 3' Oligonucleotide primer corresponding to heavy chain: 3' CH1 (558/587) 5'-GGGAGACAGCAAGACCTGCGAGGTGGCTAG-3'.
2. Reverse transcriptase of M-MLV (Gibco, BRL, Paisley, UK).
3. Enzyme buffer: 0.25 *M* Tris-HCl, pH 8.3, 0.375 *M* KCl, 15 m*M* $MgCl_2$ (Gibco, BRL).
4. 0.1 *M* DTT (Gibco, BRL).
5. dNTP: solution containing 2.5 m*M* of each dNTP (dATP, dCTP, dGTP, dTTP) (Boehringer).
6. RNase inhibitor (Boehringer).

2.3. First cDNA Amplification by PCR

1. Oligonucleotide primers for amplification of variable region of light-chain immunoglobulin:
   a. 5' VK1 (1/24) 5'-CCGGATCCGACATTCAGCTGACCCAGTCTCCA-3', containing a *Bam*H1 site (underlined)
   b. 3' CK1 (459/488) 5'-ACTGTTCAGGACGCCATTTTGTCGTTCACT-3'.
2. Oligonucleotide primers for amplification of variable region of heavy-chain immunoglobulin:
   a. 5' VH2 (2/23) 5'-GGCTGCAGAGGTC$_{/G}$A$_{/C}$ AA$_{/G}$ CTG$_{/T}$ CAGC$_{/G}$ AGTCA$_{/T}$ GG-3' containing a *Pst*1 site (underlined).
   b. 3' VH1 (414/443) 5'-GAAGTCCCGGGCCAGGCAGCCCATGGCCAC-3'.
3. Taq DNA polymerase (Appligene).
4. Enzyme buffer: 100 m*M* Tris-HCl, pH 9.0, 1% Triton X100, 15 m*M* $MgCl_2$, 0.2% BSA (Appligene).
5. DNTP: solution containing 2.5 m*M* of each (Boehringer).

2.4. Second cDNA amplification by PCR

1. Apparatus: microcentrifuge.

2. Oligonucleotide primers for amplification of variable region of light-chain immunoglobulin:
   a. 5' VK1 (1/24) 5'-CCGGATCCGACATTCAGCTGACCCAGTCTCCA-3', containing a *Bam*H1 site (underlined).
   b. 3' VK2 (303/324) 5'-TCGAATTCGTTAGATCTCCAGCTTGGTCCC-3' containing an *Eco*R1 site (underlined). VK2 corresponds to an internal oligonucleotide primer.
3. Internal oligonucleotides primers for amplification of variable region of heavy-chain immunoglobulin:
   a. 5' VH4 (1/23) 5'-GGCTGCAGCAGGTGCAGCTGAAGCAGTCAGG-3' containing a *Pst*1 site (underlined).
   b. 3' VH3 (312/345) 5'-GGATCGATTGAGGAGACGGTGACCGTGGT-3' containing a *Cla*1 site (underlined).

2.5. Cloning of PCR products into pBlueScript vector

1. Cell ject apparatus (Eurogentec, Seraing, Belgium).
2. T4 DNA ligase (Boehringer).
3. Enzyme buffer: 660 m*M* Tris-HCl, 50 m*M* $MgCl_2$, 10 m*M* dithierythritol, 10 m*M* ATP, pH 7.5.
4. Glycogen (20 mg/mL, Boehringer).
5. PCR products corresponding to each variable region of immunoglobulin chain are digested with appropriate restriction enzymes (i.e., *Bam*H1/*Eco*R1 for light chain and *Pst*1/*Cla*1 for heavy chain).
6. The pBlueScript vector is digested with restriction enzymes corresponding to those necessary for the cloning of each immunoglobulin chain.
7. Ampicillin (50 µg/mL).
8. 5-Bromo-4chloro-3indolyl galactopyranoside (X-Gal, 20 mg/mL).
9. Isopropyl-thiogalactopyranoside (IPTG, 40 mg/mL).
10. Electrocompetent DH5α bacteria.

2.6. Sequencing

1. RPM kit for preparation of DNA (Bio101, Vista, USA).
2. Solution of 2 *M* NaOH and 2 m*M* EDTA.
3. Sequenase kit (Amersham, Buckinghamshire, UK).

3. Methods

3.1. Extraction of RNA

RNA is extracted from hybridoma cell line using a modified method of Gough ***(3)*** (*see* **Note 1**).

1. 5×10^6 hybridoma cells are washed with PBS. Cells are lysed in 10 µL of Nonidet P40 13% in 200 µL of lysis buffer at 4°C.
2. After a brief centrifugation (1 min, maximal speed in a microcentrifuge), supernatant is extracted three times with phenol and twice with phenol/chloroform

isoamyl alcohol (50/48/2). Extractions are performed by addition of an equal volume of phenol or phenol/chloroform/isoamyl alcohol

Vortex briefly and centrifugate (1 min, maximal speed in a microcentrifuge). The aqueous phase containing the sample is collected by withdrawing it with a pipet.

3. RNA is precipited with ethanol. Pellet is resuspended in 200 µL H_20 containing 20 U of RNase inhibitor.
4. The integrity of RNA sample is analyzed on agarose gel before performing reverse transcription-amplification reactions (RT-PCR).

3.2. RNA Reverse Transcription

1. Incubate 5 µg of RNA with 60 pmol of each 3' oligonucleotide primer corresponding to each immunoglobulin chain (3'CK1 for light chain and 3'CH1 for heavy chain) for 10 min at 70°C (*see* **Note 2**).
2. Allow to cool to room temperature.
3. Add 1000 U of reverse transcriptase, 20 U of Rnase inhibitor, and 500 µ*M* of each dNTP in enzyme buffer. Adjust volume to 30 µL with H_2O. Reaction is catalyzed for 2 h at 37°C.

3.3. First cDNA Amplification by PCR

1. Incubate 6 µL of each reverse transcription reaction (the equivalent of 1 µg of RNA) with 2.5 U of *Taq* DNA polymerase, 200 µ*M* of each dNTP (dATP, dCTP, dGTP, dTTP), and 60 pmol of each oligonucleotide primers 3' and 5' corresponding to each immunoglobulin chain (5'VK1/3'CK1 for light chain and 5'VH2/3'VH1 for heavy chain) in enzyme buffer. Adjust vol to 100 µL. Overlay the samples with 100 µL of mineral oil to prevent evaporation.
2. Denature sample during 5 min at 94°C.
3. Perform 40 cycles of amplification. Each cycle is composed of three steps:
   a. Denaturation step: 1 min at 98°C.
   b. Annealing step: 2 min at 45°C.
   c. Extension step: 1 min 30 s at 74°C.

   At the end of the 40th cycle, extend the extension step by an additional 9 min.
4. Amplification products are analyzed on agarose gel. The oil layer will not interfere when withdrawing aliquots from the sample for analysis (*see* **Note 3**).

3.4. Second cDNA Amplification by PCR (See Note 3)

1. The second amplification reaction is performed in the same conditions as the first reaction in the presence of the oligonucleotides primers: 5'VK1/3'VK2 for the light chain and 5'VH4/3'VH3 for the heavy chain. This second reaction is performed using 1/10 vol of the first amplification reaction.
2. Amplification products are analyzed on agarose gel. To recover the whole sample, extract the sample with 100 µL of chloroform. Vortex and centrifugate the sample briefly (1 min, maximal speed in a microcentrifuge). The aqueous phase, containing the sample, is collected by withdrawing it with a pipet.

3.5. Cloning of PCR Products into pBlueScript Vector (See Note 4)

1. The second PCR products corresponding to the variable region of each immunoglobulin chain are digested with appropriate restriction enzymes (*Bam*H1/*Eco*R1 for light chain and *Pst*1/*Cla*1 for heavy chain) (*see* **Note 4**). Restriction enzymes are used following the recommandations of the manufacturer.
2. Digested products are analyzed on agarose gel and purified (*see* **Note 5**).
3. The pBlueScript vector is digested with restriction enzymes corresponding to those necessary for the cloning of each chain.
4. Incubate digested vector with the appropriate digested immunoglobulin chain PCR product (ratio 1:1) in the presence of 4 U of T4 DNA ligase. Reaction is catalyzed in a final volume of 25 µL in the presence of enzyme buffer. Ligation is performed for 16 h at 16°C (*see* **Notes 6** and **7**).
5. Precipitate ligation reaction with ethanol and in the presence of 1 µL of glycogen.
6. Transform electrocompetent DH5α bacteria with ligation product. The transformation is performed by electroporation with a Cell ject apparatus set at 2500 V and 40×10^{-6} F.
7. Bacteria are selected on LB agar plate containing 50 µg/mL ampicillin, 40 µL of X-Gal (20 mg/mL), and 20 µL of IPTG (40 mg/mL). Only efficiently transformed bacteria with vector plus insert result in white colonies.
8. On the next day, pick several colonies, and check for the presence of the variable region of light or heavy immunoglobulin chains. To do this, several clones are individually taken with a sterile toothpick. Each picked colony is incubated in the second PCR amplification reaction mixture (volume of reaction: 20 µL). After the PCR procedure the analysis of amplified DNA is performed as previously described. Clones can also be analyzed after plasmid DNA purification of each clone (*see* **Note 8**) and digestion with appropriate restriction enzyme.

3.6. Sequencing

1. Prepare plasmid DNA from at least two positive clones for each immunoglobulin chain cloned.
2. After addition of 0.1 vol of 2 *M* NaOH and 2 m*M* EDTA, the DNA is incubated for 30 min at 37°C for denaturation. The mixture is neutralized by adding 0.1 vol of 3 *M* sodium acetate (pH 4.5–5.5) and the DNA is precipitated with 2–4 volumes of ethanol.
3. Sequencing is performed with the termination method using the sequenase kit. Each clone must be sequenced on both strand. 5'- and 3'-oligonucleotide primers used for the cloning steps of each immunoglobulin chain can be used for the sequencing procedure.

3.7. Analysis of Sequences

Amino acid sequences of both heavy- and light-chain variable region of AC7 immunoglobulin are deduced from nucleotide sequences determined as described above.

Since NS1 myeloma used for AC7 hybridoma production possesses its own, but not secreted κ light chain immunoglobulin, AC7 κ light-chain sequence was confirmed by N-terminal protein microsequencing (N-terminal microsequencing was also monitored for the AC7 μ heavy variable region and confirmed nucleotide sequence data) (*see* **Note 9**). **Figure 1** shows the light- and heavy-chain variable region sequences of AC7 immunoglobulin.

We found an analogy in the sequence derived from the CDR3 of AC7 heavy-chain sequence (RQMIRGYFDV) with the RGDF region of fibrinogen. In fact, the synthetic peptide corresponding to this sequence inhibits platelet aggregation and fibrinogen binding ***(1)***.

4. Notes

1. RNA can also be extracted using the rapid total RNA isolation kit (5 prime-3 prime, Boulder, USA).
2. All oligonucleotide primers are made on a synthetizer using phosphoramidite chemistry ***(4)***. The immunoglobulin class of the antibody to be studied must be known in order to choose oligonucleotide primers with maximal homology to variable region of heavy and light chains.

   For the murine IgM (AC7), 3'- and 5'-oligonucleotide primers corresponding to the variable region of the heavy chain are choosen with maximal homology to μ chain. For light chain, 3'-oligonucleotide primer is designed wih maximal homology to κ chain.
3. In many cases, others bands are detected. The second amplification reaction results in specific PCR products.
4. PCR products can be treated with proteinase K before digestion reactions. Proteinase K permits the elimination of the remaining Taq DNA polymerase molecules fixed to DNA. This step facilitates further cloning. The reaction is as follows: incubate 50 μg of amplified DNA in the presence of 0.5% SDS, 5 m*M* EDTA, 10 m*M* Tris, pH 7.5, 20 μg proteinase K (Boehringer) in a final volume of 100 μL for 30 min at 37°C. Inactivate the enzyme by heating the sample at 68°C for 10 min. The DNA is extracted with phenol/chloroform/isoamyl alcohol and precipitated with ethanol.
5. Digested products can be agarose gel-purified by an electroelution procedure or by the use of the prepAgene kit (Bio-Rad, Hercules, USA).
6. DNA amounts of vector or inserts must be determined. An estimation of the amount of product can be done in two ways, namely, either spotting an aliquot on a plate containing 0.8% agarose and 1 μg/mL ethidium bromide together with a serial dilution of a solution of DNA with a known concentration or by determining the optical density at 260 nm.
7. Ligation can also be perfomed for 1 h at room temperature.
8. Small quantities of DNA can be prepared using the RPM kit (Rapid Pure Minipreps, Bio101, Vista, USA) or the Quiagen kit (Quiagen, Hilden, Germany).
9. After electrophoresis in SDS-polyacrylamide gel, purified antibodies are transferred onto immobilon polyvinylidene difluoride (PVDF) membrane (Millipore,

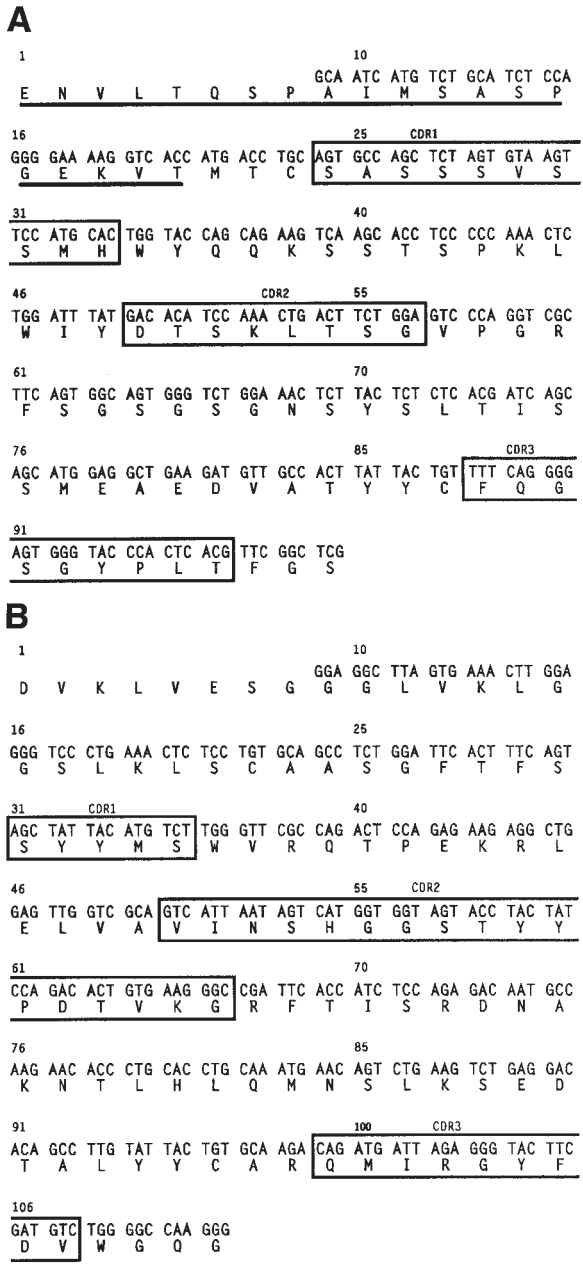

Fig. 1. Nucleotide and amino acid sequences of light **(a)** and heavy **(b)** variable regions of AC7 immunoglobulin. The sequences were segregated into CDR (boxes). Deduced amino acid sequences are shown using single-letter amino acid code. N-terminal amino acid sequences determined by protein microsequencing are underlined.

Marlborough, USA) according to the method of Matsudaira ***(5)***. Before electrophoresis, proteins are treated with 2% β-mercaptoethanol. The N-terminal amino acid sequences of both κ light and μ heavy chains were determined by automated Edman degradation methodology using an AB1 mode 470A sequenator (Applied Biosystem, Foster City, USA).

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4

Purification Through Affinity Chromatography and Microsequencing

Cecilia Garlanda

1. Introduction

Production of monoclonal antibodies (MAbs) using adhesion-involved cell types as a source of antigens allows the identification of new molecules participating in the adhesion process ***(1–3)***. Immunoaffinity purification followed by protein microsequencing is one of the techniques used to characterize the new adhesion molecule identified and to determine at least in part its amino acidic sequence ***(4,5)***.

Construction of degenerated oligonucleotides on these known amino acidic fragments permits the analysis of expression libraries and the isolation and cloning of full-length cDNA ***(6)***.

The immunoaffinity purification of large amounts of protein permits the study of its biological functions or the production of other antibodies. Essential conditions to purify a protein by immunoaffinity chromatography are:

1. The availability of reasonable amounts of tissue or cells to use as a source of antigen;
2. The capacity of one or more MAb to use for the purification to work in immunoprecipitation assays; and
3. The development of an immunoassay (as Western blot) to follow the purification steps from cell lysate to sequence.

Immunoaffinity purification is generally achieved following these steps:

1. The homogenization and lysis of the tissue or cells to use as source of antigen are performed to solubilize membrane-bound proteins.
2. A purification step, for example by affinity chromatography, may be necessary to reduce contaminants present in the total lysate and to have a good binding

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capacity of the immunoaffinity column, especially if the affinity of the MAb for the antigen is low. Since many adhesion molecules are glycoproteins, in this chapter, an affinity chromatography technique to purify glycoproteins by Concanavalin A- (Con A) Sepharose binding will be described.

3. The purified MAb is covalently coupled to a commercially available solid-phase matrix, such as protein A- or G-Sepharose or cyanogen bromide (CNBr) Sepharose.
4. The antigen binds the MAb beads matrix, and the matrix is extensively washed before antigen elution. Because the type of bonds between antigen and MAbs varies among different couples of MAb/antigen, it is generally necessary to try to find the conditions to break them and to achieve an effective elution. When trying the elution conditions, it is necessary to consider if the eluted antigen still has a recognizable conformation in the immunoassay designed to follow the purification and if the column could be used again after the elution.
5. Single-step immunopurification is seldom efficient enough to isolate a single protein, and contaminants are present in the preparation. Generally, one-dimensional gel electrophoresis is performed to isolate the antigen from contaminants. Therefore, it is often necessary to concentrate the eluted material and to change the buffer in which it is dissolved if it is not compatible with SDS-electrophoresis. This is not a trivial aspect when handling micrograms of protein that are easily lost owing to unspecific binding to plastic tubes or dialysis/filtration membranes.
6. To microsequence a purified protein, two main techniques exist: it is possible to perform the N-terminal sequence of the protein by Edman degradation, or to perform N-terminal sequence of internal peptides of the protein, obtained after digestion with an endoproteinase ***(7)***. For N-terminal sequence analysis, generally 50 pmol of purified protein are requested to obtain a 15–20 amino acid sequence (if N-terminal sequence is not blocked!). To sequence internal peptides, 100 pmol are usually requested. Since this last step is generally performed by specialized biochemists, the detailed method used will be not described in this chapter.

A unique method to purify proteins by immunoaffinity chromatography does not exist, since several steps of the procedure are conditioned by the specific protein and MAb characteristics, such as the protein expression level in the tissue or cells and its stability, the MAb affinity for the antigen and the kind of bonds between them. To compare different techniques, *see* ref. ***(8)***. In this chapter, the technique used to purify a murine glycoprotein expressed by endothelial cells will be described ***(9)***.

2. Materials

2.1. Homogenization and Lysis of the Tissue

1. Phosphate-buffered saline: PBS.
2. Homogenization buffer: 150 m*M* NaCl, 10 m*M* Tris-HCl, pH 7.4, 1 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 1 m*M* Pefabloc-SC (Pentapharm), 20 U/mL aprotinin (Trasylol, Bayer). Add Pefabloc-SC and aprotinin just before use.

3. Lysis buffer: homogenization buffer containing 1% Triton X-100.
4. Plytron homogenizer.
5. Centrifuge and fixed-angle rotors capable of centrifuging 50-mL tubes at 10,000*g*. Microcentrifuge.
6. Western blot analysis: all the material necessary for protein electrophoresis and blotting (*see* ref. ***8***).
7. Sample buffer 4 × 200 m*M* Tris-HCl, pH 6.8, 8% SDS, 40% glycerol, 0.025% bromophenol blue.

2.2 Affinity Chromatography on Con A-Sepharose

1. Con A-Sepharose (Pharmacia).
2. 1-, 10-, 30-mL columns.
3. Peristaltic pump, UV detector and recorder or spectrophotometer, fractions collector (not essential).
4. Washing buffer: 150 m*M* NaCl, 10 m*M* Tris-HCl, pH 7.4, 1 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 0.1% Triton X-100.
5. Elution buffer: Washing buffer containing 0.5 *M* methyl-α-D-mannopyranoside (Sigma), 0.04% NaN_3.

2.3. Immunoaffinity Chromatography on CNBr-Sepharose

1. CNBr-Sepharose (Pharmacia): Buffers to couple the antibody to the matrix are exactly described in manufacturer's instructions. The MAb-CNBr-Sepharose matrix is stored at 4°C in a tube at 10%, in 150 m*M* NaCl, 10 m*M* Tris-HCl, pH 7.4 containing 0.04% NaN_3, 0.04% NaN_3.
2. Protein G- or A-purified MAbs. The amount needed ranges from 2–20 mg or more, depending on the affinity for the antigen.
3. Washing buffer: 150 m*M* NaCl, 10 m*M* Tris-HCl, pH 7.4, 1 m*M* $CaCl_2$, 1 m*M* $MgCl_2$.
4. Elution buffers: *see* **Note 5**.
5. Centricon concentrators (Amicon).
6. 20 mM Tris-HCl, pH 7.0.
7. Hamilton syringe (100 µL).
8. Material for silver staining or Coomassie brilliant blue staining (*see* **ref. *8***).
9. Problott membrane PVDF (Perkin-Elmer).

3. Methods

3.1. Homogenization and Lysis of the Tissue

1. Homogenize the tissue with a polytron homogenizer in homogenization buffer in ice (*see* **Note 1**).
2. Add Triton-X 100 to a concentration of 1% to lyse the cells, and to solubilize the membrane-bound antigen, and to keep the solution in rotation at 4°C for 1–4 h. If using cells as source of antigen, wash them twice with PBS and directly lyse them in lysis buffer (*see* **Note 1**).

3. Centrifuge the material at 10,000g for 30 min at 4°C, recover the solubilized antigen in the supernatant, and eventually freeze it.

3.2. Affinity Chromatography on Con A-Sepharose to Purify Glycoproteins

Con A is a lectin with a high affinity for glycoproteins, and Concanavalin A-Sepharose is a commercially available (Pharmacia), ready-to-use matrix to purify them (*see* **Note 2** and ref. ***10***).

1. Determine the column/lysate volume ratio to use performing small-scale affinity chromatography on Con A-Sepharose with a different volume of lysate: prepare 0.5 mL of packed matrix/sample in tubes, wash it by centrifugation with washing buffer, add different volumes of lysate (i.e., 3, 9, 27 mL), incubate for 1 h at room temperature in rotation, and analyze by Western blot the amount of antigen present in the lysate material before and after (the flowthrough) the incubation with the matrix (*see* **Note 2**).
2. Pour the desired volume of Con A-Sepharose in a column, connect it to a peristaltic pump, and wash it with 10 column volumes of washing buffer at 200 mL/h. Allow the lysate to pass through the column at 20–30 mL/h threefold or continuously overnight at 4°C. Keep the flowthrough, and wash the column with 20 vol of washing buffer at 200 mL/h. Elute the glycoproteins with elution buffer at 20-30 mL/h. If a UV analyzer connected to the affinity column is available, follow the elution peak directly while collecting the fractions. If not, spectrophotometric analysis of the fractions should be performed during the elution, until the protein concentration of the eluted material is again at background.

   Analyze the fractions by Western blot, pool those containing the antigen, and eventually freeze them. When thawing the material, protease inhibitors (20 U/mL aprotinin and 1 m*M* Pefabloc) should be immediately added again.

3.3. Coupling the Antibody to CNBr-Sepharose

CNBr-Sepharose can be purchased from Pharmacia, and the coupling method is described exactly in the instructions.

The concentration of antibody generally used to prepare immunoaffinity columns is 2 mg of purified Ig/mL of CNBr-Sepharose matrix (*see* **Note 3**).

1. Begin your immunoaffinity chromatography preparing 1–2 mL of MAb-CNBr-Sepharose matrix: this will be used to test its binding efficiency and to determine the antigen elution conditions (*see* **Subheading 3.4.**).
2. Test binding efficiency and the best matrix/lysate ratio to use by performing small-scale immunoprecipitation incubating 25–50 µL of matrix/sample with different volumes of glycoproteins extract (i.e., 1, 3, 9, 27 mL). Analyze by Western blot the amount of antigen present in the lysate before and after the incubation with the matrix and the amount of antigen bound to the matrix by boiling it in 25–50 µL of sample buffer 1×.

With the same method, it is useful to determine the best condition of incubation (time and temperature) to obtain the maximal binding efficiency of the matrix.

3.4. Determination of Antigen Elution Conditions

1. Perform small-scale immunoprecipitation as described in Subheading 3.3, but instead of using sample buffer and boiling to detach the antigen, incubate the samples with 25–50 µL of different elution buffers (*see* **Notes 4** and **5** and ref. ***8***) for 30 min to 1 h, recover the eluted material with a Hamilton syringe, wash the resin with 150 m*M* NaCl, 10 m*M* Tris-HCl, pH 7.4 threefold, and boil it with sample buffer. Analyze by Western blot the protein content of the eluted material (after pH adjustment or dilution) and of the boiled resin.
2. To evaluate the degree of purity of the material obtained and to quantify the amount of antigen obtained per mL of matrix and lysate, analyze by silver staining or Coomassie brilliant blue staining the SDS-electrophoresis-resolved proteins. Lower sensibility of these techniques is about 0.1–0.2 µg of protein, and it will fit with the aim of this step. Use a higher amount of matrix and lysate until the band of the antigen is detectable.

All these preparation steps may be unnecessary when large amounts of tissue and antibody are available. In this case one immunoprecipitation might be sufficient to obtain the amount of protein and the degree of purity required to sequence, but when the material is precious or the purification hard to obtain, these small scale assays can be important to save time and material.

3.5. Immunoaffinity Purification in Large Scale

1. Prepare the necessary volume of MAb-CNBr-Sepharose, and wash it with washing buffer (*see* **Note 6**).
2. Incubate the matrix with the antigen-containing solution in a constantly mixing slurry (one or more 50-mL tubes will fit the purpose).
3. Pour the matrix in a suitable column, and wash it with 20 vol of washing buffer. Keep the lysate (*see* **Notes 5** and **7**).
4. Elute the antigen from the matrix, adding at least two column volumes of elution buffer at 20 mL/h and collect the fractions. Since the concentration of the eluted protein can be very low, it is difficult to follow the elution peak spectrophotometrically. If using low- or high-pH buffer for the elution, the pH should be adjusted immediately with a neutralizing buffer present in the collecting tubes. Wash the matrix with 150 m*M* NaCl, 10 m*M* Tris-HCl, pH 7.4, and add NaN_3 to 0.04% to store it. Analyze by Western blot an aliquot of the fractions and of the lysate before and after the incubation with the matrix. Pool antigen-containing fractions.
5. Concentrate the eluted material, and eventually change the buffer with an SDS-electrophoresis-compatible buffer using a 2-mL Centricon centrifugal concentrator. To avoid loss of material owing to unspecific binding to the filtration membrane, saturate the tube walls and the membrane with another protein of a different molecular weight (for instance, albumine or ovalbumine): concentrate 2 mL of a 2 mg/mL solution of this protein by centrifugation, discard it, and rinse

the tube several times with water. Add the eluted material, and concentrate it by centrifuging the tube in a fixed-angle rotor at 5000g for the time required (generally 30 min to 1 h/mL) at 4°C. When finished, change the buffer adding 2 mL 20 m*M* Tris-HCl, pH 7.0 and centrifuge it again. Repeat this step two to three times to be sure to remove the elution buffer completely. Recover the concentrated material (approx 100 µL) and rinse the filtration membrane with 30–35 µL of sample buffer 4×, which will be mixed with the concentrated protein if the following step is SDS-electrophoresis (*see* **Notes 8** and **9**).

3.6. SDS-Electrophoresis and Microsequencing

1. Analyze a small aliquot (0.5 µL) of concentrated protein by Western blot and in parallel by Coomassie brilliant blue staining (5 µL) to be sure to have the desired amount of purified protein.
2. The form in which the protein should be delivered to be sequenced depends on the method used. Briefly, for N-terminal sequence, in most cases SDS-electrophoresis is performed, followed by transfer on a PVDF membrane. On a separate lane, a small aliquot of the protein is charged to follow by Western blot the exact position of the antigen. The membrane is stained with Coomassie brilliant blue to identify the band to excise and to sequence by Edman degradation. For N-terminal sequence of internal peptides, after SDS-electrophoresis, the gel is lightly fixed and stained with Coomassie brilliant blue, and the stained band excised and processed for an endoproteinase digestion. Peptides obtained are extracted from the acrylamide matrix, separated by HPLC, collected, and sequenced *(7)* (*see* **Note 10**).

4. Notes

1. Analyze by Western blot different tissues and cell types to determine which is the richest antigen and the easiest to obtain. Try also different lysis buffers, in particular, different detergents and protease inhibitors. Other protease inhibitors used are: 15 µg/mL leupeptin, 0.36 m*M* 1,10-phenanthroline, 1 m*M* PMSF (which is very poorly stable in acqueous solution loosing half of its activity in 30 min), 1 m*M* DFP (which is efficient but extremely toxic). Test antigen temperature stability to know how to handle the material during purification.

   The ratio between the weight of the tissue and the volume of lysis buffer is empirically determined, depending on the tissue used. To homogenize and lyse murine lungs, 0.2 g/mL was the ratio used.

   The amount of tissue or cells to use will depend on the expression level of the antigen and on the efficiency of the purification, and it will be decided after a titration assay (*see* **Subheadings 3.3. and 3.4.**). To obtain about 100 pmol of a purified endothelial protein, 50 g of lung tissue were used.
2. To determine if an antigen is a glycoprotein, perform Western blot or immunoprecipitation analysis after deglycosilation treatments of the antigen and evaluate whether its apparent molecular mass is changed. The immunoaffinity can also desappear if the epitope is glycosilated. Substances most oftenly used for this purpose are: (a) tunicamycin, which is an *N*-glycosilation inhibitor and is added

in the culture medium of antigen-expressing cells at 1–10 μ*M* for the last 16 h before the lysis, and (b) *N*-glycanase, *O*-glycanase, and neuraminidase, which are deglycosilation enzymes detaching N-linked, O-linked and syalic acid residues, respectively. Cell or tissue lysate is treated with these enzymes following manufacturer's instructions before performing Western blot analysis.

To separate glycoproteins from 50 g of lung tissue, three 30-mL columns of Con A-Sepharose were used. Approximately twenty 4-mL fractions/column were collected, and 30 μL of one out of three of them analyzed by Western blot. About 250 mL of eluted glycoproteins were obtained from 250 mL of lung lysate: this step was not concentrating the antigen, but strongly reducing contaminants.

3. Another kind of solid matrix often used for immunoaffinity chromatography is protein A- or G-Sepharose. The main advantage of this matrix is the orientation of the antibody as it is bound by the Fc domain: the binding capacity of the MAb should be completely preserved. Besides the high cost of this matrix, the main problem is that after coupling, many free protein A or G residues are still available for binding of immunoglobulins present as contaminants in the antigen-containing lysate: for instance, if working with tissue lysate, blood immunoglobulins will bind the matrix and elute with the antigen. On the other hand, CNBr-Sepharose binds the antibody by any primary amines, even those eventually present in the antigen binding site: as a result the antigen affinity of matrix-bound antibody can be strongly reduced.
4. It is possible to have an idea about the best elution buffers to use performing ELISA assays or microscopic immunofluorescence treating fixed cells with elution buffers and evaluating their efficiency in detaching the antibody ***(11,12)***.
5. The most often used elution buffers are low-pH solutions (100 m*M* glycin, pH 2.5; 100 m*M* acetic acid, pH 2.5), high-pH solutions (100 m*M* triethylamine, pH 11.5), high-salt solutions (2 *M* NaCl, 2–3 *M* $MgCl_2$, 5 *M* LiCl), EDTA, EGTA, and denaturing solutions (0.5–2% SDS, 2–8 *M* urea). Washing the matrix with a pre-elution buffer (described in **ref. *8***) may be necessary to change the pH or salt conditions rapidly and to obtain a sharp elution peak.
6. Steps of immunoaffinity purification can be performed in column or in batch: for antigen binding in column, pour the matrix in a suitable column, connect it to the peristaltic pump, wash it with washing buffer, and then allow the lysate to pass through the column. Since the flow rate should be low (20–30 mL/h), with this method, the time required can be very long.
7. If the elution step is performed in batch, after extensive washing of the matrix with washing buffer by centrifugation, dry it as much as possible with a Hamilton syringe, add one matrix volume of the elution buffer chosen, leave them in contact for 30 min to 1 h, centrifuge the matrix at 1000*g* for 5 min, and recover the supernatant with the aid of a Hamilton syringe.
8. Protein can be concentrated with other techniques, such as trichloracetic acid (TCA) or ethanol or acetone precipitation. The problem that can arise is the low solubility of precipitated protein. Check that the method used is compatible with the elution buffer chosen.

9. To obtain about 100 pmol of 70 kD of purified protein, 12 mL of CNBr-Sepharose matrix were prepared, divided in five 50-mL tubes, mixed with about 250 mL of glycoprotein lysate. Incubation was performed at room temperature for 3 h in constant agitation on a rocker. Protein was eluted with $MgCl_2$ 2 *M* in Tris-HCl 20 m*M*, pH 7.4. Centricon-10 concentrator was saturated with 2 mg/mL ovalbumin. Owing to a very low affinity of the matrix-bound MAb for the antigen, the purification was repeated threefold with the same lysate and matrix to recover most of the antigen.
10. Since N-terminal sequence is very often blocked, it may be worthwhile to purify from the beginning a higher amount of protein to perform internal peptide sequence. Furthermore, if the cloning step that is performed after the preparation of degenerated oligonucleotides on N-terminal peptides is based on the screening of cDNA libraries prepared from poly-A+ RNA, a positive clone will be found only if the full-length insert exists. This limitation strongly reduces the chances of successful screenings. Another advantage of knowing the sequence of more than one internal peptide is the possibility to prepare longer probes for the screening from segments amplified by PCR with oligonucleotides from these peptides. Also with the second technique problems can arise: for example, if the endoproteinase used cuts the protein too often or too rarely, the peptides obtained can be too short or too long to be efficiently separated by HPLC.

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5

Functional Analysis of Cell Adhesion Molecules

David L. Simmons

1. Introduction

Screening cDNA libraries by transient expression in mammalian cells has proven to be very effective for the isolation of cDNAs encoding secreted, surface, and intracellular proteins.

The first successful applications of transient expression cloning were in the field of growth factor research. In the mid-1980s, cDNAs encoding many cytokines, such as interleukin 3 ***(1)*** and interleukin 4 ***(2)***, were cloned by transient expression of cDNA libraries in COS cells, and screening of individual COS supernatants by a sensitive bioassay.

However, the single most successful application of transient expression screening was developed by Aruffo and Seed in 1987 ***(3–5)***. It is based on transient expression of cDNA libraries in mammalian cells, and rescue of specific cDNA clones by antibody capture and panning. The efficacy of this procedure has transformed the field of cell-surface clone isolation to such an extent that once a suitable antibody or ligand or cell line has been identified recognizing a cell-surface molecule, the molecular cloning of the cDNA encoding it is now an essentially trivial process. Indeed, once one cell-surface molecule has been cloned, it is possible to clone rapidly the interacting ligand/receptor by using the extracellular domain of the first molecule, usually as an IgGlFc chimera, as an affinity reagent. This has been used very successfully to clone leukocyte molecules, e.g., the CD40 ligand gp39 ***(6)*** and the fas ligand ***(7)***.

There are many orphan receptors that have been cloned by degenerate PCR-based screens of tyrosine kinase or phosphatase domains. By using the extracellular domains of these orphan receptors, it is now possible to identify and clone their cognate ligands. A good example of this strategy is the recent cloning of the ligand for the hematopoietic flt3/flt2 tyrosine kinase receptor ***(8)***.

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Since 1987, a large number of cell-surface molecules have been cloned using monoclonal antibodies (MAb) to screen transiently expressed cDNA libraries including the T-cell adhesion/activator CD2 ***(3)*** and its ligand LFA-3 (CD58) ***(5)***; the T-cell adhesion CD28 ***(4)***; ICAM-1 (CD54) ***(9)***, and ICAM-3 (CD50) ***(10)*** recognizing LF A1 (CD11a/CD18); CD44 (11) recognizing hyaluronic acid; the endothelial intercellular adhesion CD31 ***(12)***; and the myeloid progenitor protein CD33 ***(13)*** and the hematopoietic progenitor sialomucin CD34 ***(14)***, which is a ligand for L selectin. VCAM-1 (CD106), an endothelial adhesin for VLA-4 on lymphocytes, was cloned using a variation of the panning procedure employing cells directly as the recognition reagent ***(15)***. ICAM-2 (CD102), an additional ligand for LFA-1, was cloned by using the ligand itself (LFA-1) as a direct panning reagent ***(16)***.

The technique has been extended to allow the cloning of intracellular proteins, though the number of successful examples of this category of proteins is still small ***(17,18)***.

Transient expression screens can also be used to clone genes by complementation of defective cell phenotypes. Expression of episomal-based cDNA libraries in these cells complements a defined defect, allowing selection of the rescued cell. This type of screen has been particularly successful in the field of DNA repair defects. Many of the xeroderma pigmentosa mutations have been cloned by complentation of established XP cell lines. In addition, the single genes defective in Fanconi's anemia ***(19)*** and paroxysmal nocturnal hemoglobinuria (PNH) ***(20)*** were also cloned by transient rescue.

This chapter describes the basics of cDNA library construction, and methods for transient expression screens for surface proteins, intracellular, proteins, and secreted proteins.

1.1. Basic Outline of Transient Expression Cloning

The essential elements of this technique involve the construction of a representative cDNA library in a vector capable of replication and high-level expression in mammalian cells. After transfection of the library into the cell line and transient expression of proteins encoded by it, the cells are screened in one of three different ways, depending on the compartment where the protein of interest normally resides: intracellular, surface, or extracellular secreted.

1. For intracellular proteins, the cells expressing the library are fixed and dried *in situ*, and screened with labeled ligand or antibody.
2. For surface proteins, a suspension of the cells is stained with the specific MAb or ligand and then panned on plastic dishes coated with appropriate second antibody.
3. For secreted proteins, the cells expressing the library are divided into small pools, and supernants from these individual pools are assayed for bioactivity or antibody binding.

In all cases, the selected cells are lysed *in situ*, and low-molecular-mass episomal DNA is recovered by differential precipitation (Hirt procedure). Episomes are then transformed into *Escherichia coli* and plated. This cycle of transfection/transient expression/selection/rescue usually needs to be repeated a further two or three times before individual recovered plasmids are analyzed for expression of the specific protein.

1.2. Advantages and Disadvantages of Transient Expression Cloning

The main advantages of transient expression cloning systems are:

1. Rapidity: The transient expression profile reaches a maximum at 36–48 h posttransfection. This means that each round of expression/selection and rescue only takes 3 d, so a complete three to four round library screen can be completed within 2–3 wk.
2. Isolation of full coding frame cDNA clones: By definition, only those cDNAs encoding the entire reading frame of the protein will be cloned. For surface proteins, the cognate cDNA must at least have its ATG, extracellular domain, transmembrane domain, or lipid anchor and stop transfer sequence to give rise to a properly folded and processed surface molecule. In addition, since the selection is performed with MAb or direct ligands, the expressed molecule must be substantially the correct unmutated molecule.
3. Functional studies on cloned surface molecules: The cloned cDNAs are in an efficient expression vector and can be used immediately for functional experiments, such as radioligand binding quantitation, cell adhesion studies, enzyme activity assay, and so forth.

The major disadvantages of transient expression cloning systems are:

1. Multicomponent systems: A major limitation of transient expression cloning systems is encountered by multicomponent glycoprotein complexes where the expression of any individual component of the complex requires the expression of all other members of that system. Clearly, only single molecules can be cloned by this system, and such complexes would be missed. This is a major defect, since many of the most important systems for cell recognition and signaling are multicomponent complexes, for example, the T-cell receptor αβ heterodimer requires expression of both chains to get either chain in the heterodimer to the surface. The Ti/CD3 complex γδεγη again requires multichain expression along with the TCR to get any surface expression of any of the CD3 chains. Integrins, major players in the process of cell–cell and cell–matrix adhesion, would also be missed by the expression cloning strategy, since these are αβ heterodimers where expression of the α chain requires coexpression of the β chain for surface presentation. A way out of this cloning "black hole" is the cotransfection of an existing expressing cDNA for one or all members of such complexes with the cDNA library under screen. For example, by cotransfection of integrin β chains with cDNA libraries, it is possible to clone a chains and vice versa.

It is also possible that the existing primate host cell integrins can act as "surrogate mothers" for library-derived α and β chains. Primate α chains could associate with human β chains, giving rise to a species and chain heterodimer at the cell surface. Complementation of multichain complexes, by use of host cell-surface molecules may apply beyond the integrin family, but has not yet been tested.

2. Requirement for screening ligand: A specific and high-affinity ligand is needed to screen the library. Usually, this has been an MAbs although a variety of reagents can be used to identify cell-surface molecules:
   1. MAbs
   2. Labeled ligands; and
   3. Cells.

   Ligands in a labeled form have been used directly as affinity reagents to clone their cognate receptors; for example, iodinated interleukin-1 ***(21)***, and iodinated GM-CSF ***(22)***.

Although the bias of this chapter is toward cell-surface molecules, the transient expression system has been extensively used for the direct functional cloning of secreted molecules with biological activity, such as cytokines and growth factors. IL-3, IL-4, and GM-CSF were cloned by this approach in the mid-1980s; supernatants from small pools of transfected COS cells were harvested and applied to appropriate bioassays, such as colony formation in soft agar, identification of positive pools, and repeat screening of positive pools. The cloning of all these cytokines led the way in transient expression technology. Cytokines are usually encoded by small mRNAs in high abundance, so full-length cDNA are likely to be well represented in cDNA libraries. In addition, the biological potency of many cytokines allows very small concentrations of active product to be detected in suitable mass assay systems.

Cell-surface molecules do not satisfy either of these two convenient criteria. They are certainly not abundantly expressed genes; on average, most cell-surface molecules are present at 10,000–50,000 protein molecules/cell. Most surface molecules are over 1 kb and average around 1.5–2.5 kb. There is also the problem of a sensitive assay for detecting single clones in the library.

In the mid-1980s, a high-efficiency transient expression vector system (pCDM8) was developed in the laboratory of Seed at Harvard Medical School, Department of Genetics and Department of Molecular Biology, Massachusetts General Hospital, Boston. This allows the construction of representative cDNA libraries, high levels of expression and accumulation of cell-surface molecules (15×10^6 molecules/cell surface). As CDM8 replicates to high copy number in COS cells, rescue and recovery of the episomal DNA are facilitated. Coupled with the design of a variety of ingenious screening systems, this system has greatly increased our knowledge about cell-surface molecules by allowing the rapid cloning of cDNAs encoding them.

Clearly there are two parts to this technique: (a) Construction of a cDNA library; and (b) expression and selection of that library. The protocols described below derive in large part from Seed and Aruffo.

2. cDNA Library Construction

2.1. cDNA Synthesis and Library Construction

2.1.1. Protocol 1, cDNA Library Construction

2.1.1.1. Overview

- RNA isolation.
- pA+ selection.
- cDNA synthesis.
- cDNA size fractionation.
- cDNA/vector ligation.
- General methods.

1. RNA isolation: This method is a simpler, but more effective version of the original method of Chirgwin et al. This method allows increased capacity of cell/tissue masses to be used and increased speed of preparation (much shorter centrifuge times).
   a. To each mL of cell lysate dissolve:
      i. 0.5 g guanidinium thiocyanate (Fluke) in 0.58 mL of 25% lithium chloride LiCl.
      ii. Filter through 0.45-µm filter.
      iii. Add 20 µL of stock (14.4 *M*) β-mercatopethanol.
   b. In a 50 Falcon tube:
      i. Centrifuge cells (1000–1500*g*, 5 min) or freeze tissue ground into a powder in dry ice pellets.
      ii. Disperse pellet as a paste up the walls by banging.
      iii. Add 1 mL of the GuSCN/LiCl lysis buffer for up to 5×10^7 cells.
      iv. Shear the lysate immediately in a polytron homogenizer, at top speed for 30–60 s, until DNA viscosity is completely gone. This is a very important step and cannot be overdone. It is vital to shear completely the genomic DNA to avoid contamination of the RNA and also to avoid losses of RNA yield owing to entrapment in the DNA layer at the GuSCN/CsCl interface on the gradient.
   c. For small-scale preparations ($<10^8$ cells):
      i. Layer up to 3.5 mL of the sheared lysate onto, 1.5 mL of 5.7 *M* cesium chloride (CsCl) (RNs-free; 1.36 g CsCl added to every 1 mL of 10 m*M* EDTA, pH 8.0. In a SW55 Beckman polyallomer centrifuge tube).
      ii. Spin at 50 krpm for 2 h. For large scale preparations ($>10^8$ cells):
         i. Layer 25 mL lysate onto 12.5 mL of the 5.7 *M* CsCl cushion.
         ii. Use a SW28 Beckman polyallomer centrifuge tube.
         iii. Spin at 24 krpm for 8 h.
   d. At end of the run:
      i. Aspirate off the overlay through the CsCl interface and well down into the CsCl cushion, leaving only 1 mL in the bottom of the tube.

ii. Aspirate off all residual liquid from the walls of the tube, and scour a ring just above the remaining liquid level.
iii. Invert the tube, and cut the tube just at the rounded part.
iv. Wipe off any liquid with a Q-tip or Kimwipe.
v. Dissolve the clear RNA pellet in 0.4 mL of RNase-free water by triturating in a P1000 tip 10 times or more. Clear crystals of RNA should be visible that will eventually dissolve.

e. Pipet aqueous RNA into an Eppendorf tube
i. Phenol-extract (0.5 mL).
ii. Chloroform-extract (0.5 mL).
iii. Add 10% vol of 3 *M* sodium acetate.
iv. 2.5 vol of ethanol.
v. Place on dry ice for 10–15 min.
vi. Spin in a minifuge (12,000 rpm) for 5 min.
vii. Decant supernatant.
viii. Wash twice in 70% ethanol.
ix. Decant and remove residual ethanol with a P200 tip.
x. Redissolve RNA pellet in 0.5 mL of RNase-free water and titer (OD_{260}). Store RNA at –70°C.

2. poly A^+ RNA preparation:
a. Preparation of reagents:
i. Resuspend oligodT cellulose (Collaborative Research type IV) in (0.5 mL of dry powder/1 mL of 0.1 *M* NaOH).
ii. Washed several times in RNase-free water.
iii. Place in a plastic disposable 10-mL column, previously washed in 5 *M* NaOH and rinsed with water.
iv. Rinse oligodT in two to three column vol of loading buffer (LB = 0.5 *M* lithium chloride, 50 m*M* Tris, pH 8.0, 5 m*M* EDTA, 1% SDS).

b. Binding
i. Pour oligo T cellulose slurry into sterile 15-mL Falcon tube in 4–5 mL of LB.
ii. Heat total RNA, 1–2 mg at most, at 70°C for 5 min.
iii. Chill on ice.
iv. Adjust to 0.5 *M* with LiCl.
v. Add to oligodT slurry.
vi. Rotate on a wheel for 30 min

c. Washing:
i. Decant into the disposable plastic column.
ii. Wash with 5 vol of LB.
iii. Wash with 5 vol of middle wash buffer (MWB = 100 m*M* LiCl, 50 m*M* Tris pH 8.0, 5 m*M* EDTA, 1% SDS).

d. Elution.
i. Elute pA^+RNA with serial 0.4-mL fractions of RNase-free water into Eppendorf tubes.
ii. Add l0% by volume of RNase-free sodium acetate.

iii. 2.5 vol of ethanol and place on dry ice for 30 min.
iv. Spin for 10 min.
v. Wash twice in room temperature 70% ethanol.
vi. Remove residual ethanol with a P200 tip.
vii. Redissolve in 100 mL of water.
viii. Peak fractions from 1–2 mg starting total RNA should contain 20–50 µg of pure, ribosomal RNA-free, polyA$^+$RNA.
ix. Fractions can be analyzed on non-RNase-free ultrathin 1% agarose minigels (*see below*).

3. cDNA synthesis: Double-stranded cDNA is constructed by a simplified "one-tube" version of the original Gubler and Hoffman RNaseH method.
   a. First strand: In a sterile Eppendorf tube add:
      i. 5 µg of mRNA; heat to 100°C for 1 min, and quench on ice. Adjust volume to 70 µL with RNase-free water.
      ii. Add 20 µL of 5 X RT1 buffer (RTl buffer = 0.25 *M* Tris, pH 8.8, 0.25 *M* KCl, 30 m*M* $MgCl_2$).
      iii. 2 µL of RNase inhibitor (Boehringer 40 U/µL).
      iv. 1 µL of 5 mg/mL oligodT (dT 12-18, Pharmacia).
      v. 2.5 µL of 25 m*M* dXTPs (Pharmacia Ultrapure dGTP, dTTP, dATP, dCTP).
      vi. 1 µL 1 *M* DTT.
      vii. 2 µL reverse transcriptase (the best, but unfortunately the most expensive is Life Sciences RT-XL at 25 U/µL).
      Incubate at 42°C for 40 min. and heat-inactivate at 70°C for 10 min.
   b. Second strand: To the same tube, add:
      i. 320 µL of RNase-free water.
      ii. 80 µL of RT2 buffer (RT2 buffer = 0 1 *M* Tris, pH 7.5, 25 m*M* $MgC1_2$, 0.5 *M* KCl, 50 m*M* DTT, 0.25 mg/mL BSA molecular biology-grade, Boehringer).
      iii. 5 µL of DNA polymerase I (Boehringer 5 U/µL).
      iv. 2 µL RNaseH (Boehringer 2 U/µL) Incubate at 15°C for 1 h. Switch tube to room temperature for a further hour. Stop reaction by adding 20 µL of 0.5 *M* EDTA, pH 8.0. Phenol-extract (add 0.5 mL phenol, vortex, spin, remove aqueous phase). Chloroform-extract (0.5 mL $CHCl_3$). Precipitate by adding 10% vol of 5 *M* NaCl, linear polyacrylamide carrier to 20 µg/mL, and adding 2 vol of ethanol. Place on dry ice pellets for 10 min, Spin for 2–3 min only. Wash twice with room temperature 70% ethanol. Remove residual ethanol with a P200 tip. Redissolve cDNA pellet in 240 µL of water. (I have found that addition of T4 DNA polymerase at the end of second-strand synthesis, to blunt the cDNA ends, does not appreciably or reliably increase the yield of litigable cDNA, so it is simply omitted.)
   c. Ligation of adapters: To the 240 µL of cDNA, add:
      i. 30 µL of 10X low-salt buffer (LSB = 60 m*M* Tris, pH 7.5, 60 m*M* $MgCl_2$, 50 m*M* NaCl, 2.5 mg/mL BSA, 70 µ*M* β-mercaptoethanol).
      ii. 30 µl of 10X ligation additions (LA = 1 m*M* ATP, 20 m*M* DTT, 10 m*M* spermidine, 1 mg/mL BSA, 100 m*M* $MgCl_2$).

iii. 5 µg (equimolar mixture of the *Bst*XI adapters (kinased by T4 PNK or prepared with 5'-phosphate on synthesis; *see below*).
iv. 1 µL of T4DNA ligase (New England Biolabs at 400 U/µL).

Incubate overnight at 15°C. Phenol-extract, chloroform-extract, and ethanol-precipitate as above. Resuspend final cDNA pellet in 100–200 µL of TE (TE = 10 m*M* Tris, 1 m*M* EDTA, pH 8.0). *Bst*XI adapters are available commercially. Directional cloning of cDNA is possible using an oligodT primer containing an *Not*I site for first-strand synthesis, ligating *Eco*RI adapters to the second strand, cutting with *Not*I and ligating the cDNA into *Eco*RI-*Not*I vector. Although directional cloned cDNA is obviously an advantage for expression cloning, this system has proven to be very inefficient (probably because of the inefficiency of the *Not*I), and overall yields are then much below what could be achieved by the nondirectional *Bst*XI adapters, thereby negating the advantage of 100% correct orientation with respect to the vector enhancer/promoter.

4. cDNA size fractionation: The best way we have found for achieving the dual goals of efficient nonligated adapter removal and size fractionation of the cDNA is kinetic density centrifugation on continuous gradients of 5–20% potassium acetate (KOAc).
   a. Prepare continuous linear gradients in a 5-mL gradient maker (Hoeffer SM5).
   b. Add 2.5 mL of 20% KOAc to the back chamber.
   c. Add 2.5 mL 5% KOAc to the front chamber.
   d. Fill a Beckman 5-mL SW55 polyallomer centrifuge tube.
   e. Layer the 100–200 mL of cDNA very gently onto the top of the gradient.
   f. Spin at 50 krpm for 3–4 h.
   g. Puncture near to the bottom of the tube with a 21-gage butterfly needle. Collect 0.4-mL fractions.
   h. Add 5 µg of LPA carrier, 2 vol of ethanol, and freeze on dry ice for 10 min.
   i. Spin for 3 min, and wash twice at room temperature with 70% ethanol.
   j. Remove residual ethanol with a P200 tip.
   k. Resuspend each fraction in 20 µL of water.
   l. Analyze 2 µL of each fraction on a 1% agarose minigel.
   m. Pool fractions with cDNA larger than 500–750 bp.
   n. Fractions can be kept separate for each size band, e.g., 500–1000 bp, 1000–1500 bp, 1500–2000 bp, 2–3 kb, 4 kb and larger, resulting in 5 pools of a tight size range and ligated separately from vector to make very discrete size range libraries.
5. Ligation of cDNA to vector
   a. Small-scale test ligations:
      i . Use 1–5% vol of the cDNA.
      ii. Ligate to a constant amount (10–20 ng) of vector (pCDM8, cut with *Bst*XI and the stuffer fragment removed by KOAc gradient centrifugation as above for the cDNA).
      iii. Ligations are in a small volume (10–20 µL) with 10 ng of vector for 1 h at room temperature.

iv. Transform 10% of the ligation mix (1–2 µL) into 50 µL "super-competent" MC1061 /p3 (*see below*).
v. Place on ice for 15 min, and heat-shock at 37°C for 5 min.
vi. Plate on 10-cm LB plates containing ampicillin at 10 µg/mL and tetracycline at 10 µg/mL, with a 5-mL LB agar overlay poured during the heat-shock incubation to provide a "drug-free zone" for the cells to grow and express drug resistance genes before being exposed to the antibiotics.

By using 1% of the cDNA and 10% of the ligation mix, the number of colonies per plate on this small-scale is multiplied by 10^3.

The key quality control checks on any library are primary complexity and insert size range.

1. A library size of 2×10^5–10^6 colonies should be aimed for; anything less is unsatisfactory.
2. Insert size range should be 1–2 kb, with 95% of colonies containing inserts. Standard alkaline/SDS miniprep DNA lysis should be used to analyze the inserts in at least 20–30 colonies.

b. Large-scale ligations:
i. If both of the above criteria are satisfied (library size and insert size range), proceed to large-scale ligation using most, if not all of the cDNA and proportionately more vector.
ii. The entire cDNA yield should consume no more than 1–2 µg of the *Bst*XI cut (stuffer minus) purified vector.
iii. Ligate as above, and transform into competent cells, ensuring that the ligation mix is kept at <2–4% of competent cell volume. (Spermidine in the ligation buffer severely inhibits transformation efficiency.)
iv. Plate on 24 × 24 cm dishes at 10^5 colonies/plate.
v. Harvest the resulting primary plating and maxiprep using alkaline/SDS lysis and cesium chloride density gradients.
vi. Store cDNA library as DNA at –20°C.

I have experienced no deterioration of library stocks over the 6 yr I have been making them. Also, cDNA libraries can be safely amplified by retransformation of the primary library stocks without gross loss of library complexity.

2.1.1.2. General methods

1. Ultrathin 1% agarose minigels:
a. Prepared on "old-style" blood agglutination slides (Blue Star microslides 76 × 51 mm).
b. Twenty to 30 slides are made at once, and laid out on Parafilm with fine-tooth combs (6–7 wells/slide).
c. Pipet 8–10 mL of warm (50°C) molten 1% agarose in TAE running buffer (Maniatis standard recipe). The agarose is held by surface tension as a bubble.
d. These gels hold 10 µL/well and can be run extremely fast (200 V, 15 min) allowing rapid easy monitoring of all the steps of cDNA synthesis procedure. They are extremely thin and have very low autofluoresence background, allowing 10–50 ng of cDNA to be readily visualized by trans-UV illumination.

2. Linear polyacrylamide (LPA) carrier:
   a. LPA has proven to be a reliable and completely noninjurious inert carrier, allowing efficient precipitation of picogram quantities of VNA at near zero cost. There is no risk of contamination with tRNA or rRNA and conversion to cDNA during cDNA synthesis reactions.
   b. Prepare by polymerization of a 5% acrylamide solution with ammonium persulfate (0.1%) and TEMED (0.1%).
   c. No bis-acrylamide is present, so only linear chains of polyacrylamide form. This solution is 50 mg/mL, and a working solution at 2 mg/mL is diluted from this. This is stored at –20°C, and may be frozen and thawed multiple times. Usually, 5–10 µg/precipitation reaction is sufficient.
3. Adapter preparation:
   a. Kinase adapters enzymatically: adapters at 1 mg/mL in 50 mL reaction volume, 5 mL of 10X kinasing buffer (KB = 0.5 *M* Tris, pH 7.5, 10 m*M* ATP, 20 m*M* DT, 10 m*M* spermidine, 1 mg/mL BSA, 100 $MgCl_2$), 20 U of T4 polynucleotide kinase. Incubate at 37°C overnight.
   b. The nonself-compatible *Bst*XI adapters are 5'-CTTTAGAGCACA-3' and 5'-CTCTAAAG-3').
   c. It is essential that the adapters are efficiently HPLC-purified.
   d. Before use make sure that each new batch is tested on an existing batch of "good" cDNA. I have found that good adapters are one of the keys to good library construction.
4. Super-competent cells: Many protocols exist for competizing cells. However, we have used a simple two-step chemical method, which allows the production of cells with a competency of $1–5 \times 10^8$. This level satisfies the dual need for cDNA library transformations and amplification of recovered episomal DNA from library screens (*see below*).
   a. Streak out MC1061/p3 on a fresh TYM (TYM = 2% Bacto-tryptone, 0.5% yeast extract, 0.1 *M* NaCl, 10 m*M* $MgSO_4$) plate. Incubate overnight.
   b. Pick single colonies into 5 mL of TYM, and grow on a wheel with good aeration for 3–4 h.
   c. Dilute to 100 mL in a 250-mL flask in TYM, and grow to midlog $OD_{600} = 0.5$.
   d. Dilute to 500 mL in a 2-L flask in TYM, and grow to midlog $OD600_{nm} = 0.5$.
   e. Rapidly chill cultures by swirling in water/ice.
   f. Pellet bugs in a Beckman J6 centrifuge in 1-L pots at 4 krpm for 15 min.
   g. Resuspend pellet very gently and slowly in 100 mL transformation buffer I (TFBI = 30 m*M* KOAc, 50 m*M* $MnCl_2$, 100 m*M* KCl, 10 m*M* $CaCl_2$, 15% glycerol [v/v]) on ice/water.
   h. Pellet at 2.5 krpm for 10 min at 44°C.
   i. Resuspend pellet in 20 mL transformation buffer II (TFBII = 10 m*M* Na-MOPS, pH 7.0, 75 m*M* $CaCl_2$, 10 m*M* KCl, 15% glycerol).
   j. Aliquot in prechilled Eppendorf tubes, and flash-freeze in liquid nitrogen.
   k. Store at –70°C.
   l. Competency can be tested on supercoiled plasmid standard stocks at 100, 10, 1, and 0.1-pg levels or by relative comparisons to existing tested batches of

ligated cDNA or library screen Hirts. Competent cells maintain the desired level of competency for at least 3–6 mo.

I have described the basic routine procedures used to construct cDNA libraries, even though a number of the "off-the-shelf" rapid procedures are now available from a variety of vendors that allow essentially one tube cells-to-pA^+RNA preparation, usually involving oligodT derivatized magnetic beads as the affinity isolation method. These are very quick and reliable methods, but suffer from extreme expense, especially if several libraries are to be made over a period of time. The same applies to cDNA synthesis kits. Many are now available, but if library construction is to be a routine part of laboratory skills, the cost of such an approach would be prohibitive.

Table 1 describes all of the cDNA libraries constructed in my laboratory (all freely available from me, or now distributed by the Human Genome Resource Centre, Sanger Centre, Hinksey Hall, Cambridge), in the pCDM8 expression vector. A large number of cell-surface molecules have been cloned from these libraries, and have been used by others for the isolation of many other genes by hybridization screens.

2.2. Vectors and the Basis of Transient Expression Methods

As with any library construction, the quality of the cDNA is of crucial importance to the isolation of any clones. The choice of vector into which the cDNA is ligated is linked to the choice of cell for expression. Molecular biologists have exploited elements of the genomes of mammalian DNA tumor viruses for vector construction and expression.

The two essential elements of these viruses are (1) origins of replication and (2) trans-acting DNA binding proteins that interact with the origin and the polymerase/primase complex to replicate the viral genome to high copy number in the appropriate cell line.

Two classes of virus have been exploited: papovaviruses, especially primate SV40 and murine polyoma, and ebnaviruses, especially Epstein Barr virus (EBV).

A crucial element of success in molecular cloning by expression is the copy number of the viral-based vector in the host cell for two reasons: first, it amplifies template per cell and, thus, increases the overall level of transcript production per cell; second, the amplified viral genome allows easy recovery from selected cells and thus reintroduction into *E. coli*.

This can be illustrated best by comparing vectors based on EBV vs SV40, since they differ markedly in their replication potential. EBV-based plasmids usually carry both the origin of replication (ori P) and the trans-acting origin amplifier (EBNA1), and can thus be expressed and amplified in any cell type. SV40-based plasmids usually only carry the origin of replication and have to

Table 1
cDNA Constructed in pCDM8

Human

1 HPBALL (peripheral blood, acute lymphocytic leukemia)
2 JY (lymphoblastoid, B EBV +ve)
3 HepG2 (hepatcellular carcinoma)
4 U937 (promonocytic leukemia)
5 U937/PMA-stimulated
6 K562 (erythroleukemia)
7 K562 (hemin-stimulated)
8 LAK (lymphokine-activated killer cells)
9 YT (HTLV-I +ve adult leukemia, T cell)
10 HL60 (promyelocytic leukemia)
11 HL60/β-interferon-stimulated
12 HT1080 (fibrosarcoma)
13 G361 amelanotic melanomas
14 C32 amelanotic melanomas
15 Placenta—full-term, normal pregnancy
16 Placental trophoblast (sorted 1st trimester)
17 Placental villi (1st trimester)
18 Human bone marrow (aspirate, ALL +ve, 1st remission)
19 HEL (human erythroleukemia)
20 HUVEC (umbilical vein endothelial cell line)
21 HUVEC/stimulated with IL1-β (4 h)
22 HUVEC/stimulated with HT29 conditioned medium (48 h)
23 HUVEC/stimulated with DX3 conditioned medium (48 h)
24 L920 Hodgkin's lymphoma line
25 Fetal brain, 15–16 wk
26 Normal colon
27 Colon carcinoma (solid tumor)
28 HT29 (colon carcinoma)
29 KGlmyeloblastic leukemia
30 KGlAmyeloblastic leukemia
31 KGlBmyeloblastic leukemia
32 K562 hemin stimulated
33 SU-DH-LI diffuse histiocytic lymphoma (non-Hodgkin's lymphoma)
34 Mel DS1 amelanotic melanoma CD36+
35 Mel DS1 amelanotic melanoma-X ray-induced
36 Eosinophil
37 Fetal muscle
38 Natural killer cell
39 CEM (T-cell)
40 Tonsil

(continued)

Table 1 *(continued)*

41	HU-PC (pheochromocytoma)
42	LAD (leukocyte adhesion deficiency type I patient EBV-B-cells)
43	Normal human B-cells (EBV transformed)
44	DX3 melanoma
45	HCT116 colon carcinoma
Rodent	
1	Mouse B-cells (LPS)
2	Mouse T-cells (ConA)
3	Mouse thymocytes
4	IC21 mouse macrophage cell line PMA-stimulated
5	Mouse spleen (NOD mouse)
6	Mouse bone marrow aspirate
7	Rat alveolar macrophage/βIFN-stimulated
8	Mouse serum stimulated macrophages

be introduced into cell lines containing integrated copies of crippled SV40 genomes expressing the SV40 replicator protein large T-antigen.

EBV-based plasmids only replicate to low copy number, typically 1–10 copies/cell, giving adequate levels of expression of specific molecules, but posing difficult problems for subsequent recovery of those plasmids from selected cells. Indeed, higher levels of EBV episomes per cell are often toxic to the cell and can not be tolerated. Typically, recovery of EBV episomes has had to utilize the very high efficiency of phage λ packaging extracts in order to rescue the vectors.

In contrast, SV40-based plasmids replicate to very high copy number per cell, typically 10^3–10^5, yielding very high expression of specific molecules and also relatively facile recovery of the episomes from the selected cell. SV40 replicons are a burden to the cell in the long term, and cells bearing them have elevated morbidity and eventual mortality. However, the burden can be supported for a sufficiently long time to allow expression selection and recovery of those cells.

For this reason, papovavirus-based plasmids have been the most widely utilized system for transient expression and rescue. Of the many types of papovaviruses, SV40 has been used most frequently ***(3,4)***, though the murine permissive virus, polyoma, has also been exploited ***(5)***.

There are many variants of SV40-based plasmids, and only a few will be described here. All share the same basic features: the SV40 origin of replication (a 350-bp fragment of the SV40 genome): a eukaryotic enhancer and promoter driving high-level expression of the inserted cDNA or genomic

fragment; downstream transcript processing elements (usually an intron and polyadenylation signal); and finally, a prokaryotic origin of replication and some system for drug selection in *E. coli*.

pπH3M developed by Aruffo and Seed in 1987 ***(4)***, and pCDM8, developed by Seed in 1987 ***(5)***, have been successfully employed for the cloning of numerous cell-surface molecules. pCDM8 postdates pπH3M, and has now superseded it. Consequently, pCDM8 will be described in detail. It contains the powerful cytomegalovirus (CMV) enhancer and promoter driving expression of cDNA inserted at a polylinker cloning site flanked by nonpalindromic *Bst*XI sites. Downstream of this site is an intron and polyadenylation site, allowing efficient transcript processing and transport. pCDM8 contains both an SV40 origin of replication and a polyoma origin allowing replication of this vector in either primate cell lines, such as COS-1 and COS-7 cells, or murine polyoma transformed lines, such as WOP and COP. This is particularly useful if a specific MAb crossreacts with glycoproteins on the surface of COS cells, which are after all high primate cells of fibroblast/epithelial origin. MAb raised in mice are highly unlikely to react with the surface of murine cells. The remaining elements of the vector allow replication in *E. coli* and drug selection mediated by a suppressor tRNA (supF), which suppresses amber stop codons in ampicillin and tetracycline resistance genes carried on a stable episome, p3, in the strain MC1061/p3. There is an M13 origin of replication allowing production of single-stranded templates of the plasmid when appropriate F+ *E. coli* strains are superinfected with helper filamentous f1 phage, such as M13. A T4 DNA promoter is included at the 5'-edge of the cloning site to allow in vitro production of RNA templates for transcript terminus mapping and transcript production by T4 RNA polymerase.

Recently, some alternative versions of pCDM8 have been developed by commercial companies. The modifications have been of two types: first pCDNA1, which contains a slightly improved polylinker and addition of a 3'-SP6 promoter for generation of antisense transcripts; second, pCDNA3, which is more radically altered by removal of the supF selection system and replacement with the p-lactamase ampicillin resistance gene. This allows selection of recovered plasmid in any highly competent *E. coli* strain capable of ColE1 replication, and is not confined to the MC1061/p3 system. However, this latter vector has not yet been fully tested as an efficient platform for library construction and expression screening.

A different SV40-based expression vector, pJFE14, was constructed by Elliott ***(23)***. This utilizes the SRα promoter and R and U 5'-regions of the human T-cell lymphotropic virus I. An intron from the 16S RNA is placed upstream of the *Bst*XI polylinker cloning site. The plasmid contains a ColE1 replicon and ampicillin resistance. Proponents of this vector argue that it

overcomes some of the plasmid instability observed in the pCDMS/NlC1061/p3 system.

All of these vectors only replicate to high copy number in cells bearing SV40 or polyoma genomes. In 1981, Gluzman produced SV40-transformed African green monkey kidney fibroblast (CV-1) cells bearing integrated copies of the SV40 genome, crippled by deletion of several bases at the SV40 origin. The resulting cells, COS-1 and CDS-7, express high levels of SV40 large T-antigen and permissivity factors, allowing high levels of SV40 replication per cell, but do not produce infectious viral genomes, and therefore are safe to work with under low-level containment. COS cells are eminently transfectable; with DEAE dextran/chloroquine regime (*see* **Subheading 3.2.**, ***24***), it is routinely possible to achieve 50–60% of total transfected cells expressing the introduced product. They have proven to be robust and reliable "workhorses" for transient expression.

A future development would be the construction of SV40-based plasmids that also produce SV40 large T-antigen similar to the oriP/EBNA-1 p201-p205 system. Any cell line could then be transfected, irrespective of whether it contained endogenous SV40 genomes, and would allow genetic defects in defined cell lines to be complemented by introduced libraries, and rescue of the complementing episome.

Over the past three to four years, EBV-based episomal cDNA libraries have been used very successfully to clone cDNAs by complementation. The advantage of the EBV system is that it carries its own trans-acting replication proteins (EBNA1), so it can be expressed in any cell type. Many cell lines have been established from patients with inherited genetic defects, for example, xeroderma pigmentosum (XP), ataxia telangiectasia, Bloom's syndrome, Fanconi's anemia, and paroxysmal nocturnal hemoglobinuria (PNH). Provided they can be robustly transfected, these cell lines can be used as recipients for wild-type cDNA libraries, and restoration of normal phenotype can be screened and selected. Indeed, this technique has already been successfully applied to XP, Fanconi's anemia ***(19)***, and PNH ***(20)***.

3. Screening Methods

3.1. Introduction

I will now describe the methods for screening libraries by transient expression selection and rescue for proteins in each of three cellular compartments: intracellular, surface, and extracellular/secreted. I have much more hands-on experience with cloning surface proteins by panning, so I will start with this method and use it as the basis for the other two.

3.2. Protocol 1: Screening for Surface Molecules by Panning and Rescue

cDNA libraries constructed as above in the pCDM8 vector are transfected into COS cells using DEAE dextran as a facilitator ***(24)*** and chloroquine

diphosphate to reduce lysosomal degradation of endocytosed DNA. Forty-eight to 72 h posttransfection, cells are lifted with (PBS)/2 m*M* EDTA, washed in the same buffer containing 0.02% sodium azide and 5% fetal bovine serum at 4°C and incubated with MAb as tissue-culture supernatants at minimal dilution (1/10 at most), at 4°C for 30 min, washed, and applied to bacterial Petri dishes precoated with affinity purified goat antimouse IgG. Cells are allowed to "pan" for 1–2 h at room temperature, and plates are then washed gently three to four times. It is possible to observe panned cells (10–100) per dish even at the first round of selection. However, on many occasions, no cells may be seen if the clone being sought is of very low frequency in the library. Whether panned cells are observed or not at this stage, the procedure must be continued a further two rounds before a definitive assessment of success or failure is made. The panned cells are lysed *in situ* by applying "Hirt squirt" (0.8% SDS/ 10 m*M* EDTA). The cell lysate is harvested into an Eppendorf tube, and 100 µL of 5 *M* NaCl is added, gently mixed, and placed in bath of wet ice for at least 1 h to allow precipitation of high-molecular-mass primate genomic DNA. The episomal DNA is recovered by spinning out the genomic DNA precipitate, phenol extraction, and ethanol precipitation. A fraction of the recovered episomal DNA is transformed into highly competent MC1061/p3 and plated on LB agar containing ampicillin and tetracycline. A yield of 10^3–10^4 bacterial colonies should be obtained at this stage.

It is possible to continue to introduce the selected cDNA population into COS cells by DEAE dextran facilitated transfection. However, a change of entry method is needed at this point. DEAE dextran is a very efficient method of introducing DNA into cells, so it is an ideal method for the first round of screening to maximize representation of the cDNA library in COS cells. It is estimated that up to 10^3–10^4 different cDNA clones may be taken up by each COS cell by this method.

Consequently, a panned cell expressing the clone of interest will also contain 10^3–10^4 irrelevant cDNA clones, which will be represented in the yield of bacterial colonies from the first round. Thus, if DEAE dextran were used for all subsequent rounds, a plateau of enrichment would be reached where the cDNA clone of interest would be contained within a heterogeneous population of irrelevant clones. This would mean that a very large number of individual bacterial colonies would have to be analyzed by miniprep DNA isolation, individual transfection, and MAb staining.

To prevent this, the second round of screening is initiated by introducing the bacteria into COS cells as spheroplasts or protoplasts (i.e., bacteria minus their cell walls). This is a very inefficient technique; 1–5% of COS cells are transfected, a small number of protoplasts actually fuse with each COS cell, and each protoplast obviously contains only one cDNA clone.

This means that a much smaller population of cDNA clones is introduced into each COS cell. The complexity of the resulting second-round Hirt is thus greatly reduced and enrichment for the clone of interest is greatly enhanced.

The bacterial population is grown in liquid culture, plasmids are amplified in the presence of spectinomycin and converted to protoplasts by osmotic shock, EDTA chelation, and lysozymal digestion. Protoplasts are introduced into COS cells by polyethylene glycol- (PEG 1000 or PEC, 1450) mediated membrane fusion. After another 36–48 h to allow transient expression of plasmid-encoded products, the COS cells are again incubated with the MAb, washed, and panned. As before, very few cells may be observed by visual scanning of the panning plates; although considerable enrichment has occurred as a result of the first round of selection, the switch to a more inefficient method of transfection means that a similar number of COS cells will pan at this round. A Hirt preparation is made and processed in the same way. Again, 10^3–10^4 colonies should derive from this round. A further round of protoplast fusion is needed before a definitive assessment of the success of the screening can be made. By this time, at the end of the third round of panning, COS cells should be visible on the panning dish. Individual bacterial clones are picked, DNA is isolated by standard SDS/alkaline lysis minipreparation methods, and a fraction (10%) of that DNA is transfected into COS cells by DEAE dextran facilitation. Forty-eight hours later, the COS cells are stained *in situ* with the MAb, stained with a goat-antimouse FITC second antibody, and scored by fluorescent microscopy. Results at this stage are rarely equivocal. Only a small number of individual colonies (10–20) need be analyzed at this stage, since 10–100% of these clones should be the clones of interest.

Much time and effort can be saved by screening with several MAb at once as a pool. The pool of MAb is used for the first two rounds of screening. At the third round, the COS cells are incubated with each MAb separately and panned separately.

3.3. Protocol 2: Screening for Surface Proteins By Transient Expression, Panning, and Episomal Rescue

3.3.1. Overview

- Round 1: DEAE dextran transfection, expression, screening, panning, episomal rescue, and transformation
- Round 2: protoplast fusion, expression, screening, panning, episomal rescue, and transformation.
- Round 3: repeat of round 2.
- Scoring individual clones.

1. Round 1:
   a. Transfection:
      i. Grow COS cells in DME/10% calf serum at 50–75% confluence (most conveniently in Falcon 15-cm Intergrid culture dishes).

ii. Use 10–20 µg cDNA library in pCDM8, or similar SV40-based vector, for 1 x 10^7 COS cells using 400 µg/mL DEAE dextran (Sigma M_r 400,000) as a facilitator ***(24)*** and chloroquine diphosphate at 100 µ*M*. Dilute cDNA library DNA well below 1 mg/mL in TE, add DEAE dextran, and dilute in medium either without serum or with 10% NuSerum (Collaborative Research) or a low protein concentration serum supplement, such as UltroserG from Gibco BRL. Alternatively, cells can be transfected in medium alone, although some increased mortality will occur.
iii. Leave on for up to 4 h or until the COS cells begin to look vacuolated.
iv. Aspirate medium, add 15 mL PBS/10% dimethyl sulfoxide (DMSO), and osmotic shock medium for 2 min.
v. Aspirate, and replace with regular medium.
vi. Twenty-four hours posttransfection, trypsinize cells and replate on fresh culture dishes to remove residual absorbed DEAE dextran. It is essential to do this in order to be able to lift the COS cells with EDTA the following day and achieve a monodisperse single-cell suspension.

b. Screening
i. Forty-eight to 72 h posttransfection, aspirate medium, wash twice with PBS only, and lift cells with 10 mL PBS containing 2 m*M* EDTA; put dishes at 37°C for 10–15 minutes.
ii. Wash in "panning buffer" (PB) (PB = PBS, 2 m*M* EDTA, 0.1% sodium azide, 5% fetal bovine serum) at 4°C.
iii. Incubate with MAb (either as neat, at most 1:10 dilution of tissue culture supernatants, or 1:100–1:1000 dilution of ascites, or 1 µg/mL purified antibody), at 4°C for 30 min,
iv. Wash twice in cold PB.

c. Panning
i. Apply to 10-cm plastic bacterial Petri dishes precoated with affinity purified goat antimouse IgG (*see below* for preparation of panning plates).
ii. Leave in a vibration-free part of the laboratory for 2–3 h to allow cells to pan gently at room temperature.
iii. Wash panning plate very gently using a pipet only, not a suction line. Remove cells, add 5 mL of PB to one edge of the dish held at a 30° angle, gently roll around two to three times, and remove PB from the opposite edge of the dish.
iv. Repeat washing three to five times.
v. Observe efficiency of washing under inverted microscope, gently roll dish on stage, and check for the general number of free-floating cells still remaining. Continue washing until no floaters remain. Assess the level of panned cells and whether there are large numbers of obviously dead cells nonspecifically stuck to the dish.

General methods

Preparation of panning plates

- Prepare by coating 10-cm Falcon Petri dishes with 5 mL of a 10 µg/mL solution of affinity isolated goat: antimouse IgG in 50 m*M* Tris, pH 9.5 for 1–2 h.

- Wash three times in PBS
- Block remaining sites by overnight incubation with 5 mL/dish of blocking buffer (PBS, 2 mg/mL BSA).
- Aspirate blocking buffer, and store plates at –20°C for up to 6 mo.

d. Preparation of Hirt.
   i. Lyse the specifically panned cells *in situ* in 400 µL "Hirt squirt" (0.8% SDS/10 m*M* EDTA). Gently swirl around the dish to efficiently cover.
   ii. Cut 1-2 mm from the end of a Gilson P1000 tip and pipette the lysate gently into an Eppendorf tube (this avoids shearing the genomic DNA),
   iii. Add 100 µL of 5 *M* NaCl, mix gently by inversion, and place in a bath of wet ice for at least 1 h to allow precipitation of high-molecular-mass primate genomic DNA.
   iv. Recover episomal DNA by spinning out the white genomic DNA precipitate in minifuge for 5 min.
   v. Remove clear supernatant to a fresh tube, and respin if any part of the precipitate carries over.
   vi. Remove clear supernatant to a fresh tube.
   vii. Add 0.5 mL phenol, vortex for l min, spin, and remove aqueous phase to a fresh tube.
   viii. Extract with 0.5 mL chloroform, spin, and remove aqueous phase to a new tube.
   ix. Add 5 µg of LPA carrier (*see above* for recipe) and mix.
   x. Add 2 vol of ethanol, mix, and place on dry ice for 10 min.
   xi. Spin for 3 min, wash twice with 70% ethanol, remove residual ethanol with a P200 tip, and dissolve Hirt in 50 µL TE.

e. Transformation of Hirt.
   i. 10–30% (5–15 µL) of the recovered episomal DNA is transformed into 0.5 mL highly competent MC1061/p3 (i.e., highly competent is $>10^8$colonies/µg).
   ii. Plate on 1 24 X 24 cm LB agar plate containing ampicillin and tetracycline each at 10 µg/mL.
   iii. A yield of 10^3–10^4 bacterial colonies should be obtained for the first-round Hirt. Anything less is a failure, so start again. Anything more is a bonus, so continue.

2. Round 2
   a. Expansion and amplification of round 1 population.
      i. Scrape the bacterial population from round 1 into a slurry in 20–50 mL LB containing ampicillin and tetracycline both at 10 µg/mL. Titer a 1/10 or 1/100 dilution at OD_{600nm}.
      ii. Grow this population in 100–200 mL liquid culture with vigorous shaking at 37°C from a starting inoculum of $OD_{600nm} = 0.1$ to $OD_{600nm} = 0.5$.
      iii. Amplify plasmids by overnight incubation, with shaking, in the presence of 100 mg/mL spectinomycin. This allows some amplification of plasmid copy number per bug, and also arrests bacterial growth so the fusion inoculum is not excessive.

iv. Prepare COS cells now for protoplast fusions the next day. Trypsinize COS cells and plate at 50–75% confluence in 10-cm culture dishes: 2 X 10 cm dishes/100 mL of bacterial culture.

b. Conversion to protoplasts: The overnight liquid culture is converted to protoplasts by sequential osmotic shock, EDTA chelation, and lysozomal digestion.
   i. Bacteria are pelleted (e.g., Beckman JA14/GSA rotor, 250-mL bottles) for 5 min at 10 krpm.
   ii. Resuspend the bacterial pellet in 5 mL cold 20% sucrose, 50 m*M* Tris, pH 8.0.
   iii. Add 1 mL of 10 µg/mL lysozyme freshly dissolved in 250 m*M* Tris, pH 8.0.
   iv. Incubate at 4°C for 5 min.
   v. Add 2 mL cold 0.25 *M* EDTA, pH 8.0.
   vi. Incubate at 4°C for 5 min.
   vii. Add 2 mL 50 m*M* Tris, pH 8.0.
   viii. Incubate at 4°C for 5 min.
   ix. Place in 37°C water bath for 5 min.
   x. Place on ice.
   xi. Check for percent conversion to spheroplasts by microscopy (should be 90% conversion of rod-shaped bacteria to spherical protoplasts).

c. Protoplast fusion: Perform all manipulations in a laminar flow hood.
   i. Add 20 mL cold DME/10% sucrose/10 m*M* $MgSO_4$ slowly, dropwise from a 25-mL pipet, swirling all the time.
   ii. Remove media from 10-cm dishes of COS cells at 50–75% confluency.
   iii. Add 15 mL of the spheroplast slurry to each dish.
   iv. Place dishes in bottom of buckets of a bench-top centrifuge (Beckman GPR, Sorval RC6000) with rubber bases still in. Two dishes per bucket can be accommodated.
   v. Spin at 2500 rpm for 10 min at 4°C, and decelerate without brake to avoid disruption of protoplast skins.
   vi Aspirate fluid from dishes.
   vii. Add 5 mL of 50% (w/w) PEG 1000 or PEG 1450/50% DME into the center of the dish.
   viii. After the PEG has been added to last dish, prop all the dishes up on their lids so that the PEG drains to the bottom edge.
   ix. Aspirate PEG layer.
   x. Leave for fusion to occur over 90–120 s (PEG1000) or 120–150 s (PEG1450).
   xi. Stop fusion by adding 5 mL of DME into the center of the dish. The PEG layer will be swept radially away by the medium.
   xii. Aspirate and repeat the washing.
   xiii. Aspirate, add 10 mL of DME/10% calf serum containing 10 µg/mL gentamycin sulfate, and leave for 4 h over which time the protoplast layer will gradually disintegrate.
   xiv. Swirl the dishes to disrupt the protoplast skins, aspirate, and change the medium. Gentamycin sulfate is essential for these cultures, since the

residual bacterial population is so massive that penicillin and streptomycin are completely ineffective.

d. Expression, screening, and panning
   i. Leave fused COS cells for 36–48 h to allow transient expression of plasmid encoded products.
   ii. Repeat MAb screening and panning as above for round 1.
   iii. Prepare Hirt DNA, extract, precipitate and transform in MC1061/p3.
   iv. The yield should be 10^3–10^4 colonies.

3. Round 3: Perform a further round of protoplast fusion as above (**steps 2 a–d**).
   a. At the end of this round, transform 10% of the final Hirt DNA into 50 µL of competent cells, and plate on a 10-cm Petri dish of LB + amp/tet as above.
   b. Incubate overnight.
   c. Pick 10–30 individual bacterial colonies each into 2.5 mL of LB + amp/tet, and grow at 37°C with vigorous shaking to saturation (8 h minimum culture time).
   d. Isolate plasmid DNA by standard alkaline/SDS lysis methods.
   e. Transfect 10–30% of the miniprep DNA into COS cells in six-well cluster plates by DEAE dextran facilitation protocol (*see* **step 1a** *above*).
   f. Forty-eight hours later, screen the COS cells *in situ* with the MAb at 4°C for 30 min, wash three times, and stain with a 1:100 dilution of goat-antimouse FITC second antibody. Wash three times and fix with PBS/2% formaldehyde.
   g. Score individual wells by fluorescent microscopy. The % positive clones can vary from 10–100%, depending on many variables including how abundant the original cDNA was in library, the affinity of the antibody or ligand, and the overall efficiency of the three rounds of expression, panning, and rescue.

3.3. Screening for Intracellular Proteins by In Situ *Labeling*

This technique was developed by the group of Hans Clevers in Utrecht ***(14,15)***. The cDNA library is transfected and expressed as for surface panning above. However, the COS cells are screened *in situ,* i.e., they are not lifted at d 2–3 posttransfection. In brief, the COS cells are rinsed in PBS and fixed in the culture dish for 10 min with methanol. All subsequent manipulations are performed at room temperature. The monolayer is washed twice with PBS and preincubated with PBS/5% fetal calf serum (FCS) for 10 min, followed by a 1-h incubation with antibody or labeled ligand. Plates are washed twice with PBS followed by a 45-minute incubation with peroxidase-labeled goat antimouse Ig, diluted 1:50 in 5% FCS/PBS. Peroxidase activity is subsequently visualized using a 5% dilution of a 4 mg/mL stock of 9-amino-3-ethylcarbazol in *N,N'*-dimethylformamide in 0.1 *M* NaAc (pH 4.8) containing 0.1% H_2O_2 (30–60 min). After washing with water, the plates are visually screened for positively stained (bright-red) cells with an inverted microscope. Positive cells are picked by scraping with a handheld fine tip of a Gilson tip. Next, individual scraped cells are treated with Hirt squirt as above and extracted. Plasmid DNA

is transformed into MC1061/p3, and rounds of expression and selection are repeated as above.

3.4. Screening for Extracellular/Secreted Proteins by Supernatant Bioassay

Again the basics of library transfection, transient expression, and Hirt extraction are as already described for surface panning. The only difference comes in the actual screening procedure at 2–3 d posttransfection for each of the three rounds.

Twenty-four posttransfection or protoplast fusion, the COS cells are trypsinized, pooled, and counted in a hemocytometer. Cells are then aliquoted in appropriate pool sizes, usually 10^2–10^3 cells/well in either 24- or 96-well plates, and allowed to adhere and express for a further 24–48 h. A fraction of the conditioned supernatant from each well is then harvested and applied to the assay plates. The assay will obviously be specifically designed for the protein being searched for. In the case of cytokines, growth factors, or hematopoietic colony stimulating factors, a bioassay based on cell proliferation, or colony growth or differentiation is the readout. Positive wells are identified, and a Hirt extract is made from the COS cells in the original master expression plate.

3.5. Functional analysis of cDNA Transfectants

As described in **Subheading 1.2.**, one of the advantages of the transient cloning system is that functional experiments on the cloned cDNA molecules can begin immediately. Transient expression of pure cDNA clones in COS cells can lead to the accumulation of up to 10^6 molecules/COS cell-surface, so that functional assays can be performed directly on the cells ***(25)***.

3.5.1. Protocol 3: Functional Assays on Cloned cDNAs Transiently Expressed in COS Cells

- Trypsinize COS cell stocks and replate at a density of 1 x 10^4/cm^2.
- Transfect 10–20 μg plasmid DNA into the COS cells for 4 h at 37°C using the DEAE dextran method.
- Leave cells for a further 18 h, trypsinize, and replate at a density of 10^4/cm^2 on chosen assay format; 6-well, 24-well, or 96-well plates, or 3- or 6-cm dishes. It is essential to ensure that the cell density is correct and that the distribution is even throughout the well or dish. To avoid cells "piling-up" in the center of the plate, gently rock the dishes every 1–2 h for 6 h after replating to redistribute the cells.
- Transient expression of the encoded cDNA can be measured 48 h posttransfection, but is optimal if left for 72 h. (This is especially true for double transfections, e.g., expression of two subunits of a dimeric receptor.)
- Cell-surface molecule expression can be monitored by immunocytochemistry using specific MAb or by functional adhesion assay.

- Functional assays can be performed using radioisotopically labeled cells (overnight incorporation of ^{3}[H]thymidine) or unlabeled cells coupled with visual assessment of adhesion photomicroscopically after fixing and staining COS cell/test cell rosettes in 0.2% crystal violet in 10% phosphate buffered formalin (pH 7.4).
- Allow test cells to adhere to COS transients for up to 1 h.
- Wash three to five times, monitoring for removal of floating cells.
- Fix in PB/2% formaldehyde.
- Fixed cell rosettes can be directly visualized and photographed under phase contrast using an inverted microscope.

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6

Application of Polymerase Chain Reaction for the Discovery of New Adhesion Molecule Family Members

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1. Introduction

This chapter will describe two approaches that utilize the polymerase chain reaction (PCR) to clone new members of previously known adhesion receptor families. These two approaches are useful to clone either alternatively spliced forms of previously known family members or novel molecules whose presence is suggested from protein data.

The first approach requires a combination of cDNA library screening and PCR. First, a cDNA fragment from a previously known adhesion receptor is used as a probe to screen a cDNA library and isolate clones. Then, the isolated clones are amplified by PCR to identify novel products that differ in size and sequence from the probe itself. This approach has been successfully used to isolate β_{1C}, a variant form of the integrin β_1 subunit containing a unique cytoplasmic domain ***(1)***.

The second approach utilizes degenerate primers, derived from either protein sequences or homologous sequences from previously known family members, and requires only the use of the PCR. This approach has been successfully used to clone members of the integrin and cadherin families of adhesion receptors ***(2–8)***. These genes were cloned by designing degenerate oligonucleotides from protein sequence and using those primers for PCR. Degenerate primers have also been used to clone the α_6, β_4 ***(9),*** and β_5 ***(10,11)*** integrin subunits. However, these integrin subunits were cloned by utilizing degenerate oligonucleotides to screen a library directly, rather than by utilizing PCR.

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In order to clone a gene utilizing PCR, one must first design oligonucleotide primers that flank the region of interest. When the starting point of a cloning project is peptide sequence, obtained either by protein sequencing or homologous sequence from other family members, the designed oligonucleotides must be degenerate. Because most amino acids are coded for by more than one codon, there are a number of oligonucleotide sequences that could give rise to a given peptide sequence. Two different types of oligonucleotides can be designed from peptide sequence for use in cloning. The first is a completely degenerate oligonucleotide that contains all possible permutations of nucleotides to generate a given peptide sequence. Alternatively, inosine can be included at positions where more than two nucleotides could give rise to the amino acid ***(12)***. The second is an oligonucleotide with little to no degeneracy designed by making informed guesses about which nucleotide triplet is used to code for each amino acid.

The first step in designing an oligonucleotide from peptide sequence is to determine the region of the protein of interest to use. When possible, it is best to avoid regions rich in arginine, leucine and serine owing to the fact that these three amino acids each have six codons. It is better to select a region where the amino acids have fewer codons. For example, both methionine and tryptophan have only one codon, and asparagine, aspartic acid, cysteine, glutamic acid, glutamine, histidine, lysine, phenylalanine, and tyrosine have only two (*see* **Table 1**).

The second step in designing an oligonucleotide from protein sequence is to back-translate the peptide sequence. **Table 1** lists all of the codons for each amino acid and the frequency of their occurrence in the human genome ***(13)***. Based on a codon's frequency of occurrence, one can determine which triplet is most likely to code for an amino acid. Detailed information concerning this approach can be obtained from the above-mentioned references and from an extensive review by Pytela et al. ***(12)***. However, it should be pointed out that, using this strategy, optimization is required in each case.

The materials and method necessary to use the first approach successfully (combination of cDNA library screening and PCR) are described in this chapter.

2. Materials

2.1. Library Screening

2.1.1. Determining the Titer of λ Libraries

1. Phage dilution buffer (PDB): 20 m*M* Tris-HCl, pH 7.5 (American Bioanalytical, Natick, MA), 100 m*M* $MgSO_4$ (J.T. Baker, Phillipsburg, NJ), 0.02% gelatin (Sigma, St. Louis, MO). Autoclave to sterilize. Store at room temperature.
2. Luria broth (LB): to 950 mL of deionized water add 10 g bacto-tryptone (DIFCO Laboratories, Detroit, MI), 5 g yeast extract (DIFCO Laboratories), and 10 g

Table 1.
Codon Usage Frequency in the Human Genome

Ala	Arg	Asn	Asp	Cys	Glu
GCC .41	CGG .21	ACC .56	GAC .55	UGC .57	GAG .59
GCU .26	CGC .20	AAU .44	GAU .45	UGU .43	GAA .41
GCA .22	AGA .20				
GCG .11	AGG .20				
	CGA .11				
	CGU .08				

Gln	Gly	His	Ile	Leu	Lys
CAG .75	GGC .35	CAC .60	AUC .51	CUG .42	AAG .59
CAA .25	GGA .25	CAU .40	AUU .34	CUC .20	AAA .41
	GGG .24		AUA .15	CUU .12	
	GGU .16			UUG .12	
				UUA .07	
				CUA .07	

Met	Phe	Pro	Ser	Thr	Trp
AUG 1	UUC .57	CCC .34	AGC .25	ACC .38	UGG 1
	UUU .43	CCU .28	UCC .23	ACA..27	
		CCA .27	UCU .18	ACU .23	
		CCG .11	AGU .14	ACG .12	
			UCA .14		
			UCG .06		

Tyr	Val
UAC .58	GUG .48
UAU .42	GUC .25
	GUU .17
	GUA .10

[a]Each of the codons for all 20 amino acids and their frequency of usage are indicated. The information was obtained from the Codon Usage Database ***(13)***.

NaCl (J.T. Baker). Dissolve by stirring, adjust pH to 7.0, and bring volume up to 1 L. Autoclave to sterilize. Store at 4°C or room temperature.

3. Top agarose: LB + 0.75% agarose (Bio-Rad, Hercules, CA). Autoclave to sterilize. Store at 4°C.
4. LB plates: prepare LB as described above and add 15 g/L Bacto-agar (DIFCO Laboratories). Store at 4°C.
5. 100-mm Petri dishes (Falcon, Lincoln Park, NJ).
6. 5-mL tubes (Falcon).

2.1.2. Plating the cDNA Library

1. 150-mm Petri dishes (Falcon).
2. 15-mL tubes (Corning, Corning, NY).

2.1.3. Plaque Lifts to Filters

1. Nitrocellulose filters (Millipore, Bedford, MA).
2. 3MM paper (Whatman, Fairfield, NJ).
3. Denaturing solution: 1.5 *M* NaCl, 0.5 *M* NaOH (J.T. Baker). Store at room temperature.
4. Neutralizing solution: 1.5 *M* NaCl, 0.5 *M* Tris-HCl, pH 8.0. Store at room temperature.
5. 20X SSC: Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate (J.T. Baker) in 800 mL of water. Adjust the pH to 7.0. Adjust the volume to 1 L with water. Sterilize by autoclaving. Store at room temperature.
6. Rinsing solution: 2X SSC (prepare by diluting the 20X SSC stock accordingly). Store at room temperature.
7. 3–5 cc syringe (Becton Dickinson, Lincoln Park, NJ).
8. Waterproof black india ink (Faber-Castell Corporation, Newark, NJ). Store at room temperature.
9. 21-Gage needle (Becton Dickinson).
10. Stratalinker UV crosslinker (Stratagene, La Jolle, CA).

2.1.4. Labeling and Purification of cDNA Probe

1. Geiger counter (Ludlom Measurements Incorporated, Sweetwater, TX).
2. Random Primers DNA Labeling System (Life Technologies, Gaithersburg, MD).
3. [α-^{32}P]dCTP, 3000Ci/mmol (Amersham, Arlington Heights, IL).
4. QuickSpin columns (Boehringer Mannheim Corporation, Indianapolis, IN).

2.1.5. Prehybridization, Hybridization, and Washing of Filters

1. 10X SSPE: dissolve 107 g $Na_2HPO_4 \cdot 7H_2O$ (or 56.8 g of anhydrous) (J.T. Baker), 20 mL 0.2 *M* EDTA (J.T. Baker), and 420 g NaCl in a final volume of 4 L of water. This solution can be stored at room temperature.
2. Hybridization solution: For 500 mL combine 0.25 g Heparin (Sigma), 5 g SDS (American Bioanalytical), 2.5 g powdered non-fat dried milk (Carnation, Glendale, CA), 30 g polyethylene Glycol (Sigma), 250 mL 10X SSPE and 50 mL formamide (American Bioanalytical). Bring the volume up to 500 mL with water. This solution can be aliquoted and stored at –20°C. Solutions containing 50% formamide can be used for the same purpose.
3. Gene Roller LM20 nylon mesh (Savant, Farmingdale, NY).
4. Hybridization bottle GRB 260 (Savant).
5. Gene Roller GRH10 hybridization oven (Savant).
6. Sheared sonicated salmon sperm DNA (Life Technologies, Ocala, FL).
7. Screw-cap 1.5 mL eppendorfs (USA/Scientific Plastics).

8. Wash solution #1: 2X SSC, 0.5%SDS (*see* **Subheading 2.1.3., item 5** for preparation of 20X SSC). Store at room temperature.
9. Wash solution #2: 0.3X SSC, 0.15% SDS. Store at room temperature.
10. Wash solution #3: 0.15X SSC, 0.15% SDS. Store at room temperature.
11. Shaking water bath.
12. Plastic containers (AeroPlastics, Leominster, MA).
13. Plastic wrap (Dow, Indianapolis, IN).
14. Glogos II Autorad markers (Stratagene).
15. Autoradiography cassettes (Spectronics Corporation, Westbury, NY).
16. Scientific imaging film X-OMAT-AR (Kodak, Rochester, NY).
17. Kodak M35A X-OMAT Processor (Kodak).
18. Pasteur pipet (Becton Dickinson).

2.2. Polymerase Chain Reaction (PCR)

1. DNA thermal cycler 480 (Perkin Elmer, Foster City, CA).
2. Filtered tips from USA/Scientific Plastics are strictly required to prevent a contamination from template DNA.
3. 0.5 mL microcentrifuge tubes (USA/Scientific Plastics) should be autoclaved for use in PCR.
4. *Taq* DNA polymerase, 10X PCR buffer, $MgCl_2$, dATP, dGTP, dTTP, and dCTP from Perkin Elmer.
5. Mineral oil (Sigma).
6. Agarose (Bio-Rad).
7. DNA size markers from Life Technologies.
8. Ethidium bromide (Sigma) is usually prepared as a stock solution of 10 mg/mL in water, which is stored at room temperature in dark bottles or bottles wrapped in aluminum foil. The dye is usually incorporated into the gel or the electrophoresis buffer at a concentration of 0.5 µg/mL. Ethidium bromide is a powerful mutagen and is moderately toxic. Gloves should always be worn when working with this solution.
9. TA cloning kit from Invitrogen (Carlsbad, CA).
10. Wizard minipreps DNA purification system (Promega, Madison, WI).
11. Restriction enzymes (New England Biolabs, Incorporated, Beverly, MA).
12. Sequenase version 2.0 DNA sequencing kit (United States Biochemical, Cleveland, OH).
13. Store all materials at –20°C unless otherwise indicated.

3. Method

3.1. Library Screening

A cDNA fragment (*see* **Note 1**) from a previously known adhesion molecule is used to screen a cDNA library (*see* **Note 2**) from a selected tissue or cell type. All of the following steps are carried out at room temperature unless otherwise indicated.

3.1.1. Determining the Titer of λ Libraries

1. Grow the appropriate bacterial host overnight in the required medium and at the specified temperature (*see* **Note 3**).
2. Centrifuge the cells at 2000*g* for 10 min at 4°C.
3. Resuspend the cells in 0.4 vol of 10 m*M* $MgSO_4$ by gently pipeting up and down; do not vortex the phage. These cells may be stored at 4°C for a few days.
4. Warm LB plates (*see* **Note 3**) at 37°C for approximately 1 h prior to plating.
5. Make the following dilutions of the library: 10^{-5}–10^{-7}.
6. Tap the tubes gently to mix; do not vortex.
7. Put 1 µL of each dilution into 99 µL of PDB in a 5 mL tube.
8. Add 100 µL of bacterial cells (resuspended in 0.4 vol of 10 m*M* $MgSO_4$ as indicated above).
9. Tap tubes gently to mix.
10. Incubate in a 37°C water bath for 10 min.
11. Add 4 mL of top agarose (boiled to liquefy and then cooled to 49°C) to tubes (do this in sets of three trying not to induce bubbles), and pour immediately on top of prewarmed 100 mm LB plates.
12. Let the plates stand at room temperature for approx 10 min or until the top agarose has hardened.
13. Invert plates and incubate at 37°C overnight.
14. On the following day, remove the plates from the incubator and count the number of plaques on each plate to determine the plaque-forming units (PFU)/mL (i.e., the titer of the library).

3.1.2. Plating the cDNA Library

1. Prepare two 15 mL tubes that each contain 5×10^4 PFU. Bring the volume of each tube up to 100 µL with PDB.
2. Add 600 µL of bacterial cells (resuspended in 0.4 vol of 10 m*M* $MgSO_4$ as indicated above) to each tube.
3. Tap tubes gently to mix.
4. Incubate in a 37°C water bath for 10 min.
5. Add 7 mL of top agarose (boiled to liquefy and then cooled to 49°C) to tubes, and pour immediately on top of prewarmed 150-mm LB plates.
6. Let the plates stand at room temperature for approx 10 min or until the top agarose has hardened.
7. Invert plates and incubate at 37°C overnight.
8. On the following day, remove the plates from the incubator, and either proceed with lifting as indicated below or store at 4°C.

3.1.3. Plaque Lifts to Filters

1. Label the nitrocellulose filters on one side with a waterproof marker.
2. Cut sheets of 3MM paper to fit plastic trays.

3. Place the sheets of 3MM paper in the plastic trays, and saturate the paper with the denaturing solution and the neutralization solution separately. Roll out the bubbles.
4. Fill two tubs with approx 500 mL 2X SSC.
5. Prepare a marking syringe: fill a 3–5 cc syringe with waterproof black india ink. Attach a 21-gage needle.
6. Begin to lift plaques from the plates onto the filters. Place the plates on the bench with the agar side down. Remove lids. Transfer appropriately numbered filter to the plate using blunt forceps with the numbered side up. Take care to center filters as they are applied. Let filter wet by capillary action. Once on the plates, do not move the filter. Then, stab each filter with ink syringe three to four times, marking both filter and bacterial plate. Make sure syringe is held vertically and let syringe dispense ink by capillary action. Leave the filters on the plates for 2 min for the primary lift and at least 4 min for the secondary lift (*see* **Note 4**). When marking the secondary filters, be sure to mark in the same places as the primary filters.
7. Transfer the filters to the 3MM paper saturated with the denaturing solution with the plaque side up. Do not overlap filters. Leave the filters on the denaturing solution for 10–15 min. Lift each up after about 5 min, and reposition to make sure no air bubbles are trapped.
8. Transfer the filters to the 3MM paper saturated with the neutralizing solution with the plaque side up. Leave the filters on the neutralizing solution for 10 min. Lift each up after about 5 min and reposition to make sure no air bubbles are trapped.
9. Transfer the filters to a tub with 500 mL 2X SSC and incubate for 5 min. Transfer to a second tub with 500 mL 2X SSC and incubate for 5 min. Transfer to a tub containing water and incubate for 3–5 min.
10. Dry the filters between two sheets of 3MM paper.
11. Crosslink the DNA to the filters for 30 s using autocrosslink setting on the Stratalinker UV crosslinker. Store the filters between two sheets of 3MM paper at room temperature until ready to use.
12. Store the agar plates at 4°C.

3.1.4. Labeling and Purification of cDNA Probe

1. The cDNA containing sequences from a previously known adhesion molecule (i.e., the probe that will be used to screen the cDNA library) should be labeled using a Random Primers DNA Labeling System following the manufacturer's instructions (*see* **Note 5**).
2. Purify the labeled fragment using a QuickSpin Column following the manufacturer's instructions.

3.1.5. Prehybridization, Hybridization, and Washing of Filters

1. Place the filters between two sheets of nylon mesh, and put them in a hybridization bottle. In order to prehybridize, add 25 mL of hybridization solution. Rotate the bottles at 60°C for 1–2 h in a hybridization oven.

2. While the filters are prehybridizing heat both your labeled, purified probe and an aliquot of sheared sonicated salmon sperm DNA (the final concentration of salmon sperm DNA in your probe should be 200 ng/mL) in 1.5 mL screw cap eppendorfs at 95°C for 5 min.
3. Place both the probe and salmon sperm DNA on ice after boiling.
4. Add the labeled probe and salmon sperm DNA to a 25 mL aliquot of hybridization solution (make sure that the hybridization solution has been prewarmed and that the SDS is in solution).
5. Dump the prehybridization solution from the filters.
6. Add the labeled probe solution to the filters.
7. Incubate overnight in the hybridization oven (*see* **Note 6**).
8. Remove the probe from the filters. The probe may be stored at –20°C and reused (*see* **Note 6**).
9. Wash the filters two times with 50 mL of wash solution #1 at 60°C for 15 min each time. The washes will be radioactive. Therefore, they should be disposed of in radioactivity containers (*see* **Note 5**).
10. Wash the filters two times with 2 L of wash solution #2 in a 65°C shaking water bath for 30 min each time. These washes can be carried out with the filters in Tupperware containers that are well sealed to prevent leaking of the radioactive wash solution into the water bath.
11. The filters and the wash solutions should be monitored with a Geiger counter in between each wash to make sure that the level of radioactivity is decreasing after each wash.
12. When the level of radioactivity is no longer decreasing, blot the filters briefly on 3MM paper to remove excess liquid (do not let filters dry completely), and then wrap them in plastic wrap. Attach Glogos Autorad markers to the Saran Wrap (be sure not to cover any of the filters). This will help in aligning the film after development.
13. Place the filters in an autoradiography cassette (the marked sides of the filters should face the film), and expose to film overnight at –70°C with an intensifying screen.
14. Develop film.
15. Align the developed film with filters and the Glogos Autorad markers and mark the film indicating where the ink marks are on each filter (*see* **Note 7**).
16. Positive clones will appear as a black dot on the film (*see* **Note 8**).
17. To select the positive bacteriophage, line up the original bacteria/phage plate with the film (which has been marked with the ink spots from the filters). Line up the ink marking on the plate with the ink markings on the film for each plate exactly (*see* **Note 7**). Push the wide end of a sterile Pasteur pipet (0.5–0.6 cm diameter) (it is also possible to use the wide end of a pipet tip for a P1000 Pipetman) into the bacteria/phage plate over the hybridizing plaque (which is visible on the film), place your finger over the other end of the pipet, slowly remove the plug, and place it in 500 µL of PDB. Store the phage at 4°C until ready for use.
18. To isolate an individual plaque, repeat each step of the procedure described above starting from **Subheading 3.1.1.** Use the isolated plaque to plate enough phage to generate single plaques on the bacterial plates.

3.2. Polymerase Chain Reaction (PCR)

1. The clones are amplified from the isolated bacteriophage plaque by PCR in a DNA thermal cycler 480 (Perkin Elmer). Ten microliters out of 500 µL of each phage preparation are used for PCR amplification.
2. The amplification is performed in 1X *Taq* polymerase buffer, 100 µM each of dATP, dCTP, dGTP, and dTTP, 0.1 µ*M* of each primer and 0.25 U of *Taq* DNA polymerase. The final concentration of $MgCl_2$ ranges between 1 and 4 m*M*. Optimization of the $MgCl_2$ concentration is required in each case. The final volume is 50 µL (*see* **Note 9**). Overlay each sample with 50 µL mineral oil to prevent evaporation.
3. The phage coat is denatured, and the DNA is released by heating the reactions at 95°C for 4 min prior to amplification. The specific primers (20- or 22-mers) are synthesized based on the known cDNA sequence used as a probe in the library screen. The conditions used in the PCR are: denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1.5 min. Twenty-five cycles of amplification are used (*see* **Note 10**).
4. The products are electrophoresed on a 1.5% agarose gel, and the DNA is visualized with ethidium bromide (*see* **Note 11**).
5. The amplified products are subcloned by blunt-end ligation using the TA cloning kit following the manufacturer's instructions (*see* **Note 12**). The ligation product is used to transform competent bacteria (provided by the manufacturer), and isolate individual colonies for further analysis by plasmid isolation and sequencing.
6. Small-scale preparations of plasmid DNA can be obtained using the Wizard minipreps DNA purification system according to the manufacturer's instructions. These plasmid DNA preparations generate a sufficient amount of DNA, which allows the investigator to sequence the subcloned PCR fragment.
7. Restriction analysis using enzymes whose recognition sequences are present in the TA Cloning vector is performed to confirm that the ligation has been successful.
8. Finally, the plasmid DNAs that contain the insert are sequenced using the Sequenase Version 2.0 DNA Sequencing Kit (according to the manufacturer's instructions) in order to confirm the presence of a new sequence in the subcloned PCR fragment.

4. Notes

1. Probing fragments can be generated by PCR using the "3.2 PCR Method" described in this chapter. PCR products can be used to screen either genomic or cDNA libraries ***(2–5,14)*** . Additionally, degenerate oligonucleotides can also be used to screen cDNA libraries directly ***(9–11,15)*** .
2. cDNA libraries are prepared using mRNA (0.05–0.1 mg) obtained from tissues, cultured primary cells or cell lines, or blood cells. TRIzol from Life Technologies can be used successfully for total RNA extraction from frozen tissue (TRIzol is toxic if it comes in contact with skin or if it is swallowed). The use of guanidium thiocyanate and cesium chloride is an alternative procedure to prepare high-quality total RNA ***(14)***. The mRNA can then be extracted using the oligo(dT)

chromatography technique ***(14)***. Alternatively, cDNA libraries are commercially available (Stratagene, Clontech, and others).

3. The choice of the bacterial host is dependent on what type of library is being screened. Most commercially available libraries will provide the bacterial host as well as the growth conditions. Additionally, not all bacteria require LB plates for growth. However, these conditions will also be supplied by the manufacturer.
4. Secondary filters are not necessary for library screening. However, they are highly recommended. They are useful to verify that a positive is real. If a plaque is hybridizing with both the primary and secondary filter, the chances that the positive is real are increased.
5. Because labeling of the probe requires handling of radioactive substances, all steps from here until the isolation of the phage should be carried out following all necessary precautions, i.e., lab coat, gloves, goggles, monitoring of work area, and of investigators with a geiger counter. Additionally, all waste generated should be disposed of in radioactive containers.
6. The hybridization temperature must be chosen based on the length and nucleotide composition of the probe. Low hybridization temperatures can be used to decrease the stringency of the hybridization conditions. In order to reuse a labeled probe, it should be thawed at 60°C in a water bath taking care that the tube does not submerge (put parafilm around the top of the tube). Allow the tube to remain at 60°C for at least 10–15 min after it has thawed, and then add the probe to filters for overnight hybridization as described in **Subheading 3.1.5.**
7. It is very important that the ink marks on the filters be marked precisely on the film. The film will be used as a "blueprint" of the filters to select the positive phage. It is also crucial that the film be lined up exactly with the bacteria/phage plate in order to pick the correct phage that is hybridizing with the probe. It is easier to see the markings on the film and on the plate when performing this on a light box.
8. If at this point there is high background on the filters, additional washes in more stringent conditions are performed using 2 L of wash solution #3 in a 65°C shaking water bath for 30 min. If no positive clones are detected on the film, either rehybridize at a lower temperature or wash the filters after hybridization less stringently.
9. All procedures are carried out on ice. This is to preserve the activity of the stock solutions of *Taq* polymerase and of the primers.
10. The annealing temperature is critical for successful PCR. This will have to be determined based on the length and the sequence of the primers. The recommended annealing temperature for nondegenerate primers (20–22 nucleotides in length) is 55–60°C.
11. To confirm that the newly identified cDNA is not an artifact resulting from the presence of nonmature (pre-mRNA) mRNA in the original library, several controls are required. The expression of the newly identified family member should be examined at the RNA and protein levels using cells or tissues.
12. Alternatively, the PCR products can be amplified using primers containing restriction sites at their 5' end for use in directed cloning. In this case, the final PCR products can

be subcloned into any vector suitable for sequencing or other purposes of interest to the project, providing the vector contains the selected restriction sites.

13. The sensitivity of this method is strictly dependent on the amount and stability of RNA expressed by the selected cell/tissue type used to generate the cDNA library.
14. The time scale for this technique can be approximated as follows: 1–2 wk for the library screening; 1 d for the PCR analysis; 1 wk for subcloning the PCR product in the TA vector; 1–2 d for each round of sequencing.
15. Recently, *in situ* PCR has been successfully performed ***(16)*** to localize rare sequences of DNA and RNA within a heterogeneous cell population in nondisrupted cells and tissues. This procedure can also be used to localize selected DNA sequences from formalin-fixed, paraffin-embedded tissues. This technique requires optimization, especially for determination of the magnesium ion concentration and the cycling parameters. Although the *in situ* PCR is in the early stages of development, the technique is very promising for diagnostic purposes. This indicates that cloning from pathological tissues might be performed in the near future.

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7

Construction and Purification of Adhesion Molecule Immunoglobulin Chimeric Proteins

Thomas Moll and Dietmar Vestweber

1. Introduction

Over the past few years, soluble forms of adhesion molecules and cell-surface proteins in general have become widely used tools, not only in the study of protein–protein interactions, but also as affinity probes to identify novel ligands for a given cell-surface molecule. Particularly useful in this respect have been soluble forms of proteins created by fusing the extracellular domains of the protein of interest to the Fc part of an antibody molecule ***(1)***. The resulting dimeric proteins combine many of the functional characteristics of the adhesion molecule of interest with properties of the Fc part of immunoglobulins, such as, e.g., its interaction with protein A or G. This technique has been exploited extensively, as for example for the identification and purification of ligands to all three known selectins, including the L-selectin ligands GlyCAM-1 and CD34, the E-selectin ligand ESL-1, and finally the P-selectin ligand PSGL-1 ***(2–6)***. In addition, such adhesion molecule–Ig chimeras have been useful in the functional characterization of known receptor–ligand interactions in a wide variety of experimental settings.

In broad terms, adhesion molecule–Ig chimeras are constructed by cloning the cDNA encoding the extracellular portion of the protein into a mammalian expression vector. This vector should contain an antibiotic (e.g., neomycin) resistance gene and part of the genomic sequence of the human IgG_1 heavy-chain gene. Most conveniently, the extracellular domain is placed into the intron immediately preceding the exon coding for the Ig hinge region, which is then followed by the exons for the heavy-chain CH2 and CH3 domains. The resulting plasmids are stably transfected into secretory cells as, e.g., CHO cells, and positive clones selected by enzyme-linked immunosorbent assays (ELISA).

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To do this, the culture supernatants of neomycin-resistant CHO clones are tested for the presence of human IgG Fc domains. After subcloning of positive clones by limiting dilution, adhesion molecule–Ig chimeras can then be produced in virtually unlimited amounts. Similarly to immunoglobulins, the Ig chimeras can be easily purified from cell culture supernatants by using, e.g., protein A Sepharose. Typically one obtains 0.1–1 mg of protein/litre of cell-culture supernatant (the yields may be significantly higher when using biofermenters), and the method therefore represents a true alternative to produce large amounts of recombinant proteins in a higher eukaryotic cellular system.

2. Materials

2.1. Construction of Adhesion Molecule–Ig Chimeras

1. This involves standard molecular biology methodology, reagents, and equipment, and has been described extensively *(7,8)*.
2. We are not aware of human IgG_1 genomic DNA being commercially available, but nevertheless, the gene is widely distributed among laboratories.
3. Polymerase for PCR reactions: cloned *PFU* polymerase (Stratagene Inc.), in conjunction with reaction buffer supplied with the enzyme.

2.2. Electroporation of Cells

1. Cell-culture medium: CHO(dhfr$^-$) cells are grown in MEM α medium with nucleotides and supplemented with 2.2 g/L $NaHCO_3$, 10% fetal calf serum, 2 m*M* L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin (*see* **Note 1**). Cells are incubated in a CO_2 incubator at 37°C and 10% CO_2 on Falcon tissue-culture plates.
2. Phosphate-buffered saline (PBS) is 8 g/L NaCl, 0.2 g/L KCl, 1.15 g/L NaH_2PO_4, 0.2 g/L KH_2PO_4.
3. Trypsinizing of cells: 0.05% trypsin, 0.02% EDTA (w/v) in PBS. The solution is stored at –20°C long term and at 4°C short term.
4. Bio-Rad (CA) Gene Pulser with capacitance extender and eukaryotic 0.4 cm Bio-Rad gene pulser cuvets.
5. Antibiotic resistance selection (G418): geneticin (=G418-sulfate; Gibco BRL, Eggenstein, Germany) is dissolved in ddH_2O (sterile) at 250 mg/mL and stored in aliquots at –20°C. This solution is fairly acidic, but the slight change in pH owing to its addition to cell-culture growth medium will not by itself affect the proliferation of CHO cells.

2.3. Harvest and Culture of Transfectant Clones

1. 5 mm Raschig rings (Sigma-Aldrich Techware, St. Louis, MO); autoclave before use.
2. Chemically inert and autoclavable laboratory grease (high vacuum quality); usually this type of grease is sold as accessory equipment for centrifuges (e.g., Glisseal, Borer chemie, Switzerland). Autoclave before use.

2.4. Selection (ELISA) and Subcloning

1. 96-Well MaxiSorp Immuno plates (Nunc GmbH, Wiesbaden, Germany) for ELISA.
2. Affinipure F(ab')$_2$ rabbit antihuman IgG fragments, Fc-specific (Dianova, Hamburg, Germany); peroxidase-conjugated affinipure F(ab')$_2$ goat antihuman IgG fragments (Dianova).
3. PBS, 0.1% Tween-20; PBS, 10% fetal calf serum; 3 *M* hydrochloric acid.
4. Peroxidase substrate reaction mix: 0.1 *M* citrate, 0.2 *M* K_2HPO_4, store stock solutions at room temperature; *o*-phenylendiamindihydrochloride (OPD, Sigma); hydrogen peroxide (30%). The reaction mix should always be freshly prepared: Mix 25 mL 0.1 *M* citrate, 25 mL 0.2 *M* K_2HPO_4 and 50 mL ddH_2O; add 30 mg OPD and dissolve in the dark. Immediately before use, add 40 µL hydrogen peroxide, mix thoroughly.
5. ELISA reader for 96-well plates equipped with a 492 nm filter.

2.5. Purification of Chimeric Proteins

1. 0.22- and 0.45-µm sterile filtration membranes (Millipore).
2. Wash buffer: 20 m*M* Tris-HCl, pH 8.5, 130 m*M* NaCl, 0.04% NaN_3.
3. Protein A Sepharose (Pharmacia, Uppsala, Sweden) is swollen in wash buffer, washed extensively in this buffer (until the supernatant is no longer acidic), and stored in this buffer as a 50% (v/v) slurry at 4°C.
4. Empty 1 mL chromatography columns; peristaltic pump.
5. 0.1 *M* glycine/HCl, pH 2.5, 0.04% NaN_3; 1 *M* Tris-HCl, pH 9.5.

3. Methods

3.1. Construction of Adhesion Molecule-Ig Chimeras

1. Clone the genomic sequences of the human IgG_1 heavy-chain gene, containing the intron with the splice acceptor site preceding the hinge region as well as the CH2 and CH3 domains, into the multiple cloning site of a mammalian expression vector. This vector should carry a strong promoter (as, e.g., a CMV or a SV40 promoter) as well as ideally an antibiotic resistance gene (as, e.g., a neomycin resistance gene) (*see* **Note 2**).
2. Generate by PCR the DNA fragment coding for the extracellular domain of the protein of interest. For this purpose, primers with suitable restriction sites should be used, and the 3'-primer should be designed such that it contains an artificial splice donor site. Such an artificial splice site could, e.g., look like this: . . . *N'NN*A'G↓GTAAGT*NNN* . . ., where splicing occurs after the first AG (↓), and A is the last base of the last codon of the extracellular protein domain, and G is the first base of the first codon of the following exon encoding for the Fc hinge region (*see* **Note 3**).
3. Clone the produced PCR fragment into the mammalian expression vector, upstream (5') of the Ig Fc part. Since in this type of construct the extracellular domains and the Ig Fc domains are combined by splicing, the two parts of the chimeric gene do not have to be in frame with respect to their individual open reading frames.

4. Check the construct by sequencing, and prepare plasmid DNA according to standard procedures (*see* **Note 4**).

3.2. Electroporation of Cells

1. Culture CHO cells in αMEM with 10% fetal calf serum (*see* **Subheading 2.** and **Note 1**). Each individual electroporation is carried out with 1×10^7 cells.
2. Remove cells from the tissue-culture plates by mild trypsinization for 2–3 min at 37°C (*see* **Subheading 2.**).
3. Rinse cells twice with ice-cold phosphate-buffered saline (PBS) and finally resuspend the cells in 0.6 mL.
4. Add 20 µg of the plasmid DNA to be transfected to the cells and incubate on ice for 10 min.
5. Transfer the cells to an eukaryotic 0.4 cm electroporation cuvet, and electroporate cells at 0.25 kV and 960 µF (*see* **Note 5**).
6. Transfer cells to 10 mL prewarmed (37°C) cell-culture medium, and split cells onto 100 mm tissue-culture plates (*see* **Note 6**). Usually cells are seeded at varying densities in between 2.5 and 10×10^4 cells/mL, and incubated at 37°C.
7. After 24 h, add G418 to a final concentration of 0.8 mg/mL.
8. After another 72 h, change the cell-culture medium, and read G418 to a final concentration of 0.8 mg/mL. This may have to be repeated one or two more times until single clonal colonies become apparent by eye.

3.3. Harvest and Culture of Transfectant Clones

Once individual colonies are easily detected (with a diameter of 1–2 mm), these colonies are individually harvested and subcultured:

1. Remove cell-culture medium, and rinse plates once with PBS.
2. Dip one side of an individual Raschig ring (basically a glass cylinder, 5 mm in diameter and approx 5 mm high) in grease (*see* **Subheading 2.**) and lay down on tissue-culture plate with the greasy side down, such that an individual CHO colony is effectively "sealed" from the rest of the plate (*see* **Note 7**).
3. Trypsinize the individual colonies by adding 50–100 µL of trypsin/EDTA solution to the inside of the Raschig rings and incubating for 2–3 min at 37°C.
4. Pipet off the cells and seed the individual colonies onto 24-well plates that contain 1 mL cell-culture medium supplemented with 0.8 mg/mL G418.
5. Once the 24-wells are confluent, individual clones are tested for the production of the adhesion molecule–Ig chimeric protein by ELISA.

3.4. Selection (ELISA) and subcloning

1. Coat 96-well ELISA plates with 0.1 mL rabbit anti-human IgG (5 µg/mL in PBS) for 1h at 37°C.
2. Rinse wells three times with PBS, and subsequently incubate with 0.2 mL PBS and 10% fetal calf serum for 30–60 min at 37°C (*see* **Note 8**).
3. Rinse wells once with PBS, and add 0.1 mL of the cell-culture supernatants from the transfectants. Incubate at room temperature for 1 h.

4. Rinse wells four times with PBS, 0.1% Tween-20, and another two times with PBS.
5. Incubate with 0.1 mL horseradish peroxidase-coupled secondary goat antihuman IgG (1:5000 in PBS, 10% fetal calf serum) for 30–60 min at room temperature.
6. Rinse wells four times with PBS, 0.1% Tween-20, and another two times with PBS.
7. Develop by adding 0.1 mL horseradish peroxidase substrate reaction solution (*see* **Subheading 2.**). After a few minutes, an orange staining should become apparent in the positive wells, and the reaction is then stopped by adding 50 µL of 3 *M* hydrochloric acid.
8. The results can be quantified by using an ELISA reader at 492 nm (*see* **Note 9**).
9. Positive transfectants are subsequently subcloned by limiting dilution: To do this, positive colonies are harvested and replated onto three to four 96-well plates at approx 0.3 cells/well and cultured in cell-culture medium in the presence of 0.8 mg/mL G418. Once the single clones are confluent, the respective cell-culture supernatants are again tested for the production of Ig chimeras by repeating the above-described construct ELISA (*see* **Note 10**).

3.5. Purification of Chimeric Proteins

1. Grow up large-scale cell cultures of selected subclones expressing the fusion protein of interest (*see* **Note 11**).
2. Collect the cell-culture supernatants after the cells have reached confluence.
3. Centrifuge the supernatants to remove leftover cells (4500*g* for 12–15 min). Subsequently, sterile-filtrate the medium containing the Ig chimeras, first through a 0.45 µm and subsequently through a 0.2 µm filter membrane (*see* **Note 12**). If possible, the culture supernatants should be concentrated by ultrafiltration (*see* **Note 13**).
4. Add 0.4 mL of protein A Sepharose slurry (50% v/v in wash buffer; *see* **Subheading 2.5.3.**)/L of supernatant (or the respective volume of concentrated supernatant), and incubate with slight agitation, e.g., by end-over-end rolling, overnight at 4°C. All subsequent steps should be performed at 4°C.
5. Collect the protein A Separose beads by centrifugation, and transfer to an empty chromatography column.
6. Wash the loaded column extensively with approx 100–150 mL of wash buffer. Most conveniently, this is done with a peristaltic (or FPLC) pump at a flow rate of 1.0–2.0 mL/min.
7. Elute bound Ig-chimera proteins at a flow rate of 1.0–2.0 mL/min with 0.1 *M* glycine/HCl, pH 2.5, 0.04% NaN_3 in 5 × 1 mL fractions, followed by 5 × 1 mL of wash buffer. The pH in the glycine containing fractions is immediately neutralized with 60 µL 1 *M* Tris-HCl, pH 9.5 (*see* **Note 14**).
8. Determine the protein concentration in the fractions eluted (*see* **Note 15**). The identity and concentration of the proteins eluted should be confirmed by SDS-PAGE and Coomassie brillant blue staining, since preparations are usually not completely free of immunoglobulins stemming from the fetal calf serum. For comparison, a defined amount of bovine serum albumine (BSA) can be separated on the same gel.
9. Aliquots of the purified adhesion molecule–Ig chimeric protein should be snap-frozen and stored at –80°C.

4. Notes

1. The cell-culture medium given is suitable for CHO(dhfr$^-$) strains and may vary with different CHO strains. In addition to CHO cells, the mouse myeloma cell line J558L has also been successfully used to produce soluble Ig chimeras ***(1)***.
2. The Fc part of the human IgG_1 molecule (***9,10***; EMBL accession number J00228) was chosen because it binds well to protein A Sepharose, and the resulting chimeric proteins can therefore easily be purified. If the expression vector by itself does not contain an antibiotic resistance gene, the stable transfection can still be carried out by cotransfecting a vector carrying this gene.
3. For the PCR, preference may be given to *PFU* polymerase as compared to *Taq* polymerase. The yields of amplified DNA may be lower, but this enzyme has a 3'-5' exonuclease proofreading activity, and in our hands, the fidelity of this polymerase is quite superior to the one of *Taq* polymerase.
4. We find that for most purposes, including transient or stable transfections, CsCl-purified DNA is not required, but that the quality of, e.g., standard Qiagen maxiprep DNA is sufficient for this DNA to be used directly in transfection experiments (Qiagen, CA).
5. The values given work well for CHO as well as J558L cells, but may differ for other cell types and may have to be empirically determined.
6. As a consequence of the electroporation, a fairly large number of cells will be lysed and the DNA released. Therefore, clumps may become apparent in the cell suspension. These should not be pipeted onto the cell-culture plates.
7. Once the PBS has been removed, care should be taken that the cells do not become dry, so the Raschig rings and the trypsin solution should be added with as little delay as possible. With some practice, usually up to 8 or 10 colonies can thus be picked from a single plate. We typically pick 48 colonies (two 24-well plates)/transfection, which is usually sufficient to obtain several good producers.
8. The incubations in steps 1 and 2 may as well be performed at 4°C overnight.
9. The color reaction is light-sensitive, and even after the reaction has been stopped, the 96-well plates should be kept in the dark and the results quantified immediately.
10. CHO cells grow well as single cells in standard cell-culture medium without any further supplements or feeder cells. After 4 to 5 d of incubation, the clonal growth of the cells in the individual wells should be checked, and confirmed by microscopic examination.
11. The way to do this will vary with the laboratory equipment available. In principle, CHO cells are very robust and any methodology suitable to grow up large numbers of, e.g., antibody-producing hybridomas should be applicable, like, e.g., the use of roller bottles. Alternatively, of course, considerable amounts of cell-culture supernatants can also be obtained simply by using 150 mm cell-culture petri dishes as required. The equipment mentioned as well as buffer volumes and others in **Subheading 3.5.** are for the purification of Ig chimera from approx 0.5–2.0 L of cell-culture supernatants.
12. In principle the supernatants containing the Ig chimeric protein can be stored at 4°C at this stage for several days or even weeks. The actual stability of the respective chimera will vary though and should be individually tested.

13. For large volumes of chimera-containing supernatants, it may be useful to concentrate these supernatants prior to the incubation with protein A Sepharose by using some sort of ultrafiltration device (e.g., from Amicon, MA, or Filtron, MA). Since the protein A Sepharose incubation represents an affinity-purification step, the concentration step is optional, but strongly recommended. Given the large volumes of medium vs relatively low amounts of beads, collecting the protein A Sepharose from nonconcentrated medium is somewhat tricky and yields may be low. To avoid an excessive loss of beads, the bead-containing medium should be centrifuged in 50 mL Falcon tubes at 700*g* for 5 min and most of the supernatant discarded. At this point, not too many protein A Sepharose beads may be apparant, but they will be at the bottom of the tubes. The leftover slurry should be combined and centrifuged again at 700*g* for 5 min.
14. The fastest way of doing this is by adding the Tris-HCl, pH 9.5, to the respective collection tubes prior to the glycine elution. The protein A Sepharose can be reused repeatedly to purify Ig chimeras. Nevertheless, to avoid cross-contamination, the same batch of sepharose should not be used for different Ig chimeras. When not in use, the Sepharose can be stored in wash buffer at 4°C.
15. The simplest means of doing this is measuring the UV absorbance of the fractions at 280 nm. Since the proteins mesured are Ig-like molecules, 1.35 A_{280} will roughly correspond to a protein concentration of 1 mg/mL. The yields of protein obtained will vary considerably. This is not only owing to different transfected clones producing different amounts of protein, but is also strongly influenced by the nature of the respective Ig chimeric protein.

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8

Immunofluorescence of Cultured Cells

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1. Introduction

The development and the extensive use of immunofluorescence microscopy on cultured cells has greatly improved the knowledge of cell structure. Immunofluorescence microscopy has become a major tool that any cell and molecular biologist cannot afford to ignore.

The technique is easy and rapid, giving easily interpreted information even when applied by scientists with limited experience in microscopy. However, it may become a powerful tool when the microscopical observation is done by experienced morphologists. Unfortunately, experience in observation is not easily achieved, and years of dedicated attention at the microscope eyepiece are required to obtain the most from a single slide.

Immunofluorescence is based on the recognition of an antigen operated by an antibody solution on substratum-attached cultured cells. First a distinction should be made between (1) direct immunofluorescence microscopy and (2) indirect immunofluorescence microscopy. In the first case, the antibody is directly coupled to a fluorochrome, whereas in the second, the antibody is revealed by a secondary antibody that has been previously tagged with a fluorochrome. The latter technique is now almost universally adopted because the fluorescent signal is greatly enhanced.

To be successfully used, immunofluorescence requires three major features:

1. An antibody with high affinity for its antigen;
2. A protocol that optimizes the rate of interaction of the antibody with its antigen; and
3. An excellent microscope provided with narrow bandwidth illumination through the lens obtained by appropriate filters (epifluorescence microscope).

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Finally, pictures should be taken on films designed to provide high sensitivity, good resolution, and appropriate contrast.

The characteristics of the optimal immunofluorescence antibody cannot be dealt in detail in this chapter. A good antibody should be used at Ig concentrations of or below 10 μg/mL. At higher Ig concentrations, unspecific Ig binding could blur the specific binding sites, and render the microscopic image difficult or impossible to interpret. It is almost useless to say that any antibody tested in immunofluorescence should go side by side with the appropriate control. When using monoclonal antibodies (MAbs), the control should be provided by an irrelevant antibody of the same Ig subclass; with polyclonal sera or purified polyclonal Igs, the control may be provided by the relevant reagent obtained from the same animal prior to immunization (preimmune serum) or by a pooled reagent obtained from the nonimmunized animals of the same species. When the antigen used for immunization is available in pure form, as in the case of synthetic peptides, an excellent control is provided by serum aliquots preadsorbed with an excess antigen.

Immunofluorescence protocols largely depend on which cellular structure one should visualize. For example, to visualize a membrane antigen exposed on the outer aspect of the cell, cells may be exposed to the antibody and then fixed before exposure to the secondary fluorochrome-tagged antibody. Alternatively, a very mild formaldehyde fixation should precede exposure to the antibody to prevent possible endocytosis of the Ig. Prevention of endocytosis may be also obtained by incubation at +4°C; however, in the latter case, the cell is not likely to keep its original shape because of cytoskeletal damage.

The visualization of internal antigens, either nuclear or cytoplasmic, requires permeabilization of the plasma membrane to gain access to large hydrophylic Ig molecules. Permeabilization can be obtained in different ways by different organic solvents or by different detergents. In this chapter, only the protocols that are in use in the laboratory will be reported. Readers who have different requirements may consult specialized books ***(1)***.

One further major feature of immunofluorescence is that it may visualize two antigens within the same cell. This potential may be achieved by using Igs of different subclasses when dealing with MAbs or by a combination of a mouse MAb and a polyclonal antibody raised in a different animal followed by secondary antibodies tagged with different fluorochromes. The results of double-label immunofluorescence are usually excellent provided some simple rules are observed. Finally, in the authors' laboratory, even when we are visualizing a single antigen, we stain cells with labeled phalloidin that ligates F-actin with very high affinity: this facilitates the focusing of the fluorescent image and gives important information on the cell localization of the antigen under scrutiny. This approach has been widely used in human endothelial cells ***(2)***.

Immunofluorescence procedures represent powerful tools in cell biology provided simple rules are observed and protocols carefully followed, exactly as any scientist would do with a complex molecular biological procedure. One word of caution should be given here about washing procedures that should be accurate enough to get rid of any unspecific antibody binding, since the ultimate goal is to obtain the highest possible signal/background ratio.

2. Materials and Preparative Work

1. Any 24-well tissue-culture plates.
2. Glass coverslips 10–12 mm diameter (*see* **Note 1**). Number 5 Dumont type watchmaker's tweezers (available at any hardware shop) are used to handle coverslips.
3. Standard microscope slides (*see* **Note 2**).
4. Automatic pipets of any type (the authors use Gilson).
5. Phosphate-buffered saline (PBS).
6. PBS supplemented with bovine serum albumin: (PBS—0.2% BSA, PBS—2% BSA; *see* **Note 3**).
7. Fresh solution of formaldehyde from paraformaldehyde (electron microscope grade) in PBS adjusted at pH 7.6 containing 2% sucrose (*see* **Note 4**).
8. Permeabilization buffer (HEPES-Triton buffer): 20 m*M* HEPES, 300 m*M* sucrose, 50 m*M* NaCl, 3 m*M* $MgCl_2$, 0.5% Triton X-100, pH 7.4. The solution should be prepared in batch and kept at +4°C.
9. Mounting media: Polyvinyl alcohol (Mowiol, Hoechst, Frankfurt, Germany), 20% in PBS, pH 7.6 containing sodium azide to prevent bacterial growth. Alternatively, glycerol-PBS (1/1, [v/v]; *see* **Note 5**).
10. Microscope observation: Modern epifluorescence microscopes are being continuously developed along with increasing popularity of immunofluorescence. This is not a handbook of microscopy but the reader should be conscious that optimal results can be obtained only with high-power, high-numerical-aperture lenses. Only these lenses offer the sharpness required for interpreting and recording immunofluorescence images.

3. Methods

N.B. This section does not list all the possible different fixation procedures that have been employed in the authors' laboratory in the last years. It just describes the routine procedures that the authors currently use to cover a significant part of the needs encountered in a non specialized laboratory.

3.1. Fixation Procedure

In our laboratory, we currently use formaldehyde fixation, which represents a good compromise for most immunostainings.

1. Add 1.5 mL formaldehyde solution to coverslips sitting at the bottom of culture wells that had previously been deprived of the culture medium. Avoid pipeting

over the coverslip-attached cells to prevent detachment; rather, flow fixative gently along the well wall. Standard fixation time is about 5 min at room temperature (25°C). Avoid fixation at +4°C, which changes cell shape dramatically because of microtubule depolymerization.
2. Remove fixative thoroughly by sucking from tilted wells, and wash 3× with PBS-0.2% BSA (*see* **Note 6**).

3.2. Permeabilization Procedure

1. Put plates on crushed ice, and gently add 1 mL of chilled HEPES-Triton buffer for 3 min.
2. Rapidly remove the permeabilization solution, and wash cells 3× with PBS-BSA/0.2 at room temperature (*see* **Note 7**).

3.3. Fixation-Permeabilization Procedure

1. After sucking the culture medium, remove individual coverslips from wells, and rapidly place them in a clay or metal rack without allowing them to dry out.
2. Immerse the rack in a vessel containing absolute methanol chilled at –20°C, and incubate at this temperature for 5 min.
3. Extract the rack from methanol, drain excess methanol and without warming, immediately soak for about 5 s in absolute acetone chilled at –20°C.
4. Air-dry coverslips in a gentle air stream and directly rehydrate them with the relevant first antibody solution. Incubation and all the following steps follow the protocol described below (*see* **Note 8**).

3.4. First Antibody Incubation

1. Completely remove the washing solution from tilted wells to avoid excess dilution of the antibody.
2. Add the Ig solution onto each coverslip usually in a volume of 10 µL. The antibody drop should be gently smeared on top of the wet coverslip to facilitate diffusion by capillarity.
3. Incubate at 37°C for 30 min in a water- saturated tight plastic box (those used to keep food in a refrigerator do excellent job with some water-soaked paper lining the bottom). Others prefer longer incubation times at room temperature or even at +4°C overnight.
4. Remove the first antibody by washing 3x with PBS–0.2% BSA at room temperature.
5. Add 2 mL of PBS–2% BSA, and incubate for 15 min at 37°C to remove traces of unspecifically bound Ig (*see* **Note 9**).

3.5. Secondary Antibody Incubation

1. Smear the fluorochrome-tagged secondary antibodies on coverslips as described above at a concentration that varies for each batch and depends on how extensively is the commercial reagent labeled.
2. Incubate for 30 min at 37°C.

3. Thoroughly wash 3× with PBS–0.2% BSA at room temperature. We use to employ a 15–30 min incubation at 37°C to remove unspecifically bound secondary antibodies. This step usually gives a clean background.
4. To visualize nuclei, dilute Hoechst 33342 1:3000 in PBS, and incubate for 3 min at room temperature.
5. Wash 3× in PBS (*see* **Note 10**).

3.6. Mounting

1. Remove each coverslip from the well with tweezers, invert it onto a microscope slide, and immerse in a mounting medium. Routinely, we use a drop of Mowiol 4-88 onto which each coverslip, after a brief dipping in distilled water, is mounted, and let dry (*see* **Notes 11** and **12**).

4. Notes

1. Coverslip thickness should be factory-controlled and must not exceed 150 μm, since thicker glass will prevent focusing with high-power lenses. Plastic coverslips (e.g., Thermanox) cannot be used because of their focus-incompatible thickness.
2. Slides with frosted ends are desirable to allow pencil writing. Do not use permanent glass markers, since their inks are immersion oil-soluble and often highly fluorescent.
3. Two types of PBS-BSA are used: one containing 0.2% BSA and one containing 2% BSA. The lower concentration is used for ordinary rinsing, whereas the PBS-BSA/2 is used for intermediate incubations aimed at reducing unspecific antibody binding (*see* **Note 6**).
4. Paraformaldehyde should be dissolved under continuous stirring at 60°C. If some paraformaldehyde remains in suspension, add 5–10 μL of 1 *N* NaOH to 100 mL of paraformaldehyde-PBS-sucrose, and the solution will immediately become clear. Allow the solution to reach room temperature, and adjust pH if required with 1 *N* HCl.
5. The standard mounting medium is polyvinyl alcohol. The solution, which has a final appearance similar to light honey, should be prepared by slight warming (40–50°C) and under stirring. The aliquots are stable for months at room temperature. The great advantage of this mounting medium is that it self-solidifies. It may occasionally be dissolved to unmount a coverslip being water-soluble. If coverslip unmounting is routine (e.g., the coverslip has to be restained after observation) or in special observing conditions (e.g., when interference reflection microscopy or phase contrast is routinely employed along with fluorescence), the mounting medium is glycerol-PBS. In this case, however, no spontaneous solidification occurs, and the coverslip must be fixed with ordinary nail varnish (be careful because the latter products are usually intensely autofluorescent!).
6. When even traces of formaldehyde have to be removed, add 2 mL of PBS-2% BSA, and incubate for 15 min at 37°C. This step should be adopted when background reduction is critical. Fixed coverslips could be kept at +4°C at this stage

for weeks under PBS. Plates may also be shipped to other laboratories after tight double wrapping in Parafilm.

7. When one antigen has to be recognized by antibodies within the cell or at the tightly attached adhesion surface, the hydrophobic membrane has to be partially dissolved to become permeable to large Ig molecules. The extent of membrane permeabilization depends on how long and at which temperature the Triton X-100 detergent soaks the cells. Again, when minute traces of formaldehyde have to be removed also from the cell's inner compartment, add 2 mL of PBS-BSA/2 and incubate for 15 min at 37°C. This step should be adopted only when background signal reduction is very critical.
8. Some cell structures, such as microtubules, do not like the formaldehyde-HEPES-Triton procedure that is not suitable for their correct preservation. In this case and in others that cannot be discussed in detail, formaldehyde fixation and HEPES-Triton permeabilization can be replaced by a combination of organic solvents that dissolve the membrane lipid bilayer and dehydrate cells. The procedures are multiple and adapted to specific needs. Here we just mention a simple one that works with microtubules and intermediate filaments.
9. When two antigens are simultaneously detected, each antibody, obtained from different animals (usually rabbit immune Ig and a mouse MAb), is smeared at the same time on the coverslip (7–10 μL each) and then incubated simultaneously. Make certain that the final concentration of the reagents is corrected for the reciprocal dilution. Incubation and washings take place as above.
10. We prefer to use rhodamine-tagged secondary antibodies when a single antibody is employed in the previous step (*see below*). Obviously, when the previous step involved a mouse and rabbit antibody, coverslips should be reacted with secondary antibodies tagged with either rhodamine or fluorescein. Typically, we use a rhodamine-tagged goat antimouse Ig and a fluorescein-tagged swine or donkey antirabbit Ig (do not use tagged Ig raised in mouse or rabbit for obvious reasons!). The standard of our laboratory for single antibody staining is to add 200 n*M* fluorescein-labeled phalloidin (F-PHD; Sigma) in the secondary incubation step. F-PHD binds F-actin with extremely high affinity and allows a good visualization of the cell shape by highlighting its microfilament cytoskeleton. The strategy of checking cell shape by looking at cytoskeleton often allows the tracing of the topography of the unknown antigen with greater accuracy than simple phascontrast observation. The reason why we choose F-PHD and not rhodamine-tagged PHD (R-PHD) is that the latter has a much stronger signal, which during photography, may blur the usually weaker signal that comes from the first antibody. When required, the nucleus can be visualized by a dye called Hoechst 33342 that binds chromatin. Hoechst requires an UV light source to be visualized and emits a bright blue fluorescence. It seems useless to us to say that each of the above steps should be carefully subjected to a control of the specificity of each reagent. For example, the primary antibodies must be replaced by control nonimmune Ig. Similarly, the specifity of the secondary antibodies should be checked in cells that have not been incubated with any primary reagent.

11. When observations in phase contrast or other optical techniques, like interference reflection microscopy, are expected Mowiol is not a suitable mounting medium and should be replaced by 50% glycerol in PBS or by plain PBS.
12. Slides that have been stained with any immunofluorescence procedure may be stored in trays or boxes for at least 2–3 mo provided they are kept at +4°C in the dark, i.e., wrapped in foil. In our experience, longer storage involves loss of fluorescence intensity, but occasionally, slides that have been correctly stored for 1 yr may still be observed.
13. The observation of immunofluorescence preparations requires a good microscope, a lot of patience, and considerable experience. With patience and experience, immunofluorescence gives information that a superficial observer does not appreciate and does not even see. A careful observer must know what he or she should look at. This is one of the reasons for suggesting staining cells for F-actin routinely, even if the problem is not at all related to the microfilament cytoskeleton.

    Observation must be done sitting on a comfortable seat and in complete darkness; the observer's eye should be adapted to darkness for about 10 min before beginning observation and photography. A weak red light is permitted to fight darkness fear.

    As stated above, high-numerical-aperture lenses should be used to appreciate details of an immunofluorescence staining. Do not use low-power lenses that just provide scattered light spots and not real cells.
14. Good photography is a difficult task in immunofluorescence. It requires the same conditions that are essential for good observation plus some knowledge of photographic tricks that stem from taking pictures of bright objects in a dark field.

    In the last 10 years, there has been a tremendous improvement in the quality of black-and-white and color films. Routinely, we use a very sensitive Kodak T-Max 400 black-and-white panchromatic film that we expose automatically at a 1000 ISO setting using a spot exposure program where the spot of the light meter is centered on a bright object. Experience will teach the photographer to avoid spotting an excessively bright object, and thus underexposing the frame or vice versa, a very dull object that will cause overexposure.

    We combine underexposure of the film (1000 ISO setting for a nominal 400 ISO film) with overdevelopment at a value of 1600 ISO. With modern films, this extreme treatment does not increase the grain of the emulsion significantly and permits good prints up to a magnification of 7×. The advantage of underexposing is that exposure time is short and bleaching of the fluorescence source is minimized.

    The same applies to color films. We use Kodak Ektachrome 1600 films exposed at 1000–1250 ISO and routinely overdeveloped at 1600 ISO. To obtain sharp double or triple exposures, set the exposure at 1250–1600 ISO and develop at 1600 ISO. Prints from slides are usually good up to 6×.

Conventional photography is progressively being replaced by magnetic or optical recording of digitized images obtained by microscope-fitted Charged

Couple Device (CCD) cameras. This approach is not yet widespread, but has the enormous advantage of yielding files that can be elaborated and immediately used for electronic publishing. The description of its technology is outside the scopes of this chapter.

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9

Detection of Adhesion Molecules by Immunohistochemistry on Human and Murine Tissue Sections

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1. Introductrion

1.1. Principles

Immunohistochemistry allows detection of antigenic molecules *in situ* in tissue samples using a standard light microscope. This technique has been used widely in recent years in diagnostic pathology and in research. The large popularity of immunohistochemistry came after the discovery of monoclonal antibodies (MAbs) which made available unlimited amounts of identical antibodies.

The antigen presence is demonstrated through immunoenzymatic techniques, which give a visible reaction product. These procedures were first described in the 1960s, were accomplished by "direct technique" using enzyme-conjugated antibodies, and are mainly based on the activity of horseradish peroxidase (the immunoperoxidase technique) ***(1)***. The fortune of this enzyme derives from the fact that the molecule is easy and inexpensive to purify, is enzymatically stable, and can be detected by a number of cytochemical reactions, which give sharply localized insoluble reaction products. The most common substrates used for peroxidase contain diaminobenzidine and hydrogen peroxide, or aminoethylcarbazole and hydrogen peroxide. More recently, a number of laboratories have used calf intestinal alkaline phosphatase as an alternative enzyme for peroxidase (the immuno-alkaline phosphatase technique). Commonly used substrates for alkaline phosphatase contain naphtol phosphate together with hexazotized New Fuchsin or Fast Red.

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Vehiculation of the enzyme to the site of antigen–antibody reaction is usually achieved using the avidin-biotin complex (ABC) technique ***(2,3)*** or the "unlabeled antibody" method ***(4,5)***. The rationale for ABC technique is the very high affinity of biotin for avidin. The procedure involves three incubation stages prior to addition of substrate. The first step is the addition of the primary antibody to the tissue section followed by repeated washings for removal of unbound antibody; the second step is an incubation with a biotin-labeled secondary antibody directed against immunogloblulins of the primary antibody species; the third step is the addition of preformed complexes of avidin and biotinylated enzyme (peroxidase or alkaline phosphatase), which will strongly bind to biotin present on secondary antibody. The "unlabeled antibody" method was first developed in the late 1960s, and its principle is that the enzyme is bound by an antibody that has been raised against the enzyme ***(6)***. The immune complexes of enzyme and antibody can be linked to the primary antibody by a bridging antibody, provided that the antienzyme antibody has been raised in the same species as the primary antibody. The most widely used of these techniques are the peroxidase/antiperoxidase technique (PAP) and the alkaline phosphatase/antialkaline phosphatase technique (APAAP) ***(6,7)***. Several immunohistochemistry kits are now available that are based on these techniques, or on slight modifications, are easy to use, and give excellent results. Some of them defined as "universal" can be used with primary antibodies of different species ***(8)***. In fact, they contain as secondary antibody a mixture of affinity-purified biotinylated antibodies directed against Igs of different species. Immunohistochemistry kit reagents are designed to minimize preparation time and to reduce the possibility of mistakes.

1.2. Paraffin vs Frozen Sections

Immunohistochemistry can be applied to sections of formalin-fixed, paraffin-embedded material, or to frozen sections of fresh cryopreserved tissues ***(9)***. Paraffin sections have the advantage of a good preservation of tissue morphology, but formalin fixation and the high temperature of paraffin embedding mask or denature most antigenic molecules present in tissues, and thereby restrict the scope of immunohistological analysis ***(10)***. Antigenic reactivity can in some instances be restored by treating paraffin sections with proteolytic enzymes (i.e., trypsin, pronase) ***(11)***. In other instances, antigen retrieval is achieved through preheating of the sections in a microwave oven or in boiling water ***(12)***. Even using these procedures, the number of antigens that can be demonstrated on paraffin sections is much lower than that which can be investigated on frozen sections. When an antigen has more epitopes recognized by different MAbs, as is the case for most proteins, it is possible that at least one of them is

preserved in paraffin-embedded material; paraffin-resistant epitopes are more often present in cytoplasmic proteins or in the cytoplasmic tail of membrane proteins ***(10)***.

Frozen sections of cryopreserved samples of fresh tissues are the best substrate for immunohistochemistry, because most antigens are preserved during the freezing procedure ***(13)***. The quality of tissue morphology in frozen sections is poorer than that of paraffin sections, but providing that some cautions are taken, it is still highly satisfactory. The quality of frozen sections depends on specific property of the tissue, on the use of a proper freezing and storing procedure, and on cryostat cutting. Tissues or organs rich in connective stroma (skin, breast, ovary, muscles), and with a low content of H_2O give the best results; other tissues, such as brain and lymphoid organs, are much more difficult to use. Most morphological alterations in frozen sections are caused by freezing of the water contained in the tissue. Embedding the tissue fragment in cryopreserving substances, and speeding the freezing by dropping the sample in liquid nitrogen reduce greatly the morphological alterations; in addition, optimal preservation of frozen samples is achieved when they are stored at –80°C. Tissue morphology is crucially dependent also on the quality of the section. Optimal sectioning requires a cryostat with an intact blade and a good practice of the operator.

1.3. Interpretation of the Results

The most difficult part of immunohistochemistry is the interpretation of the staining ***(14)***. The best results are obtained when the immunostained sections are interpreted by someone who has good experience in histology, histopathology, and immunohistochemistry. Knowledge of histology and histopathology is necessary for proper identification of immunostained structures. Experience in immunohistochemistry is fundamental for distinguishing artifacts from specific reactions.

1.4. Immunohistochemistry in Murine Tissues

Although the most common application of immunohistochemistry is diagnostic pathology of human tissues, it is becoming increasing popular to use this technique for research purposes in murine experimental models. Good results are much more difficult to achieve in mouse immunohistochemistry for the high content of biotin and alkaline phosphase in mouse tissues, and for the need to use only affinity-purified secondary antibodies, especially when directed against specific immunoglobulin subclasses, to avoid crossreactions within rodent immunoglobulins.

2. Materials

2.1. Formalin Fixation for Paraffin Embedding

1. Optimal fixation is obtained in neutral buffered formalin, 10% v/v in PBS, pH 7.0, or 4% w/v paraformaldehyde in PBS pH 7.0. Standard acid formalin fixation is also good, and it is easier to achieve in a pathology laboratory (10% v/v formalin in water, pH 5.7). Milder fixations are obtained with Bouin's, Zenker's fluid, and B5. These mercuric chloride-containing fixatives have to be removed prior of the application of the antibodies.
2. Fixed tissue specimens are dehydrated in graded alcohols, xylene or xylene substitute, and then included in low-melting-point paraffin wax using a standard Automatic Tissues Processor and a Paraffin Working Station.
3. Sections 3–5 μm thick are cut from the paraffin block using a microtome and are harvested on poly-L-lysine-coated glass slides.
4. For poly-L-lysine coating, slides have to be washed in 0.1% w/v SDS, abundantly rinsed in tap water and then in distilled water, dehydrated in 95% ethanol, air-dried, dipped for 20 min in 1% w/v poly-L-lysine in distilled water, and air-dried at room temperature. Poly-L-lysine coated slides have to be kept in air-tight boxes at 4°C.

2.2. Tissue Freezing and Cryostat Sectioning

1. Cryopreserving compound (OCT, Miles; tissue-freezing medium, Leica Instrument).
2. Cryomolds.
3. Liquid nitrogen for 15–30 s.
4. Poly-L-lysine-coated glass slides.
5. Absolute acetone.

2.3. Immunostaining Reagents

1. Washing solution: TBS: 0.15 *M* NaCl, 0.1 *M* Tris-HCl, pH 7.2–7.6, or PBS: 0.137 *M* NaCl, 2.7 m*M* KCl, 10 m*M* Na_2HPO_4, 1.7 m*M* KH_2PO_4.
2. Blocking solution: Carrier protein (2% v/v serum from the animal source of the bridging antibodies, 1% w/v BSA, or 3% w/v nonfat dry milk diluted in PBS or TBS. (Carrier proteins are usually prepared in x10 concentrated stock solutions in water containing 15 m*M* sodium azide).
3. Primary antibodies and negative control antibodies: Most monoclonal and polyclonal antibodies against adhesion molecules are commercially available; in addition, many specific hybridomas are commercialized by the American Type Culture Collection (ATCC). The working condition of each antibody has to be determined using serial dilutions with a standard staining procedure. In general, MAbs work at a concentration between 250 ng and 1 μg on a cm^2 section, and polyclonal antibodies from 2–100 ng for section. Hybridoma supernatants may be used from undiluted to 20X diluted. The dilutions of purified commercial

MAbs are made in PBS or TBS. The dilutions of monoclonal supernatants and antisera are made in PBS added with carrier proteins. The presence of sodium azide in the antibody working dilution may inhibit the reaction. One hundred µL of the antibody solution are needed for each section.

4. Bridging antibodies are immunoglobulins raised against rabbit, mouse, rat, or hamster immunoglobulins. They are biotinilated and diluted in PBS or TBS without sodium azide. Working dilutions and incubation time are usually reported by the manufacturing house. Concentrations of the bridging antibody vary from 1:20 for affinity-purified antibodies against immunoglobulin subclasses to 1:1000 for antisera. Cover with 100 µL of bridging antibody dilution each section. Incubation time is between 10 and 40 min.
5. ABCs or streptoavidin conjugated with horseradish peroxide or alkaline phosphatase is diluted in TBS. The manufacturer reports the working concentrations and incubation time. The reaction has to be performed in humid chambers. These are commercially available, but lidded boxes bottomed with PBS- or TBS-soaked paper with sustains to keep the slides in a flat position are most commonly used.

2.4. Cromogen Substrates

Most cromogen substrates are potent carcinogens. All the reactions have to be carried under an aspiration hood while wearing gloves.

1. Peroxidase conjugated ABC or streptoavidin is developed in a solution of 0.3% w/v 3,3'-diaminobenzidine 0.6% H_2O_2 in PBS or TBS for 5 min, or in a 1% v/v AEC, 0.6% H_2O_2 in PBS or TBS for 5–10 min.
2. Alkaline phosphatese-conjugated streptoavidine is developed in a cromogen solution of 20 mg of Fast Blue BB salt, Fast Red Violet LB salt, Nitro blue tetrazolium (NBT), or 0.6 mL of 0.2% w/v Neofuchsin dissolved in 0.2 N HCl and hexazotized with an equal volume of 4% w/v sodium nitrate. Each cromogen is dissolved in 50 mL 0.1 *M* Tris-HCl, pH 8.7, solution containing 90 mg of levamisole and 20 mg of Naphtol AS-BI Phosphate freshly dissolved in 0.6 mL of *N*,*N*-dimethylformamide. Bring the pH of the staining solution to 8.7 with veronal buffer; lower pH develops the endogenous acid phosphatase activity.
3. Endogenous alkaline phosphatase activity of endothelial cells is inhibited by levamisole.

2.5. Counterstaining and Mounting

1. Hematoxylin (Mayer's).
2. When DAB chromogen is used, sections can be mounted with a nonaqueous mounting media (Canadian balsam) which is very stable. When alcohol-soluble substrate chromogens are used, such as AEC, Fast Blue, Red Violet, NBT, or neofuchsin, only aqueous mounting media are recommanded to preserve the immunostaining.

3. Methods

3.1. Formalin Fixation for Paraffin Embedding

1. Antigen survival may depend on the type and concentration of the fixative, on fixation time, and on the thickness of the tissue specimen. Whenever possible, use thin specimens and short fixation time. It is important to maintain an optimal standard fixation method in order to obtain reproducible results. Optimal fixation is obtained in neutral buffered formalin, 10% v/v in PBS, pH 7.0, or 4% w/v paraformaldehyde in PBS, pH 7.0. Tissue blocks of approx $1 \times 1 \times 0.5$ cm have to be immediately placed in 5–10 mL formalin, and should not remain in the fixative for longer than 24 h.
2. Fixed tissue specimens are dehydrated in graded alcohols, xylene, or xylene substitute, and then included in low-melting-paraffin wax using a standard Automatic Tissues Processor and a Paraffin Working Station. During the process, the temperature must be kept under 60°C to preserve antigenic properties better. Rapid high-temperature processing destroys the antigens.
3. Sections 3–5 μm thick are cut from the paraffin block using a microtome, are harvested on poly-*L*-lysine-coated glass slides in a cold distilled water bath, and are then distended by dipping the slide in a 60°C water for few seconds.
4. Sections have to be deparaffinized and rehydrated before use in immunohistochemistry. Dewaxing is obtained at room temperature by a 2X repeated 10-min xylene or xylene substitute bath in a glass jar, followed by an absolute ethanol 2X repeated 10-min bath, 95% ethanol 2X repeated 10-min bath, 70% ethanol 2X repeated 10 min bath, and finally 5 min in distilled water and 5 min in PBS where slides have to be kept until use. The dewaxing procedure has to be made under an aspiration hood because of the toxic vapors of xylene.
5. Antigen retrieval may be achieved by protein digestion. Deparaffinized rehydrated sections are wiped for excess of PBS, are covered with 200 μL of a solution containing 0.1 *M* Tris-HCl, pH 7.2–7.6, 0.025% $CaCl_2$, 0.025% Protease Type XXIV for 5 min at room temperature, and then are rinsed abundantly with Tris-HCl.
6. Microwave antigen retrieval is obtained by boiling the tissue slides in a microwave oven in a 0.01 *M* salt solution, pH 6.0. Dewaxed dehydrated sections are placed in a plastic jar filled with 10 m*M* citric acid, pH 6.0. The jar is irradiated 3X for 5 min at 600 W in a microwave processor. The jar has to be refilled after each boiling step. After treatment, sections have to be cooled at room temperature prior to processing for immunohistochemistry. Microwave treatment has the additional advantage of destroying most endogenous enzymatic activities of the tissue. A possible disadvantage is detachment of the sections from the glass slide; special glues are commercially available and should be used for this purpose.

3.2. Freezing of Tissue Samples

1. Cut the tissue sample in pieces not larger than 1×1 cm and not higher than 0.5 cm.
2. Place the slice of tissue in a cryomold of appropriate size, and cover with Optimal Cryopreserving Tissue compound.

3. Place the carrier in a recipient containing liquid nitrogen for 15–30 s (until you can hear a sizzle)
4. Write with a pencil on a small tag of hard paper for identification of the sample.
5. Staple the tag on the carrier containing the frozen sample
6. Store the frozen samples in a –80°C refrigerator (*see* **Note 1**).

3.3. Preparation of Tissue Sections

3.3.1. Pretreatment of Glass Slides

1. Use pretreated slides for immunohistochemistry, or clean the slides with alcohol to remove lipids. Slides have to be washed in 0.1% w/v SDS, abundantly rinsed in tap water and then in distilled water, and finally dehydrated in ETOH (*see* **Note 2**).
2. Dip the slides for 20 min in a 1% w/v poly-*L*-lysine solution in distilled water, air-dry the slides at room temperature, and preserve in air-tight boxes at 4°C. Poly-*L*-lysine-coated slides are specially recommended when antigen retrieval by proteolitic digestion is used (*see* **Note 3**).

3.3.2. Cryostat Sectioning

1. Cryostat sectioning should be done by experienced people because the informative content of the immunostaining is strictly related to the quality of the section.
2. Let the frozen samples reach the cryostat temperature of –20 to –25°C.
3. It is advisable to use disposable blades to optimize cutting conditions.
4. Cut 5–8 µm sections, and place them on poly-*L*-lysine-coated glass slides.
5. Place one section for each slide.
6. Dry the sections at room temperature for at least 2 h or, better, overnight and use them on the following day. Alternatively, wrap each individual slide in aluminum foil and store at –80°C (*see* **Note 4**).

3.3.3. Fixation

1. Dip the slides in absolute acetone in a glass jar with a cover for 10 min at room temperature (*see* **Note 5** and **6**).
2. Air-dry the slides at room temperature.
3. Draw a circle around the section with a glass pencil.
4. Put two pipets in parallel on the top of a basin, one 5 cm apart from the other, or use an incubation chamber for immunohistochemistry (*see* **Note 7**). Place the slides flat on the sticks with the section on the top. Be sure that the basin is in plane in order to avoid incubation liquids sliding out of the section.

3.3.4. Quenching of the Endogenous Peroxidase

It has to be performed after fixation and before the beginning of the immunostaining only when the peroxidase activity of the tissue is very high (inflamed tissues rich in neutrophils, bone marrow sections). It is generally accepted that endogenous peroxidase activity of most cells is lost after 12 h at

room temperature. Apply 200 µL of 3% hydrogen peroxide in PBS on the section at room temperature for 5 min, rinse gently with PBS, and place in fresh PBS for 5 min.

3.4. The Immunohistochemistry Reaction in Human Tissues

3.4.1. The Primary Antibody

1. Determine the working concentration of the primary antibody in preliminary experiments. Store prediluted aliquots at –20°C. Use storage concentration 10- to 20-fold higher than working concentration to bypass possible loss of activity. It can be estimated that 50–100 µL of working dilution are needed for each section.
2. Cover the section with enough solution of the primary antibody to reach the glass pencil circle drawn around the section. Incubation time with primary antibody is generally 30 min.
3. Remove the solution of the primary antibody by gently flushing PBS or TBS on the section using a pipet.

3.4.2. The Development Kit

1. Commercial kits for ABC-peroxidase generally contain:
   a. Blocking solution.
   b. Biotinylated secondary antibody.
   c. Avidin or streptavidin-horseradish peroxidase (HRP) conjugates.
   d. Substrate buffer.
   e. Chromogen dilution.
   f. Hydrogen peroxide.
   g. Hematoxylin solution.
   h. Mounting solution (*see* **Note 8**).
2. Follow the instructions, and apply to the section all the reagents in the proper sequence. Standard incubation times are 30 min for the secondary antibody, 30 min for enzyme conjugate, and 5 min for chromogen/substrate. However, incubation times may be shortened when high-sensitive reagents are used.
3. Wash the sections extensively with PBS using a pipet after each incubation step.
4. Do not let the sections dry during the whole procedure.
5. Dry the slide around the section anytime you start a new incubation. This step is important to avoid that the drops of the new reagent will dilute in the surrounding residual PBS.

3.4.3. Cromogen Substrates

Most cromogen substrates are potent carcinogens. All the reactions have to be carried under an aspiration hood while wearing gloves.

1. Peroxidase-conjugated ABC or streptoavidin is developed in a solution of 0.3% w/v 3,3'-diaminobenzidine 0.6% H_2O_2 in PBS or TBS for 5 min. The reaction gives an alcohol-resistant strong brown color. Alternatively, 1% v/v AEC, 0.6%

H_2O_2 in PBS or TBS for 5–10 min, may be used, which gives an alcohol-soluble brownish red color. The latter procedure is at least 10X less potent than diaminobenzidine. Apply 500 µL of the filtered staining solution over each section, rinse gently with washing solution, then wash for 5 min with distilled water.

2. Alkaline phosphatese-conjugated streptoavidine is developed in a cromogen solution of 20 mg of Fast Blue BB salt, Fast Red Violet LB salt, NBT,or 0.6 mL of 0.2% w/v Neofuchsin dissolved in 0.2 *N* HCl and hexazotized with an equal volume of 4% w/v sodium nitrate. Each cromogen is dissolved in 50 mL 0.1 *M* Tris-HCl, pH 8.7, solution containing 90 mg of levamisole and 20 mg of Naphtol AS-BI phosphate freshly dissolved in 0.6 mL of *N*,*N*-dimethylformamide. Bring the pH of the staining solution to 8.7 with veronal buffer; lower pH develops the endogenous acid phosphatase activity. Endogenous alkaline phosphatase activity of endothelial cells is inhibited by levamisole (*see* **Note 9**). Place 500 µL of filtered staining solution to cover the section and incubate at 37°C. The staining time is temperature-dependent and varies from 5–30 min at 37°C. The reaction color depends on the chosen dye, blue for Fast Blue, brilliant crimson for Fast Red Violet, black granules for NBT, and brown red for neofuchsin. All the reactions are alcohol-soluble.

3.4.4. Counterstaining

1. Immunostained sections are usually counterstained with hematoxylin (Mayer's) for 1–5 min depending on the strength of the hematoxylin used, rinsed in tap water for 5–10 min, or gently washed in distilled water, and dipped 8–10X in 37 m*M* ammonium hydroxyde in distilled water freshly prepared from a 15 *M* ammonium hydroxyde stock solution maintained at room temperature in tightly capped bottle (*see* **Note 10**).
2. Sections have to be covered with a coverslip. When DAB chromogen is used, sections can be dehydrated in ethanol 95%, absolute ethanol, xylene, and mounted with a nonaqueous mounting media (Canadian balsam), which is very stable. When alcohol-soluble substrate chromogens are used, such as AEC, Fast Blue, Red violet, NBT, or neofuchsin, only aqueous mounting media are recommanded to preserve the immunostaining (i.e., 4% v/v glycerol in distilled water). For better preservation, keep the immunostained slides in the dark.

3.4.5. Controls and Interpretation of the Results

1. False-negative results may be owing to technical problems during the procedure ***(14)***, to poor quality of the reagents, or to amounts of antigen under the threshold of detection of the technique. Use as positive controls serial sections of the investigated tissue immunostained for vimentin (the intermediate filament present in mesenchimal cells), CD31, or vWf (endothelial antigens). Since mesenchimal cells and blood vessels are ubiquitous, immunostaining for these antigens will provide information on the state of preservation of the tissue, on proper fixation, and on the technical procedure. Immunostaining of a tissue known to contain the investigated antigen will provide a control for the primary antibody.

2. False-positive results derive from nonspecific binding of the reagents to tissue components and from noninhibited endogenous enzymatic activity (myeloperoxidase, alkaline phosphatase) ***(15,16)***. Recognition of nonspecific binding may sometimes be difficult. Coexistence in the same area of negative and positive cells or structures, colocalization of the staining with a definite biological structure, proper location on the staining within the cell (membrane, cytoplasm, nucleus) according to the specificity of the antibody used, and proper type of reactivity (granular vs diffuse) are all elements that have to be checked for recognizing a reaction as specific. Other elements favoring a specific reaction are the use of the primary antibody in a concentration range commonly employed in immunohistochemistry and the reproducibility of the findings when other samples of the same type of tissue are stained. Nonspecific binding of primary or secondary antibody is owing to poorly characterized mechanisms, or to binding of the reagents to Fc receptors exposed on the section. Both these events are commonly eliminated by a preliminary blocking step in which nonspecific binding sites are saturated with a preincubation of the section with a nonimmune serum. Endogenous peroxidase and alkaline phosphatase activities present in tissue cells can be blocked by a preincubation of the sections with H_2O_2 or levamisole, respectively. A good control for effective blocking of endogenous enzymatic activity is a parallel staining of a serial section in which the primary antibody was omitted.

3.4.6. Immunohistochemistry of Adhesion Proteins

1. Immunohistochemistry is a valuable technique for detection of adhesion proteins in tissue sections. In fact, most selectins, immunoglobulins, integrins, and cadherins have been successfully visualized.
2. Immunohistochemistry can provide reliable information concerning different issues including:
   a. Expression of the investigated molecule in various cell types and in different tissues.
   b. Detection of polarized expression.
   c. Location of the molecule at cellular level (cell membrane, cytoplasm, nucleus).
   d. Altered expression of the molecule in pathological conditions.
3. Tissue specificity of some adhesion molecules (i.e., cadherins, CEA) may find some application in diagnostic pathology for determining proper histogenesis of poorly differentiated tumors ***(17)***.
4. Anti-CD31 is commonly used for visualizing vascularization in tissue sections ***(18,19)***.
5. In some tissues, expression of a determined adhesion molecule is associated with malignant transformation. For example, VCAM-1 is expressed rarely in normal epithelia, but it is detected very often in neoplastic cells of malignant mesothelioma ***(20)*** and of undifferentiated nasopharyngeal carcinoma ***(21)***.
6. Altered expression of β1 integrins and cadherins has been detected in a proportion of human malignant tumors of different organs ***(22,23)***.

3.5. Immunohistochemistry in Murine Tissues

3.5.1. Primary Antibodies

Most of the antibodies raised against human adhesion proteins react also with the mouse counterparts, but usually they are not reliable in tissues. Mouse-raised MAbs need antimouse secondary antibodies, which react also with the endogenous immunoglobulins; even when a subclass specific secondary antibody is used the crossreaction may be very intense. Polyclonal rabbit antibodies may be used with affinity-purified Fab2 secondary antibodies, but the background staining of these reagents is very high, especially on macrophages and endothelial cells. Good results are obtained with rat- or hamster-raised antibodies. Many of these antibodies are now commercially available or hybridomas may be obtained by ATCC ***(24)***.

3.5.2. Secondary Antibodies

Best results are obtained with biotinilated Fab2 affinity-purified antirat immunoglobulins. Hamster MAbs require antistrain-specific affinity-purified immunoglobulins, depending on the hamster strain, that give rise to the primary MAb. They are commercially available and can be used following the manufacturer's instructions.

3.5.3. Endogenous Biotin in the Tissue

Murine tissues have a high content of biotin. In most tissues, biotin activity is markedly decreased after air-drying the frozen sections overnight. Endogenous biotin reaction is kept low using TBS at a pH not higher than 7.2. In some organs, such as kidney, liver, intestine, and skin glands, the avidin binding activity can be suppressed with sequential incubation with 0.1% avidin and 0.01% biotin in TBS, pH 7.2, immediately after fixation ***(25)***.

4. Notes

1. Some –80°C refrigerators have special drawers designed to contain cryomolds.
2. Removal of the fat from the slide is crucial, because it may act as a repellent to the aqueous solutions containing the primary antibody and the other reagents.
3. Polylysine increases the stickiness of the section to the slide.
4. It is advisable to use frozen sections within 24 h. After 72–96 h at room temperature, most antigens are lost. Storage of the sections at –80°C prevents antigen loss to some extent, but the best results are usually obtained with sections cut on the day before.
5. Fixation is crucial for the immunohistochemical reaction. Poor fixation may derive from a lower concentration of acetone present in long-standing bottles.
6. Certain antibodies requires fixation in buffered formalin (10% v/v formalin in PBS, pH 7.0) or buffered paraformaldehyde (4% w/v in PBS, pH 7.0) followed by a wash in distilled water. These latter fixatives are not optimal for preservation of frozen tissue morphology, and are not used for membrane-bound antigens.

7. Incubation chambers for immunohisochemistry are Plexiglas boxes with a cover and with bars for sustaining the slides in a flat position. The cover is important for avoiding drying of the sections during the incubation steps or profound alterations in the salt concentration of the incubation buffers owing to evaporation of the water.
8. Numerous commercial kits are available based on ABC peroxidase, ABC alkaline phosphatase, PAP method, APAAP method, or on slight modifications of these techniques. Most of them give excellent results. In the opinion of the authors, the best visualization of the reaction product is obtained when peroxidase/diaminobenzidine is used. Moreover, sections stained with these reagents can be mounted with Canadian balsam and, therefore, are stable for years. The disadvantage of diaminobenzidine is that it is a potent carcinogen for humans. Sections stained with peroxidase/aminoethylcarbazole or with alkaline phosphatase/New Fuchsin have to be mounted with an aqueous mounting media whose dehydratation will cause deterioration of the sections.
9. Because of the high levels of endogenous alkaline phosphatase activity, it is advisable to use peroxidase-benzidine for immunostaining of endothelial cells.
10. Darkening of the brown staining obtained with peroxidase/diaminobenzidine can be obtained with a short incubation in ammonium sulfate prior of counterstaining with hematoxylin.

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10

In situ Hybridization with RNA Probes

Georg Breier

1. Introduction

Immunohistochemistry or immunofluorescence analyses are ideal techniques for localizing adhesive molecules in organs and tissues if reliable, well-characterized antibodies are available. *In situ* hybridization with RNA probes represents an alternative method for detecting mRNA encoding adhesive proteins ***(1)***. In comparison to immunological methods, *in situ* hybridization is more time-consuming and has a lower resolution at the cellular level. However, it is the method of choice if cloned cDNA probes for adhesive molecules have been isolated, but suitable antibodies are not available. Furthermore, *in situ* hybridization is often more sensitive than immunological methods and, thus, allows the detection of adhesive molecules that are expressed at low levels. Finally, the detection of mRNA allows their inambiguous determination of the site of synthesis of secreted molecules or of molecules whose extracellular domains can be proteolytically processed, and that, on release from their producer cells, may travel to distant locations in the tissue ***(2)***.

The protocol presented here describes a procedure for *in situ* hybridization of frozen tissue sections with single-stranded RNA probes. The method is relatively simple and sensitive; however, the histological preservation of tissue is not ideal. Better histological results, however, at the expense of a higher background and longer exposure times, may be obtained with sections of paraformaldehyde-fixed (PFA) tissue embedded in paraffin. This technique has been described in detail elsewhere ***(3)***.

2. Materials

2.1. Tissue Embedding

1. 10X Phosphate-buffered saline (PBS) stock solution. Dilute to 1X PBS with ddH_2O and autoclave (*see* **Notes 1** and **2**).

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2. Plastic molds of different sizes dependent on the size of the specimen.
3. Tissue Tek O.C.T (Miles Scientific).
4. Dry ice.

2.2. Sectioning and Postfixation

1. Cryostat.
2. Silane-coated glass slides: clean standard glass slides (e.g., 26 × 76 mm) by wiping with 70% ethanol. Autoclave and store in a stainless-steel rack. Silane coating and subsequent washing of slides are performed in glass staining jars (e.g., 80 × 110 × 70 mm) in a fume hood as follows: Immerse slides in a 2% solution of 3-aminopropyl-trimethoxysilane in acetone for 5 min. Wash slides for 3 × 5 min in acetone. Air-dry. Store in a glass container. Silane coating binds the tissue sections, which otherwise tend to flow from the glass surface during the washes.
3. Thermostat heating plate (50°C).
4. Plastic staining boxes with racks for slides (e.g., black boxes 80 × 100 × 50 mm). These should be cleaned with 70% ethanol before use.
5. 4% PFA solution: Dissolve PFA in 1X PBS at 80°C with constant stirring in a fume hood (takes approx 1 h). PFA fumes are toxic. Cool to room temperature. Should be made fresh before each use.
6. 1X PBS.
7. Graded ethanols: 30, 60, 80, 95, and 100% EtOH. Use sterile ddH_2O to dilute absolute EtOH.
8. Silica gel: Wrap in paper wipers.

2.3. Preparation of RNA Probes

1. Microcentrifuge for eppendorf tubes.
2. Restriction enzymes: Store at –20°C.
3. Phenol/chloroform/isoamyl alcohol (PCI; 50: 50:1) saturated with TE buffer (10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA). Store at –20°C. Phenol is very toxic on inhalation and skin contact, and should be handled with care in a fume hood.
4. 3.3 *M* Na-acetate, pH 5.5. Autoclave.
5. Absolute ethanol: 80% ethanol.
6. ddH_2O: Autoclave.
7. Agarose.
8. T3-, T7- or SP6 RNA polymerase (e.g. Stratagene) and 5X transcription buffer. Store at –20°C. RNA polymerases are used at 10 U/ mL. If necessary, dilute RNA polymerase in enzyme dilution buffer (also supplied by the manufacturer).
9. 1 *M* DTT and 0.2 *M* DTT. Dissolve in water. Filter sterilize and store in aliquots at –20°C. Avoid repeated freeze-thaw cycles.
10. RNase inhibitor (e.g., RNAguard, Pharmacia): Store at –20°C.
11. 10 m*M* mix of ATP, CTP and GTP solutions (e.g., Pharmacia) in ddH_2O: Store at –20°C.
12. Uridine 5'-[α-^{35}S]thiotriphosphate ([^{35}S]UTPαS), SA > 1000 Ci/mmol.

13. 1 *M* $MgCl_2$: Autoclave.
14. DNase I (10 U/µL): Must be RNase free, e.g., Pharmacia or Boehringer Mannheim. Store at –20°C.
15. Yeast tRNA (50 mg/mL in sterile water): Store at –20°C.
16. 6 *M* Ammonium acetate: Filter-sterilize.
17. Deionized formamide (e.g., BRL): Store at –20°C.
18. 2 *M* NaOH.
19. 2 *M* Acetic acid.
20. Liquid scintillation counter: Liquid scintillant.

2.4. Section Pretreatment

1. ddH_2O: Autoclave.
2. 20X SSC: 3 *M* NaCl, 0.3 *M* Na-Citrate; pH 7.0; stock solution. Autoclave. Dilute to 2X SSC with ddH_2O.
3. Pronase (e.g., Boehringer Mannheim). This should be predigested at 40 mg/mL for 4 h at 37°C in 1X Pronase buffer: 50 m*M* Tris-HCl, pH 7.5, 5 m*M* EDTA, pH 8.0, to destroy RNase. Store in aliquots at –20°C.
4. 2 *M* Triethanolamine-HCl, pH 8.0, stock solution. Autoclave.
5. Acetic anhydride.
6. 0.2% Glycine in PBS: Autoclave.
7. 4% PFA solution in PBS (*see* **Subheading 2.2.**).
8. Acetic anhydride in 0.1 *M* triethanolamine: Dilute triethanolamine-HCl stock solution to 0.1 *M* in 1X PBS. Add $^1/_{400}$ vol of acetic anhydride in a fume hood immediately before use.
9. Graded ethanols: 30, 60, 80, 95, 100% EtOH (*see* **Subheading 2.2.**).

2.5. Hybridization

1. Incubator at 48–60°C.
2. Hybridization chamber (e.g., metal or plastic box) with racks for orienting slides in a horizontal position. A humid atmosphere is generated by adding a paper wiper soaked with 50% formamide.
3. Hybridization buffer is made from stock solutions:

Deionized formamide (e.g, BRL)	10 mL
Autoclaved 1 *M* Tris-HCl, pH 7.5	200 µL
Autoclaved 0.2 *M* Na-phosphate, pH 6.8	1 mL
Autoclaved 0.5 *M* EDTA, pH 8.0	200 µL
50 mg/mL yeast tRNA	60 µL
100 m*M* UTP solution	20 µL
50% Dextrane sulfate	4 mL
Autoclaved ddH_2O to a total volume of	20 mL

Dissolve dextrane sulfate (e.g., Pharmacia or Sigma) in ddH_2O with heating and store at –20°C. Hybridization buffer is stored in 1-mL aliquots. Before use, add $^1/_{100}$ vol each of 100 m*M* ADPβS, 1 m*M* ATPγS, 1 *M* DTT, 1 *M* 2-mercaptoethanol. These compounds are stored in aliquots at –70°C.

4. Pieces of Parafilm cut to appropriate size can be used as coverslips without any pretreatment. Alternatively, glass coverslips treated with siliconizing solution (repelsilane) may be used.

2.6. Post-Hybridization Washes and RNase Digestion

1. A separate set of plastic staining boxes must be used for the post-hybridization treatments because they will be contaminated with RNase.
2. Shaking water bath at 37°C.
3. Wash buffer: 50% formamide, 2X SSC, 10 m*M* 2-mercaptoethanol.
4. RNase buffer: 0.5 *M* NaCl, 10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, pH 8.0.
5. 1 *M* DTT: Dissolve in water. Filter-sterilize. Store in aliquots at –20°C or –70°C.
6. RNase A (10 mg/mL) in H_2O. Store in aliquots at –20°C.

2.7. Autoradiography

1. A darkroom that can be locked.
2. Standard or high-sensitivity X-ray films or a high-resolution Phospho-Imager.
3. Kodak NTB-2 emulsion.
4. Water bath at 42°C.
5. Large light-proof box (e.g., 40 × 40 × 40 cm) with a slide rack for drying slides in an upright position.
6. Plastic boxes for slides (*see* **Subheading 2.2.**).
7. Silica gel.

2.8. Developing and Staining of Slides

1. Kodak D19 developer: Store protected from light.
2. 1% Acetic acid.
3. 30% Sodium thiosulfate: Freshly prepared solutions are very cold and must be brought to room temperature before use.
4. Timer.
5. 0.2% Toluidine blue in 0.2 *M* Na-acetate, pH 4.2.
6. 0.2 *M* Na-acetate, pH 4.2.
7. Glass coverslips (e.g., 24 × 50 mm).
8. Mounting fluid (e.g., Entellan or Eukitt).
9. Microscope with bright-field and dark-field illumination.

3. Methods

3.1. Tissue Embedding

1. Dissect out tissue and wash in PBS (*see* **Notes 1, 2,** and **3**).
2. Carefully blot off excess liquid and orient tissue in a plastic mold filled with Tissue Tek. Avoid air bubbles. Cover tissue with Tissue Tek.
3. Transfer mold onto dry ice and hold steady until frozen. Store at –70°C. Blocks stored frozen for several months have given satisfactory results.

Fixation of tissue with PFA prior to embedding (*see* **Note 3**) results in a better histological preservation of the tissue, however signal strength can be significantly reduced.

3.2. Sectioning and Postfixation

1. Bring tissue blocks from –70°C to cryostat (approx –20°C). Wait until tissue has adapted to cryostat temperature (approx 1 h). Mount onto cryostat chuck using Tissue Tek and cooling in the cryostat. Orient block and trim with a razor blade.
2. Cut sections at approx –20°C at 8–10 µm (*see* **Notes 4** and **5**).
3. Collect sections onto silane-coated glass slides at room temperature (2 sections/ slide: one each for antisense and for sense control probe).
4. Place slides on a heating plate at 50°C (approx 5 min).
5. Fix sections in 4% PFA solution (20 min at room temperature).
6. Wash slides in PBS (5 min at room temperature).
7. Dehydrate sections in graded ethanols: 30, 60, 80, 95, and 100% EtOH (2 min each).
8. Air dry slides (approx. 1 h) and store dessicated in a box containing silica gel at –70°C (*see* **Note 6**).

3.3. Preparation of RNA Probes

Plasmid vectors used for cloning template DNA contain promoters for bacteriophage RNA polymerases, such as T3-, T7-, or SP6 RNA polymerase (*see* **Note 7**). Ideally, plasmids are used in which the multiple cloning site is flanked by a T3 on one and by a T7 promoter on the other side, e.g., pBluescript (Stratagene). T3 and T7 polymerases are preferable over SP6 RNA polymerase, which is less active. Plasmids are linearized by restriction enzyme digest ***(4)*** on either side of the insert in order to generate templates for in vitro transcription of antisense and sense RNA probes, respectively. Linearized templates are incubated with the respective RNA polymerase. Antisense RNA probe will hybridize with the corresponding mRNA. Sense RNA transcripts are generated for use as a negative control, since they do not hybridize specifically with the corresponding mRNA.

3.3.1. Preparation of the Linearized DNA template

1. Digest 10 µg of plasmid DNA downstream of the DNA insert using 20 U of restriction enzyme in a final volume of 20 µL. This template is used for generating antisense RNA probe. In a second reaction, digest plasmid DNA upstream of the DNA insert. This DNA serves as a template for generating sense RNA probe. Avoid enzymes that generate 3' protruding ends.
2. Extract with phenol/ chloroform/ isoamyl alcohol (PCI, 50:50:1).
3. Add 0.1 vol of 3.3 *M* Na-acetate, pH 5.5, mix, and precipitate DNA with 2.5 vol of ethanol. Spin for 20 min in a microcentrifuge at maximum speed, and pipet off supernatant. Wash precipitate as follows: add 100 µL 80% EtOH without disturbing the pellet, spin 5 min, and pipet off supernatant.

4. Dissolve DNA in 20 µL of ddH_2O (RNase-free), i.e., at 0.5 µg/µL. Check a 1 µL aliquot of the DNA by gel electrophoresis in a 1% agarose gel. Store DNA at –20°C.

3.3.2. In Vitro Transcription

Radioactivity security guidelines for working with [^{35}S] must be followed.

1. Mix at room temperature in an eppendorf tube:

5X Transcription buffer	4 µL
0.2 *M* DTT	1 µL
RNase inhibitor (33 U/ µL)	0.3 µL (10 U)
10 m*M* GTP, ATP, and CTP	1 µL
Template DNA (0.5 µg/ µL)	2 µL
[^{35}S] UTPαS (10 mCi/mL)	10 µL
RNA polymerase (10 U/µL)	1 µL

2. Incubate at 37°C for 60–90 min

3.3.3. Removal of the DNA Template

1. Add:

ddH_2O	80 µL
1 *M* $MgCl_2$	1 µL
DNase I (10 U/µL)	1 µL

2. Incubate at 37°C for 10 min

3.3.4. Probe Purification

All centrifugations are made at maximum speed in a bench top centrifuge.

1. Mix with 100 µL PCI by vortexing. Spin for 5 min. Transfer the upper aqueous phase to a fresh eppendorf tube.
2. To the aqueous phase, add:

Yeast tRNA (50 mg/ ml)	4 µL
6 *M* Ammonium acetate	50 µL.
Mix and add:	500 µL ethanol.

Mix and incubate at –20°C for at least 60 minutes. Spin for 20 min in a microcentrifuge. Pipet off supernatant (*see* **Note 8**).

3. Dissolve RNA pellet in 100 µL ddH_2O.

Add 6 *M* Ammonium acetate	50 µL. Mix.
Add ethanol	500 µL. Mix.

Incubate at –20°C for at least 60 min. Spin for 20 min, and pipet off supernatant. Take care not to disturb the pellet.

4. Add 100 µL 80% ethanol. Spin for 5 min and pipet off supernatant.

For efficient hybridzation, probes should be only 100–800 nucleotides in length. Longer in vitro transcripts are subjected to alkaline hydrolysis. If hydrolysis of probes is required, omit the 80% ethanol washing step and proceed as described below (**Subheading 3.3.5.**).

5. Dissolve RNA pellet in 100 µL ddH_2O. Add 2 µL of 1 *M* DTT and 100 µL deionized formamide.
6. Measure incorporation of radioactive nucleotide by counting a 1 µL aliqout in liquid scintillant. Do not vortex scintillant. Expected radioactive concentration is 500,000–1,000,000 cpm/µL.
7. Store probes at –20°C. Probes can be used for up to four weeks.

3.3.5. Hydrolysis of RNA Probe (Optional)

1. Chill probe (dissolved in 100 µL ddH_2O, from **Subheading 3.3.4.**) on ice (*see* **Note 9**). Add 10 µL ice-cold 2 *M* NaOH. Incubate on ice for the required time period (5–20 min). Incubation time varies depending on the original length of the transcript. As a rule of thumb, incubation time in minutes is probe length (number of nucleotides) divided by 200, e.g., 10 min for a 2000-nucleotide transcript.
2. Neutralize by mixing with 10 µL 2 *M* acetic acid.
3. Precipitate RNA with 750 µL ethanol at –20°C for at least 60 min. Spin 20 min in a microcentrifuge. Pipet off supernatant. Take care not to disturb the pellet.
4. Add 100 µL of 80% ethanol. Spin for 5 min. Pipet off supernatant.
5. Dissolve RNA pellet in 100 µL ddH_2O. Add 2 µL of 1 *M* DTT and 100 µL formamide.
6. Measure incorporation of radioactive nucleotide by counting a 1 µL aliqout in liquid scintillant. Do not vortex the scintillant. Expected radioactive concentration is 500,000 –1,000,000 cpm/µL.
7. Store probes at –20°C. Probes can be used for up to 4 wk.
8. It is recommended to control the size of the hydrolyzed transcripts by electrophoresis in a formaldehyde-containing 1% agarose gel or in a denaturing polyacrylamide gel **(4)**.

3.4. Section Pretreatment

Bring slides (still desiccated in the box) to room temperature. Do not open the boxes before the slides have adapted room temperature (this takes at least 1 h) (*see* **Note 6**). Slides are transferred to racks, and the following incubations are performed in plastic staining boxes at room temperature unless otherwise stated:

1. ddH_2O	(1 min)
2. 2X SSC	(30 min at 70°C in a water bath)
3. ddH_2O	(1 min)
4. 40 µg/mL Pronase in 1X Pronase buffer (*see* **Note 10**)	(10 min)
5. 0.2% Glycine in 1X PBS	(30 s to block Pronase digestion)
6. 1X PBS	(30 sec)
7. 4% PFA in PBS	(20 min)
8. 1X PBS	(3 min)

9. Acetic anhydride (1:400 in 0.1 *M* triethanolamine) (10 min)
10. 1X PBS (2 min)
11. 30, 60, 80, 95, and 100% EtOH (2 min each)
12. Air-dry for 30 min or longer. Use for hybridization.

3.5. Hybridization

1. Prewarm slides in a hybridization chamber in an incubator at 60°C.
2. Dilute probe to 50,000 cpm/µL in hybridization buffer. Mix well. Use approx 20 µL for each section.
3. Denature probe in hybridization buffer at 95°C for 2 min. Do not chill on ice.
4. Apply probe. In general, antisense and sense probes are applied to serial sections on the same slide. This allows comparison of the signals of sense and antisense probes on serial sections. Depending on the size of the section, 10–30 µL of hybridization probe are used. Lower a piece of parafilm precut to the appropriate size and press gently with forceps. Avoid trapping air bubbles (*see* **Note 11**).
5. Hybridize in a humid chamber overnight (12–20 h) at 48°C (*see* **Note 13**).

3.6. Posthybridization Washes and RNase digestion

Washing and RNase A digestion are important procedures for achieving specific signals. RNase A will remove all single-stranded RNA probe that does not form a hybrid with mRNA. All washing steps are performed in plastic staining jars at 37°C in a water bath with gentle agitation. A separate set of boxes must be used for these treatments. Prewarm all washing buffers.

1. Wash in wash buffer for at least 2 h until the coverslips float free.
2. Wash in RNase buffer for 15 min.
3. Digest with RNase (20 µg/mL in RNase buffer) for 15 min.
4. Wash in RNase buffer for 15 min.
5. Wash in wash buffer supplemented with 5 m*M* DTT overnight.
6. Dehydrate sections in graded ethanols at room temperature: 30, 60, 80, 95, and100% EtOH (2 min each). Air-dry slides for at least 1 h.

3.7. Autoradiography

1. Expose slides on X-ray film for 2–3 d at room temperature.
2. Develop X-ray film. The image should give an impression of whether the experiment was successful and how long the slides should be exposed with the photographic emulsion which is much less sensitive than X-ray film (*see* **Note 14**).
3. In a dark room, dilute Kodak NTB-2 emulsion 1:1 with dH_2O, and melt in a water bath at 42°C. The emulsion is applied and evenly distributed with a plastic pipet tip. Alternatively, the slides are dipped in emulsion, which is then wiped from the back of the slides. Avoid air bubbles in the emulsion.
4. Air-dry slides in an upright position in a light-tight large box for at least 2 h.
5. Expose slides desiccated in a black plastic box with silica gel at 4°C. Seal box in a plastic bag or aluminium foil. The standard exposure time is 2 wk (*see* **Note 15**).

3.8. Developing and Staining of Slides

1. Bring slides to room temperature.
2. In a darkroom, develop slides in Kodak D19 developer for 3 min at room temperature. Stop developing in 1% acetic acid. Fix in 30% sodium thiosulfate for 3 min. Rinse with dH_2O and several times in tap water.
3. Stain with 0.2% Toluidine blue (in 0.2 *M* Na-acetate, pH 4.2) in a staining jar for 1–5 min at room temperature. Destain for several minutes in 0.2 *M* Na-acetate, pH 4.2. Rinse with tap water and finally with dH_2O. Air-dry (at least 1 h).
4. Mount coverslips using mounting medium (e.g., Entellan or Eukitt).

View the localization of silver grains under the microscope. A dark-field illumination is very helpful for detecting weak signals.

4. Notes

1. Extreme care must be taken to avoid contamination of all glassware and solutions with RNase. Wear gloves during all steps of the procedure. It is also a good idea to have a separate clean bench space that is used only for RNA *in situ* hybridization and other RNA techniques.
2. All aqueous solutions should be sterilized, if possible, by autoclaving or by filtration through a 0.45 µm filter. Solutions that cannot be sterilized (e.g., hybridization buffer) are made up of sterile solutions. Use sterile tubes and autoclaved pipet tips throughout the whole procedure.
3. It is recommended to perform fixation of tissue in PFA solution prior to embedding only if a strong hybridization signal is expected and to avoid long fixation times. Tissue fixation and embedding are performed as follows:
   a. Dissect out tissue and wash in PBS.
   b. Fix in fresh 4% PFA solution (*see* **Subheading 2.2.**) at 4°C. Short fixation times are preferable. The fixation time depends on the size of the sample and should be determined empirically. Try 2 h for a 5-mm piece of tissue. Lower concentrations of PFA (e.g.,1%) may also be useful.
   c. Rinse in PBS.
   d. Transfer to 0.5 *M* sucrose in 1X PBS (filter sterilized) and incubate at 4°C for 24 h.
   e. Carefully blot off excess liquid and orient tissue in a plastic mold filled with Tissue Tek. Avoid air bubbles. Cover tissue with Tissue Tek.
   f. Transfer mold onto dry ice, and hold steady until frozen. Store at –70°C. Blocks stored frozen for several months have given satisfactory results.
4. Optimal sections are a prerequisite for a successful experiment. For best preservation of tissue, it is essential that the tissue is frozen slowly on dry ice. Do not be tempted to use liquid nitrogen for freezing tissue blocks.
5. For best results, the knife has to be in an excellent condition. The optimal cutting temperature must be determined empirically. For most tissues, temperatures between –14 and –20°C are optimal. The tissue temperature must be raised in 1° steps if the tissue ruptures. Soft tissues are cut at lower temperatures than more solid ones. The optimal temperature of the knife is often several degrees

lower than the temperature of the tissue. Lower the temperature if the section adheres to the knife.

6. Slides can be repeatedly thawed and frozen without loss of quality provided that great care is taken to avoid humidity. Boxes for storing slides must contain silica gel wrapped in wiping paper. Before freezing slides, check that the silica gel is still desiccated, as indicated by the blue color.
7. The result of the *in situ* hybridization protocol is largely dependent on the use of a suitable hybridization probe. Templates may range in size between <100 and more than several thousand base pairs. Probes should be derived from the coding region of a protein. Repetitive sequences (which may be contained in noncoding mRNA regions or in introns) must be excluded. It is also advisable to avoid sequences encoding conserved protein domains, since these might hybridize also with related mRNAs. Sequences with extremely high or low GC contents should not be used. Two or more different probes derived from a single gene may be required if alternative mRNA splicing occurs.
8. It is advisable to monitor the radioactivity contained in supernatants and in pellets with a sensitive radioactivity contamination monitor. This helps to minimize unintentional loss of ^{35}S-labeled probe. In general, approx 60% of ^{35}S-labeled nucleotide should be contained in the pellet after the first ethanol precipitation. No significant loss of radioactivity should occur in all subsequent ethanol precipitation and washing steps. It is a good idea to collect all supernatants until probe purification is finished.
9. It is important that alkaline hydrolysis of in vitro transcripts is performed on ice and that all reagents are precooled on ice because, the reaction proceeds much faster at higher temperatures, which may result in fragments of too small sizes.
10. Optimal Pronase concentration varies for different tissues. Incomplete Pronase digestion may result in high-hybridization background levels. In this case, increase Pronase concentration to 80–120 µg/mL.
11. Removal of air bubbles trapped under parafilm is easier following incubation of the slides for a few minutes in the hybridization incubator.
12. While applying the hybridization probe, cooling of slides to room temperature should be avoided. Return slides to the incubator from time to time.
13. The optimal hybridization temperature is dependent on the GC content of the probe. Probes with lower or higher than average GC content require lower or higher hybridization temperatures, respectively. Optimizing the hybridization temperature can greatly improve results.
14. Ideally, antisense probe generates a strong specific hybridization signal, whereas sense control probe gives only weak background levels. However, some sense probes generate relatively high unspecific background, which can only be judged under the microscope.
15. Very strong signals may require shorter exposure times (e.g., 10 d), and weak signals may require longer exposure times (e.g., 3 wk). Sections of PFA-prefixed fixed tissue generally require longer exposure times.

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11

Purification of Fibronectin from Human Plasma

Saverio Francesco Retta, Piercarlo Ferraris, and Guido Tarone

1. Introduction

Fibronectin is an ubiquitous extracellular glycoprotein that exists in a soluble form in body fluids and in an insoluble form in the extracellular matrix. It plays a major role in many important physiological processes, such as embryogenesis, wound healing, hemostasis, and thrombosis ***(1)***. Fibronectin is secreted as a dimer, with a monomer molecular weight of approx 220–250 kDa; the monomers are joined by two disulfide bonds near the carboxyl terminus of the protein ***(2)***. Like many proteins of the extracellular matrix, fibronectin is a mosaic protein composed of modular units ***(3)***. Fibronectin is composed almost entirely of three types of modules (types 1–3). The modules are organized into functional domains that are resistant to proteolysis and contain binding sites for extracellular matrix proteins such as collagen and thrombospondin, cell-surface receptors such as integrins, circulating blood proteins such as fibrin, and glycosaminoglycans such as heparin and chondroitin sulfate ***(4)***. In general, fibronectin is synthesized by and is present around fibroblasts, endothelial cells, chondrocytes, glial cells, and myocytes. Fibronectin is abundant in the connective tissue matrix and in basement membranes ***(5)***. The distribution is quite characteristic for each cell and tissue. Although blood cells, such as lymphocytes, myelocytes, and erythrocytes, lack fibronectin, this glycoprotein is abundant in the plasma (about 30 mg/100 mL). Actually, this body fluid represents the main source for fibronectin purification. The most popular approach to purify plasma fibronectin relies on its specific, high-affinity binding to denatured collagen ***(6–10)***. After affinity chromatography of plasma on columns containing covalently coupled denatured collagen (usually gelatin), the bound protein is eluted by 1 *M* KBr, 1–8 *M* urea, or amines ***(7–11)***. In this chapter the authors describe a simple purification procedure for fibronectin

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based on a preparative affinity chromatography of plasma on gelatin coupled to Sepharose, as proposed first by Engvall and Ruoslahti ***(7)***. This method gives electrophoretically and immunologically pure fibronectin in high yields (*see* **Notes 1–3**).

2. Materials

2.1. Purification of Fibronectin

1. Outdated human plasma (100 mL) (*see* **Note 4**).
2. Centrifuge capable of centrifuging 50 mL tubes at 10,000*g*.
3. Pre-column: Sepharose 4B (10 mL bed volume) (*see* **Note 5**).
4. Affinity chromatography column: Collagen (Gelatin)-Sepharose (5 mL bed volume) (*see* **Note 6**).
5. Chromatography apparatus (*see* **Note 7**).
6. 0.2 *M* phenylmethane sulfonyl fluoride (PMSF) (Sigma, Pierce, or BRL) in ethanol (*see* **Note 8**).
7. 0.5 *M* NaCl in 10 m*M* Tris-HCl, pH 7.4.
8. Tris-buffered saline (TBS): 0.15 *M* NaCl, 10 m*M* Tris-HCl, pH 7.4.
9. 5 m*M* EDTA in TBS.
10. 1 *M* urea in TBS.
11. 4 *M* urea in TBS.

2.2. Quantitation, Electrophoretic Analysis, and Dialysis of Purified Fibronectin

1. UV/visible spectrophotometer for measuring protein concentration.
2. Apparatus for protein electrophoresis (e.g., Mini-PROTEAN II from BioRad).
3. Standard reagents for sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) ***(12)***.
4. Coomassie brillant blue R250 staining solution: dissolve 0.259 of Coomassie brillant blue R250 in 90 mL of methanol:H_2O (1:1 v/v) and 10 mL of glacial acetic acid; filter the solution through a Whatman No. 1 filter to remove any particulate matter.
5. Destaining solution: methanol:H_2O:glacial acetic acid (45:45:10).
6. Dialysis tubing (*see* **Note 9**).
7. TBS: 0.15 *M* NaCl, 10 m*M* Tris-HCl, pH 7.4.

3. Methods

3.1. Purification of Fibronectin

1. All procedures are performed at room temperature.
2. Plasma is brought to 0.5–1 m*M* in the PMSF and centrifuged at 10,000*g* for 15 min.
3. The supernatant is applied to the Sepharose 4B pre-column (2 mL/min flow rate), which has been previously washed with TBS containing 5 m*M* EDTA (*see* **Note 10**).

4. The flow-through material is applied to the gelatin affinity column (2 mL/min flow rate), which has been previously equilibrated with TBS (*see* **Note 11**).
5. The column is washed with at least two column volumes of 10 m*M* Tris-HCl, pH 7.4, containing 0.5 *M* NaCl, then with three column volumes of TBS (*see* **Note 12**).
6. The bound fibronectin is eluted with 1 *M* urea in TBS. The elusion consists of at least 1.5 column bed volumes, collecting fractions of roughly 1/20 to 1/10 of the column bed volume (e.g., 0.5–1 mL per fraction) (*see* **Note 13**).

3.2. Quantitation, Electrophoretic Analysis, and Dialysis of Purified Fibronectin

1. The peak of eluted protein can be determined by Coomassie blue protein assay (BioRad) or by absorbance at 280 nm ($E_{1\ mg/mL} = 1.2$).
2. The level of fibronectin purification is assessed by discontinuous PAGE (stacking gel: 5%; running gel: 7.5%) followed by Coomassie Brillant Blue staining of the gel ***(12)***. Fibronectin appears as a doublet of closely spaced bands with apparent mol wt of 220–250 kDa.
3. The appropriate fractions are pooled and the pool is dialyzed against TBS at 4°C for 18 h, with three changes.
4. The fibronectin final concentration is measured by absorbance at 280 nm ($E_{1\ mg/mL} = 1.2$) (or by Coomassie blue protein assay [BioRad] and aliquots are stored at –70°C) (*see* **Note 14**).

4. Notes

1. Heparin affinity columns can also be used to purify fibronectin **(13)**. However, many other plasma proteins can also bind to heparin, and heparin affinity columns appear to be most useful when used as a second affinity step following a gelatin affinity column.
2. The fibronectin purified by one-step gelatin-Sepharose procedure is relatively pure. However, substantial contaminants are sometimes present, e.g., fibrinogen. For immunological studies, a preferable approach is to purify further the fibronectin preparations. Methods used include applying the preparations to the gelatin affinity columns a second time or gel filtration chromatography. An alternative strategy is to purify the fibronectin by gelatin affinity chromatography, then to elute and further purify by heparin affinity chromatography in the presence of 4 *M* urea, which prevents reassociation of fibronectin with contaminating proteins to which it may bind ***(14)***.
3. A comparison of the recovery and biological activity of fibronectin eluted in various ways from collagen in terms of ability to mediate cell adhesion suggests that urea is the safest eluting molecule for recovering fully active fibronectin ***(15)***.
4. Outdated human plasma can be obtained from blood banks, anticoagulated by either citrate or EDTA. Plasma that contains any visible clotted material appears to be less satisfactory, since subsequent contaminants often appear in the purified preparations, perhaps due to the binding of fibrin monomer to fibronectin and isolation of the complexes during affinity chromatography. Other sources include

fresh-frozen plasma or human serum, although the latter source is less satisfactory because of the reduced amount of plasma fibronectin usually found in serum after clotting.

5. The pre-column serves to remove proteins or protein complexes that bind nonspecifically to Sepharose 4B columns.
6. Porcine skin gelatin can be coupled to commercially available CNBr-activated Sepharose 4B according to the procedures recommended by the manufacturer (e.g., Pharmacia or Pierce). An example of an effective coupling protocol is the following. A 40 mg/mL solution of gelatin stored at –20°C is warmed to room temperature, then melted by brief heating in a boiling water bath and cooled to approx 40°C. CNBr-activated Sepharose (Pharmacia) is washed on a Buchner funnel with a medium-porosity fitted glass bottom in 10^{-3} *M* HCl. An appropriate volume of gelatin solution (e.g., 0.7 mL/10 g of moist CNBr-activated Sepharose beads) is added to the gel beads immediately after the addition of coupling buffer (e.g., 7 mL of 0.2 *M* $NaHCO_3$, 1 *M* NaCl, plus water to bring the total solution volume to 14 mL/10 9 of moist gel). The mixture is vigorously shaken after these additions, then placed in a plastic bottle on an end-over-end mixer for 2 h at room temperature. The amount of gelatin that is finally covalently bound to the beads can be determined by the absorbance of the supernatant solution. The reaction is stopped by incubation in 1 *M* ethanolamine, pH 8.0, for 2 h. The gelatine-Sepharose is then washed on a Buchner funnel with three sequential cycles of low and high pH washes, using 1 *M* NaCl, 0.1 *M* sodium acetate, pH 4.0, then 1 *M* NaCl, 0.1 *M* sodium borate, pH 8.0, in order to remove absorbed but noncovalently bound gelatin. The washed gel beads can be stored at neutral pH, e.g., in PBS or TBS at 4°C as a 50% (v/v) suspension. Sodium azide (0.02%) should be added to prevent the growth of microorganisms during long-term storage.
7. A typical low pressure, preparative, chromatography system consisting of a column, a peristaltic pump and a fraction collector (e.g., GradiFrac™ System from Pharmacia Biotech).
8. PMSF is a very toxic compound. Wear suitable protecting clothing, gloves, and eye/face protection. Do not breathe dust. Stock solutions of PMSF are prepared immediately before use, since hydrolytic breakdown of this compound occurs over a period of hours in acqeous solution. A stock of 0.2 *M* is prepared in 95% ethanol, and a 1 to 200 dilution is added to the plasma with vigorous stirring.
9. Cut the cellulose membrane dialysis tubing into pieces of convenient length (10–20 cm). Boil for 10 min in a large volume of 2% (w/v) sodium bicarbonate and 1 m*M* EDTA (pH 8.0). Rinse the tubing thoroughly in distilled water. Boil for 10 min in 1 m*M* EDTA (pH 8.0). Allow the tubing to cool, and then store it at 4°C. Be sure that the tubing is always submerged. From this point onward, always handle the tubing with gloves. Before use, wash the tubing with distilled water.
10. Even anticoagulated plasma will sometimes unpredictably initiate clotting during column chromatography, resulting in poor flow rates and contaminated preparations. This problem is completely prevented by the addition of 2 U/mL hirudin

(Sigma), to both the plasma and to the washes of pre- and gelatin- Sepharose columns, to inhibit thrombin activity.

11. The ratio of plasma to gelatin-Sepharose is approx 100 mL/10 mL of packed gel. The capacity of the gelatin-Sepharose adsorbent is high: 40 mg of gelatin binds 20 mg of fibronectin.
12. Before the elution step, check absorbance at 280 nm of flow through: it should be below 0.1.
13. With a correct gelatin affinity chromatography, up to 100% of fibronectin from plasma could be recovered. The gelatin-Sepharose column can be reused after extensive washing with 4 *M* urea in TBS, then TBS alone.
14. Purified fibronectin in TBS can be stored in polyethylene containers at 0–4°C for at least several days with no apparent deterioration. Use of phosphate buffers or glass or wettable plastic containers may result in precipitation or nonspecific losses by binding to vessel walls. For routine long-term storage, quick-freeze aliquots in polypropylene tubes, e.g., by immersion in liquid nitrogen, and store the samples at –70°C or colder. Storage at –20°C is not satisfactory, since eventual loss of activity or even grossly visible aggregation is observed. For use, samples are quickly defrosted by placing vials in room temperature water. Occasionally, a small precipitate is visible which is either dissolved by heating to 37°C or by centrifugation.

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12

Adhesion to Matrix Proteins

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1. Introduction

Cells in tissues exist in a structural and informational context that involves other cells and extracellular matrix (ECM), as well as growth factors, hormones, and ECM-remodeling enzymes. The ECM is a complex mixture of collagenous and noncollagenous glycoproteins and proteoglycans that are secreted locally and assemble into an organized meshwork. Among glycoproteins, collagens of various types, fibronectin, laminin, and vitronectin have been isolated from ECM and partially characterized, both structurally and functionally. Adhesive interactions between cells and the insoluble meshwork of ECM play a vital role in embryonic morphogenesis ***(1,2)***, and in the regulation of gene expression in cells of the adult organism ***(3,4)***. Although in recent years there have been major advances in deciphering the overall phenomenology of ECM effects on cell adhesion events underlying processes, such as embryogenesis, immune response, invasion, metastasis, thrombosis, inflammation, signaling and/or changes in gene expression, the biochemical and molecular bases for these effects have remained elusive ***(5–7)***. It is clear that many of the interactions between cells and the ECM are mediated by the integrin family of cell surface receptors ***(8,9)***. The integrins are transmembrane heterodimeric proteins and are comprised of at least 14 distinct α-subunits and 8 or more β-subunits that can associate in various combinations; each integrin subunit has a large extracellular domain, a single membrane-spanning region, and generally a short cytoplasmic domain ***(9–12)***. The α/β associations determine the ligand binding specificities of the integrin heterodimers for various ECM glycoproteins, including fibronectin, vitronectin, laminin, and collagens: integrins can be monospecific (e.g., α5/β1, the "classic" fibronectin receptor, or α6/β1, which recognizes laminin only) or polyspecific (e.g., α3/β1, which recognizes fibronectin, laminin, epiligrin, and collagen) ***(9)***. The substratum preference of

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a particular integrin on a specific cell line does not necessarily predict the general behavior of that integrin. Indeed, the ligand specificity of certain integrins is variable between cell lines ***(13)*** and even within the same cell lineage ***(14,15)***. Furthermore, cells often display multiple integrins capable of interacting with a particular ECM protein ligand ***(9,16)***. In some cases two integrins that share a ligand actually recognize different regions of the ligand molecule, as is true for the α5/β1 and α4/β1 fibronectin receptors ***(17)***, or the α1/β1 and α6/β1 laminin receptors ***(18)***; in other cases, such as α5/β1 and α3/β1, two integrins bind to the same region of the ECM protein (Arg-Gly-Asp adhesion sequence) ***(19)***. This overlapping and apparently redundant expression of integrins seems paradoxical. However, two integrins that bind the same region of the same ligand may not necessarily serve identical functions in the cell. This suggestion is based on the fact that the cytoplasmic domains of integrin subunits display divergent sequences, indicating that each subunit can be capable of specifically transducing signals from the ECM to the cell interior and, therefore, contribute to discrete intracellular function. The integrins differ from cell-surface receptors for hormones and for other soluble signaling molecules in that they bind their ligand with relatively low affinity ($K_a = 10^6–10^8$ L/mol) and are usually present at about 10- to 100-fold higher concentration on the cell surface. This suggests that these matrix receptors might function cooperatively and that cells may respond to an organized group of ligands in the extracellular matrix rather than to individual molecules. Cellular adhesion, however, is a complex and highly regulated phenomenon that can not be completely understood by considering only the density of integrins on the plasma membrane and the availability of ligand molecules in the extracellular matrix. Many recent experimental observations indeed indicate that integrins exist in different affinity states regulated by intracellular signals ***(20,21)***. It is likely that the lowest affinity state corresponds to the unoccupied integrin, incompetent for ligand binding, whereas the highest affinity state corresponds to the ligand-occupied integrin engaged in the transmission of an outside-in signal ***(22)***.

To assess cell adhesion to ECM proteins, different methods have been used, depending on the specific experimental purpose. Actually, to date, a standard, universal method for cell adhesion assay does not exist. In this chapter, the simplest procedure for evaluating the attachment of cells to extracellular matrix ligands will be described in detail. Other approaches will be presented in **Subheading 4**.

2. Materials

1. Tissue-culture 96-well microtiter plates (e.g., Falcon 3912 from Becton Dickinson).
2. Purified extracellular matrix protein.

3. Phosphate-buffered saline (PBS): 0.15 *M* NaCl, 10 m*M* Na-phosphate buffer, pH 7.4.
4. 1% (w/v) Bovine serum albumin (BSA) in PBS.
5. 5 m*M* EDTA in PBS.
6. 3.7% Paraformaldehyde in PBS.
7. Coomassie brillant blue R250 staining solution: dissolve 0.25 g of Coomassie brillant blue R250 in 90 mL of methanol:H_2O (1:1 v/v) and 10 mL of glacial acetic acid; filter the solution through a Whatman No. 1 filter to remove any particulate matter.
8. Inverted optical microscope.
9. Microtiter plate reader (e.g., Bio-Rad 450 from Bio-Rad Laboratories).
10. CO_2 incubator.
11. Tissue-culture medium (e.g., DMEM).

3. Methods

1. Coat tissue-culture 96-well microtiter plates by overnight incubation at 4°C with various concentration of purified matrix proteins (*see* **Note 1**).
2. Remove the protein solutions and saturate the wells with BSA (1% w/v in PBS) for 1 h at 37°C.
3. Detach cells, at confluence on 90-mm culture dish, by 5 m*M* EDTA treatment in PBS for 10 min, and wash them twice in serum-free tissue-culture medium.
4. Resuspend the cells in serum-free tissue-culture medium, plate in triplicate (5×10^4 cells/well) and allow them to attach on the coated tissue-culture wells for 1 h at 37°C (*see* **Note 2**).
5. Rinse microtiter plates twice with PBS to remove unbound cells, and then fix adherent cells with 3.7% paraformaldehyde in PBS for 10 min at room temperature (*see* **Note 3**).
6. Wash microtiter plates three times with PBS, and stain adherent cells with Coomassie brillant blue for 1 h at room temperature.
7. After three washes with PBS, evaluate cell adhesion by optical microscope analysis (*see* **Note 4**) or by reading the absorbance at 540 nm of stained cells in a microtiter plate reader (*see* **Note 5**).
8. Alternative methods (*see* **Notes 6, 7, 8,** and **9**).
9. Inhibition of cell adhesion (*see* **Notes 10** and **11**).
10. Divalent cation sensitivity of integrin-mediated adhesion ***(19)*** (*see* **Note 12**).

4. Notes

1. Typically, a cell attachment assay is performed on 96-well microtiter plates coated with various concentrations of purified ECM proteins. Usually, the coating concentration range is 0.5–10 μg/mL.
2. To eliminate the contribution of protein synthesis and secretion during cell adhesion, cells can be pretreated with the protein synthesis inhibitor cycloheximide (20 μ*M*) for 2 h before detachment, and plated in the adhesion assay in the presence of monensin (1 μ*M*), which inhibits protein secretion.

3. Alternatively, the attached cells can be fixed with 70% ethanol.
4. For simple purposes, cell adhesion can be evaluate by counting stained cells at 200× magnification in 10 microscope fields.
5. Cell adhesion to ECM proteins can be expressed as percentage of a control (e.g., the number of cells bound to serum-coated wells). Results must be calculated from the average of triplicate samples and must be representative of several separate assays. Nonspecific cell adhesion (attachment to wells coated with BSA) must be used as "blank" and, thus, subtracted from the results determined for the ECM substrata.
6. Crystal violet staining **(23)**. Use the same procedures as described in **Subheading 3.** with the following exceptions. Fix the cells with 96% ethanol for 10 min, and stain them with 0.1% crystal violet in H_2O for 30 min. Wash away the excess stain with H_2O, and after lysis of the fixed cells with 0.2% Triton X-100 in H_2O, read the absorbance of cell-bound stain in an microtiter plate reader at 595 nm.
7. Colorimetric assay for acid phosphatase activity **(24)**. Use the same procedures as described in **Subheading 3.** with the following exceptions. After allowing the cells to attach on the coated tissue-culture wells for 1 h at 37°C, wash adherent cells two times with PBS and measure acid phosphatase activity in each well by incubating cells for 90 min at 37°C with 200 µL of 10 m*M* *p*-nitrophenyl phosphate in 0.1% Triton X-100, 0.1 *M* sodium acetate, pH 5.5, developing the colorimetric reaction with 20 µL of 1 *M* NaOH, and reading the absorbance at 405 nm in a microtiter plate reader. The optical density should be linearly correlated with the number of viable cells.
8. ^{51}Cr labeling ***(19,25)***. Incubate 5×10^6 adherent cells with ^{51}Cr (0.5 µCi; 1 Ci = 37 GBq) in tissue-culture medium for 4–6 h at 37°C, wash sequentially with PBS, followed by 1 m*M* EDTA in PBS, and finally serum-free tissue-culture medium supplemented with 1% BSA. Then detach cells by 5 m*M* EDTA treatment in PBS for 10 min, and wash them twice in serum-free tissue-culture medium. Resuspend the cells in serum-free tissue-culture medium supplemented with 1% BSA, and plate them in triplicate (5×10^4 cells/well) for 20–30 min at 37°C on 96-well microtiter plates that have been previously coated with ECM proteins (*see* **Subheading 3.**). After aspirating unbound cells and washing the plates three times with serum-free tissue-culture medium supplemented with 1% BSA, bound cells are lysed with 0.1% sodium dodecyl sulfate (SDS), and ^{51}Cr present in cell lysates is measured using a γ-counter. The percentage of adherent cells can be determined from a comparison of the residual radioactivity with the total input. Background binding of radiolabeled cells to BSA-coated control wells must be subtracted during calculation of specific attachment.
9. ^{35}S-labeling ***(26)***: Partially depleted cultured cells of methionine by incubation for 2–4 h in tissue-culture methionine-free medium, containing PBS-dialyzed serum, and then label them by incubation with 250 µCi of [^{35}S]methionine overnight. The cells are then washed three times with serum-free medium, harvested by 5 m*M* EDTA treatment in PBS for 10 min, washed twice in serum-free medium, and added to coated microtiter wells. After 30–60 min at 37°C, the cells

are washed three times and solubilized by 0.1 *M* NaOH treatment for 60 min at room temperature. Bound cells are quantitated by counting β-emissions in a liquid scintillation analyzer, and the results can be expressed as percent of a control. Background binding of radiolabeled cells to BSA-coated control wells must be subtracted during calculation of specific attachment.

10. For inhibition experiments by integrin MAbs, add dilutions of inhibitory MAb (1–5 µg/well) to ligand-coated microtiter plates before addition of cells, and conduct the adhesion assay exactly as described above.
11. To analyze the effect of Arg-Gly-Asp (RGD) peptides on cell adhesion, use the same procedures as described above, with the exception of the use of a GRGDSP peptide (0.5–1 mg/mL) instead of inhibitory MAbs. The GRGESP peptide can also be used as control.
12. To analyze divalent cation sensitivity of integrin-mediated adhesion, first wash the cells with 1 m*M* EDTA in PBS to deplete extracellular levels of pre-existing divalent metals. Subsequently, equilibrate the cells separately with 1 m*M* each of either $CaCl_2$, $MgCl_2$, or $MnCl_2$ in 20 m*M* Tris, pH 7.4, 135 m*M* NaCl, 5 m*M* KCl, 2 m*M* *L*-glutamine, 1.8 m*M* glucose, and 1% BSA, and plate them on ECM-coated dishes to carry out adhesion assays as indicated above.

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13

Leukocyte-Endothelial Monolayer Adhesion Assay (Static Conditions)

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1. Introduction

Peripheral blood leukocytes interact with the endothelial lining of the cardiovascular system in a wide variety of physiologic and pathophysiologic situations. In various types of acute and chronic inflammatory reactions, for example, polymorphonuclear leukocytes (neutrophils, basophils, eosinophils) and mononuclear leukocytes (monocytes, lymphocytes) adhere to and then migrate across the locally activated endothelial lining to form an inflammatory exudate. This process involves the ligand–receptor pairing of a series of adhesion molecules expressed by both circulating leukocytes and the endothelial cell ***(1)***. The sequential and coordinated interaction of these leukocyte-endothelial adhesion molecules, in conjunction with the localized generation of leukocyte-selective chemoattractant cytokines, confers a cellular selectivity to these reactions ***(1,2)***.

Much of our knowledge of these complex in vivo phenomena is based on mechanistic studies performed in simplified in vitro model systems. The leukocyte-endothelial monolayer adhesion assay described below allows freshly isolated human peripheral blood leukocytes to adhere to the surface of cultured human vascular endothelial monolayers, under static conditions, and then utilizes inversion/centrifugation to separate nonadherent leukocytes ***(3,4)***. This monolayer adhesion assay has been useful in defining adhesion-stimulatory or inhibitory substances that might act on either the leukocyte or endothelial cell, as well as characterizing the ligand–receptor pairs involved (e.g., through the use of specific antibodies or other blocking reagents incorporated into the assay system) ***(5–7)***. Although described here for leukocyte-endothelial adhesion, this monolayer adhesion assay can be readily adapted to the study of

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adhesive interactions of other cell types (e.g., tumor cells adhering to endothelial or nonendothelial monolayers) ***(8)***. The more complex assay geometry of leukocyte adhesion to endothelium under fluid mechanical conditions that simulate blood flow is described elsewhere in this volume.

2. Materials

2.1. Isolation of Peripheral Blood Leukocytes

1. Peripheral venous blood obtained from normal human volunteers.
2. Anticoagulant (100 m*M* sodium citrate in 130 m*M* dextrose, pH 6.5).
3. Lymphocyte separation medium (Ficoll and sodium diatrizoate, sterile solution, density = 1.077–1.080 g/mL) (Organon Teknika # 50494) (LSM).
4. Hank's balanced salt solution without calcium and magnesium (Gibco BRL #14170-112) (HBSS).
5. 2% (w/v) Dextran (Sigma #D5251) in HBSS.
6. Ice-cold pyrogen-free tissue-culture-grade water.
7. HBSS with 0.5% (w/v) bovine serum albumin (BSA) (Sigma #A3350) and 25 m*M N*-(2- Hydroxyethyl) piperazene-*N*'-(2-ethane sulfonic acid) (HEPES), pH 7.3–7.4 (HBSS/BSA/HEPES).
8. 50 mL conical polypropylene centrifuge tubes.

2.2. Loading of Leukocytes with Fluorescent Probe

2',7'-Bis-(2-carboxyethyl)-5(and-6) carboxyfluorescein, acetoxymethyl ester (BCECF-AM) stored at –70°C as a 1 m*M* stock in endotoxin-free, tissue-culture-grade dimethyl sulfoxide (DMSO) (Sigma #D2650).

2.3. Adhesion Assay

1. Human umbilical vein endothelial cells (HUVEC), passage level 1–3, cultured in 96-well microtiter tissue-culture plates (Costar), precoated with 0.1% gelatin, plated to be confluent at the time of assay.
2. RPMI 1640 culture medium containing 25 m*M* HEPES (Gibco BRL #22400-071) and 1% v/v fetal bovine serum (HyClone #A-1111-L, defined fetal bovine serum, endotoxin tested).
3. Acetate sealing tape for microtiter plates (Dynatech #001-010-3501).
4. Plastic-backed absorbant paper (VWR# 52857-120).
5. Centrifuge fitted with rotor to accept microtiter plates.
6. Lysis buffer (50 m*M* Tris-HCl, 0.1% SDS in water, pH 8.2–8.4).
7. Fluorimeter to read microtiter plates (e.g., Pandex FCA Screen Machine).

3. Method

3.1. Isolation of Peripheral Blood Leukocytes

1. Collect 50 mL venous blood in anticoagulant by sterile venapuncture (final volume 10% anticoagulant).

2. Centrifuge whole blood 4 min at 1000*g* in a 50 mL conical tube, and then carefully aspirate supernatant (plasma) leaving buffy coat on red cell pellet.
3. Dilute remaining packed cells to 50 mL in HBSS, and mix gently.
4. Gently layer cell suspension onto LSM (25–30 mL blood/10 mL LSM).
5. Centrifuge for 40 min at 500*g* at room temperature.
6. For mononuclear cell isolation, collect band at gradient interface (predominantly lymphocytes and monocytes).
7. To isolate neutrophils, aspirate remaining fluid, and resuspend large pellet in 25 mL HBSS. Add 25 mL 2% dextran and mix gently. Incubate for 30 min at room temperature to allow red blood cells to sediment.
8. Transfer the supernatant (containing the neutrophils) to a fresh tube, and centrifuge at 800*g* for 5 min at room temperature.
9. Aspirate the resulting supernatant. In order to lyse remaining red blood cells, add 3 mL ice-cold water to the cell pellet, and mix gently for 20–30 s.
10. Immediately fill the tube with HBSS/BSA/HEPES. Centrifuge 3 min at 400*g*.
11. Resuspend cell pellet (purified peripheral blood neutrophils) in HBSS/BSA/HEPES buffer to a final volume of 10 mL and count an aliquot to determine cell concentration and total cell yield.

3.2. Loading Leukocytes with BCECF

1. Resuspend the leukocytes at 10^7 cells/mL in HBSS/BSA/HEPES, and add BCECF-AM to a final concentration of 2μ*M*.
2. Cover the tube with aluminum foil, and incubate at room temperature for 20 min with intermittent mixing.
3. Spin at 400*g* for 5 min, remove supernatant, and resuspend the pellet in 50 mL of the HBSS/BSA/HEPES.
4. Incubate for an additional 10 min at 37°C.
5. Centrifuge at 400*g* for 5 min, aspirate and discard supernatant, and resuspend the pellet in RPMI 1640 with 1% FBS to desired final concentration (usually 2×10^6cells/mL).

3.3. Adhesion Assay

1. Confluent HUVEC monolayers in microtiter plates should be examined microscopically, prior to assay, to check for integrity and uniformity of the monolayers in each test well. HUVEC can be activated by treatment with a cytokine or other stimulus prior to assay to induce the expression of adhesion molecules of interest ***(2–5)***. Alternatively, metabolic inhibitors or other endothelial-directed antagonists can be incorporated into this pre-incubation step ***(6–7)***.
2. Endothelial-monolayers should then be washed once (200μL/well) with RPMI + 1%FBS at 37°C.
3. Aspirate wash fluid, and add 100 μL of the BCECF-labeled leukocyte suspension to each well. (Reserve triplicate 100 μL aliquots of the fluorescent cells to be used to determine the average fluorescence per cell.) If leukocyte-directed agonists or antagonists are to be used, they can be added to the appropriate wells at

this point (or in a preincubation step, to the leukocyte suspension). Alternatively, monoclonal antibodies (MAbs) or other adhesion blocking reagents can be incorporated into the assay at this time ***(3–7)***.

4. Incubate plates at 37°C for 10 min, and then carefully fill the wells to the top with RPMI + 1% FBS.
5. Seal the plate with acetate tape. Wrap the plates in absorbant paper, invert, and centrifuge at 350*g* for 5 min at room temperature.
6. Holding the plate in the inverted position, carefully strip off the acetate tape seal. Keeping the plate inverted, with a flick of the wrist, expel the fluid from the wells. Aspirate any residual medium left in the wells, taking care not to disturb the cell monolayer.
7. Solubilize the contents of the well in 150 µL lysis buffer, and then quantify the fluorescence in each well using a microplate fluorimeter, at excitation 485 nm, emission 530–540 nm.

4. Notes

1. Both cultured human endothelial cells and human blood are considered biohazardous (potentially infectious) materials. All procedures therefore should be performed in compliance with appropriate institutional protocols and regulations for biocontainment.
2. Although HUVEC are used in this example, other sources of endothelial cells and, indeed, other types of cells that grow as confluent monolayers can be used in this assay system.
3. Neutrophils can be easily activated during isolation, leading to uncontrolled increases in adhesion. Activated neutrophils have a rough, irregular shape and a ruffled appearance, and tend to form clusters, whereas unactivated neutrophils are round, smooth spheres, and remain unclumped in suspension. Care should be taken to ensure that all buffers are low in endotoxin (essentially "endotoxin-free") and prepared as sterile solutions. Avoid bubbles during pipeting and resuspension. Also take care not to exceed the recommended incubation times during the neutrophil preparation. Be especially careful during the red cell lysis step that the water is ice-cold and the 30-s time is not exceeded. Always monitor the morphology of the final leukocyte preparation before using.
4. BCECF-AM is the acetoxymethyl ester form of the dye. It is lipophilic and thus freely crosses cell membranes. Once inside the cell, BCECF-AM is hydrolyzed to BCECF, which is retained in the cytosol. Retention of the dye is an indication of cell membrane integrity.
5. BCECF-AM should be dissolved in DMSO as a 1 m*M* stock solution and stored in single-use aliquots (20–25µL) at –70°C. The solution should be colorless with no evidence of precipitate.
6. For the BCECF loading step, another buffer may be used, but the inclusion of a small amount of protein in the buffer solution is recommended to enhance the viability of the cells. Once dissolved in aqueous buffer, the BCECF should be used immediately. If the BCECF is successfully loaded, the cell pellet will be an

obvious yellow color. Although it is not necessary to be scrupulous about protecting the BCECF-labeled cells from light, prolonged exposure to bright light will quench the fluorescent signal. Therefore, it is advisable to wrap the test tube containing the labeled cells with aluminum foil.

7. Leukocyte cell lines can be fluorescently labeled and used in the same way as isolated peripheral blood leukocytes, although different cell types may vary in their ability to take up the BCECF-AM. The number of labeled leukocytes added per well in the assay should be optimized, taking into account the specific activity of labeling and the need to avoid piling up the added cells on the well bottom. Ideally, there should be adequate fluorescent signal per well at less than a saturating concentration of monolayer-bound cells. Alternatively, blood leukocytes and leukocyte cell lines can be radioactively labeled, e.g., with ^{111}Indium-oxine, to obtain a potentially more sensitive assay system ***(3)***.
8. The end point of the adhesion assay can be changed by altering the incubation time, but note that adequate time (usually at least 5–7 min) should be allowed for the leukocyte suspension to settle uniformly through the fluid in a microtiter well to reach the monolayer at the well bottom.
9. To avoid leakage problems during the centrifugation step at the end of the assay, certain precautions need to be taken. When filling the microtiter wells with medium, fluid should be added until a convex "dome" is formed. Attach the sealing tape securely, first to the straight (not notched) short side of the plate, and then attach the film across the plate, using a steady motion to force excess fluid ahead of the sealing edge. For this reason, avoid plating cells in the first and last rows of the plate (rows 1 and 12). Be sure the plate is firmly sealed on all four sides of the plate; in addition, wrap the sealed plate in absorbant paper to prevent splattering inside the centrifuge if a leak does occur. Avoid bubbles, but never try to remove tape from a plate if a bubble appears. Simply note the position of the well(s) for later reference.
10. BCECF has a pH-dependent spectrum with a maximum emission above pH 8.0; thus, the lysis buffer is adjusted to pH 8.4 to optimize the fluorescence output.
11. There are different manufactured brands of microplate fluorimeters. Some styles take the reading from the bottom, through the well, whereas others read from above the well. If your plate reader reads from above the well, it may be useful to transfer the solubilized contents of the well to a black, opaque plate to optimize the reading, since tissue-culture plastic can interfere with the reading.
12. Endothelial cells can be plated in wells of larger dimension, and the aliquots of solubilized fluorescent material then transferred to a 96-well plate for reading.
13. Fluorescence units per cell (specific activity of label) is determined by reading the fluorescence of the reserved 100 µL aliquots of labeled cells and dividing by the number of cells in the aliquot. Assay results are usually expressed as "number of adherent cells per well" or "number of adherent cells per mm^2 of target monolayer" ***(3,4)***.

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14

Cell–Cell Adhesive Interactions in an In Vitro Flow Chamber

Douglas J. Goetz, Daniel M. Greif, Jian Shen, and Francis W. Luscinskas

1. Introduction

A critical step in the recruitment of leukocytes to a site of inflammation is leukocyte adhesion to the vascular endothelium in the fluid dynamic environment of the microcirculation. This process involves a cascade of adhesive events, including initial attachment, rolling, spreading, and ultimately transendothelial migration (reviewed in ***1,2***). To elucidate the biochemistry and biophysics that govern these processes, in vitro flow chamber assays have been developed. These assays allow the study of leukocyte adhesion under well-defined fluid flow conditions that mimic the in vivo fluid dynamic environment. The parallel plate flow chamber is the design most frequently used for this purpose. Such a device was used in the late 1980s to study human neutrophil adhesion to cultured human umbilical vein endothelial monolayers ***(3)***. This group ***(3,4)*** has recently published a report that details the chamber design and operation. In general, this method involves perfusing a suspension of purified leukocytes over a coverslip containing a monolayer of cells or a region of purified adhesion molecules. Adhesive events are monitored in live time and recorded by videomicroscopy for later analysis. We present here the methodology of using the flow chamber currently used by our group to study leukocyte and tumor cell adhesion to vascular endothelium or purified adhesion molecules under defined laminar flow conditions.

2. Materials

2.1. Flow Chamber

A sketch of the flow chamber as well as a photo of the top and bottom plates are shown in **Fig. 1A** and **B**. It consists of an upper and a lower plate made of

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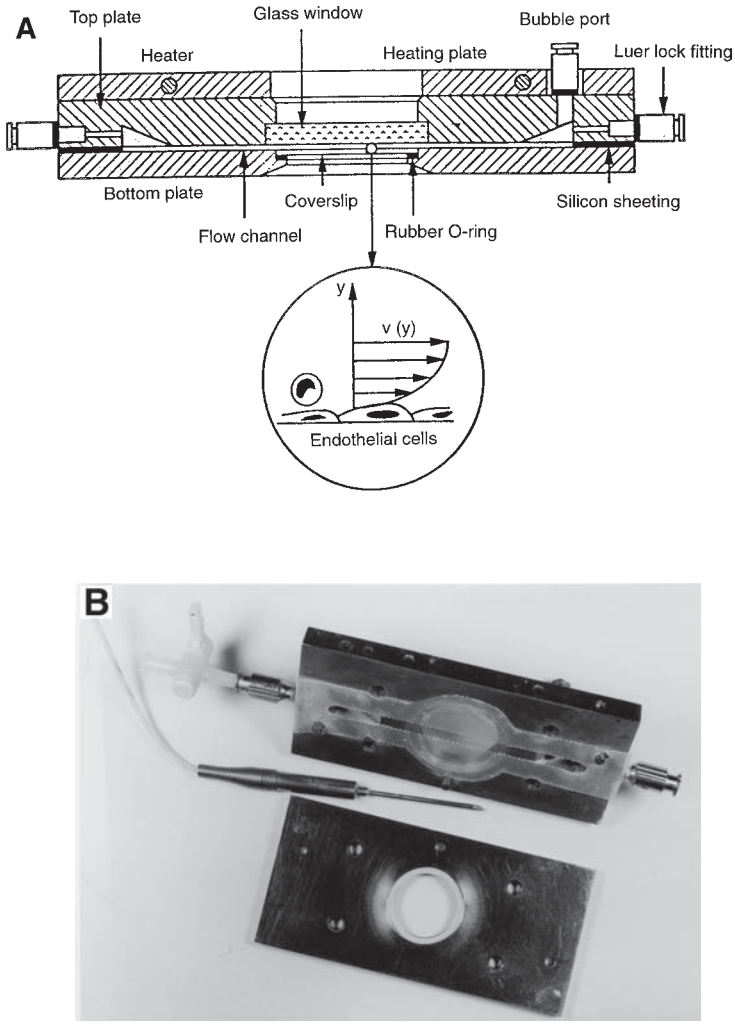

Fig. 1. **(A)** Schematic of the parallel plate flow chamber used to perfuse leukocytes across cultured endothelium under defined laminar flow. **(B)** Photograph of the top (top of figure) and bottom plates that are assembled to give the flow plate. The temperature probe pictured is inserted into the top plate. The top plate is pictured with the gasket in place.

stainless steel. The upper plate has an inlet and outlet for delivery and removal of media, a bubble trap (*see* **Note 1**), an opening for a temperature probe, and a circular cutout containing a piece of quartz glass, which allows direct visualization of events within the flow chamber. The quartz glass and glass coverslip

give efficient passage of a wide spectrum of light, making it possible to perform quantitative ***(5-7)*** fluorescence and bright-field microscopy. The holes bored for the inlet, outlet, and bubble ports have 1-cm, 12-gage needles (Z12,677-2; Sigma, St. Louis, MO) soft soldered into them. The lower plate has a circular cutout where a 25-mm diameter glass coverslip, which is coated with a cellular monolayer (the adhesive substrate), is placed. An O-ring is placed between the lower plate of the flow chamber and the coverslip to prevent fluid from leaking out of the flow chamber. A heating plate of the same dimensions as the flow chamber is used to maintain the appropriate temperature. The heating plate is made of copper and has two heating elements (10W, 120V, SC12-1, Atlantic Thermal Sales; Hopedale, MA) imbedded within its surface, which are connected to a temperature controller (4201A; Omega Corp; Stamford, CT). A thermal probe (HYP-4; Omega) is inserted into the upper plate of the flow chamber and connected to the temperature controller. Together, the heating plate and the controller maintain the temperature of the upper plate of the flow chamber at the desired temperature. The heating plate, and the upper and lower plates of the flow chamber are held together by four bolts. The upper and lower plates of the flow chamber are separated by a silastic gasket (Allied Biomedical; Paso Robles, CA), which defines the flow area, 5.0 × 80 mm, and height, 0.25 mm (*see* **Note 2**), of the flow chamber. The position and orientation of the silastic gasket are maintained by locator pins, which are imbedded in the upper plate of the flow chamber. This particular flow chamber was designed by Jian Shen, Richard Fenner, and C. Forbes Dewey at the Fluid Flow Laboratory at The Massachusetts Institute of Technology (M.I.T.) and built at M.I.T.'s machine shop. Fluid is drawn through the flow chamber at defined volumetric flow rates with a syringe pump (Harvard Apparatus, model 44; Natick, MA). We use 50-mL glass syringes (Fisher Scientific, Pittsburgh, PA).

2.2. Video Equipment

The flow chamber is mounted on an inverted phase-contrast microscope (Nikon Diaphot TMD) equipped with 10, 20, 40, and 60× phase objectives and camera/videoports. Leukocyte attachment and rolling can be observed with a 10× objective but such events as spreading and migration are best observed at higher magnification (40× or 60× objective). The videoport allows live-time viewing via a video camera connected to a monitor and a VCR. Video recording of the entire experiment allows later off-line analysis. We currently use a black- and-white Panasonic video camera (WV-1800) and black- and-white Panasonic (WV-5470) and Sony (PVM-1342Q) monitors with 16- and 12-in diagonal screens, respectively. We have found that these size screens allow visualization of neutrophil spreading and extravasation. Depending on the

application, either low- or high-end VCRs may be used (e.g., commercial grade, Panasonic NV-8050 or Sony SVO-9500). The SVO-9500 VCR, which supports digitized frame-by-frame analysis, has an RS-232 port and is connected in line with a millisecond scale time-date generator (Panasonic WJ 810), which together allow for quantification of adhesive events, such as cell rolling. Recording on Super VHS (S-VHS) mode is not necessary for most applications. To facilitate these analyses, the SVO-9500 VCR is connected through an RS-232 port to a serial port on a 486 IBM-PC running the Windows-compatible image analysis software Optimas.

2.3. Buffers

A variety of perfusion buffers can be used. It is important that the buffers contain Ca^{2+} since some of the adhesion processes, i.e., selectin mediated interactions, are Ca^{2+} dependent. We prepare, fresh for each experiment, a buffer that consists of DPBS containing 0.75 m*M* Ca^{2+}, 0.75 m*M* Mg^{2+}, and 0.5% human serum albumin ***(8–10)***. The 0.75 m*M* Ca^{2+} and Mg^{2+} DPBS are made by mixing DPBS 1:1 (vol:vol) with DPBS containing 1.5 m*M* Ca^{2+} and Mg^{2+}. Both DPBS solutions are purchased from BioWhittaker (Walkersville, MD) and are endotoxin-free.

2.4. Cell Preparation

Human umbilical vein endothelial cells, and various cell lines, such as Chinese Hamster Ovary or murine L-cells stably expressing E-, P-selectin, VCAM-1, or ICAM-1, have been used as adhesive substrates ***(9,10)***. Culture conditions for these cell types have previously been given ***(9,10)***. Chapter 13 of this volume contains a protocol for the isolation of human neutrophils from whole blood. Techniques for isolating human lymphocytes ***(9)*** and monocytes ***(8)*** from blood have been given previously.

2.5. Disposable Supplies

Three-way stopcocks, plastic couples and $^{1}/_{16}$th in inner diameter tygon tubing (Fisher Scientific) are used to create the lines that deliver and remove buffer from the chamber.

3. Methods

3.1. Preparation for the Flow Chamber Assay

1. Place parafilm around microscope objectives for protection from excess flow buffer, which may cause deposit of salts on microscope components and corrosion of the objectives in the turret.
2. Warm flow buffer to desired temperature by incubating in water bath for several hours (*see* **Note 3**). Preheat the flow chamber by placing the heating plate on top of the upper plate. Insert the thermal probe into the upper plate and heat upper plate to the desired temperature (*see* **Fig. 1B**).

3. Place the silastic gasket on the underside of the upper plate (as shown in **Fig. 1B**). Use the locator pins to position the gasket and align the gasket such that the short edges of the gasket's rectangular space are centered on the plate's inlet and outlet. Ensure that the gasket is smooth and its width is constant.
4. Assemble tubing connections to the outlet, inlet, and bubble ports:
   a. Place a three-way stopcock in the outlet port;
   b. Connect one end of tubing to the inlet port and the other end to a three-way stopcock. Attach a 10-mL syringe full of buffer (inlet syringe #1) to the port of the three-way stopcock that is 180° from the port connected to the tubing, and attach an empty 10-mL syringe, without plunger (inlet syringe #2), to the third port of the three-way stopcock;
   c. Connect a three-way stopcock to a separate piece of tubing, and attach the tubing to the bubble port.
5. Secure outlet 50-mL glass syringe in syringe pump and set syringe pump to desired flow rate.
6. Place a videotape in the VCR, and record the dimensions of the screen via a microscope stage micrometer. Record under objective magnifications to be used in the experiments.

3.2. Using the Parallel Plate Flow Chamber

1. Place a minimal amount of vacuum grease on four spots on the O-ring on the lower plate. Use forceps to position the cell-coated coverslip on the O-ring with cells facing up. Gently place a few milliliters of flow buffer on the coverslip and across the flow path on either side of the coverslip.
2. Gently and carefully place upper plate (with heating plate on top) on lower plate. Gradually tighten screws connecting the plates, alternating between the screws (*see* **Note 4**).
3. Close outlet port, and gently push buffer through inlet syringe #1 connected to the inlet port. Buffer should go through bubble port and stay at a steady level in tubing. If buffer level decreases, there is a leak in the system: tighten screws further as in previous step. When buffer level is steady in the bubble line, use the three-way stopcock to close off bubble port.
4. Open outlet port, and use inlet syringe #1 to push buffer through chamber. Remove bubbles, but do not disturb monolayer (*see* **Note 5**).
5. Close inlet and outlet ports, dry off underside of coverslip completely with an absorbent disposable paper (kimwipe), and transfer the assembled flow chamber to inverted microscope stage. Connect outlet port three-way stopcock to outlet syringe via tubing. Move the microscope stage in order to determine the number of monitor screens between the edges of the gasket and, thus, more precisely measure the width of the flow field.
6. Fill inlet syringe #2 with desired cell suspension. Open outlet and inlet three-way stopcocks. Turn on pump. Record experiment on videotape. When experiment is concluded, disconnect outlet port from tubing, and remove chamber from microscope stage. Disassemble chamber and repeat.

3.3. Analyses of Leukocyte-Endothelial Adhesion Events

3.3.1. Adhesion

Adhesion data are usually reported as a function of the shear stress at the bottom surface of the flow chamber (*see* **Note 6**). Routinely, we perfuse the leukocyte suspension for 10 min and determine the number of leukocytes bound per unit area. Quantification of spreading and migration has been given previously ***(8–10)***.

3.3.2. Rolling Velocities

A feature of leukocyte-endothelial cell interactions under flow is that the adherent leukocytes routinely exhibit a translational velocity in response to the fluid shear. This phenomena is commonly referred to as rolling and is characterized by a low-velocity, high-variance translation **(11)** (*see* **Note 7**). Rolling velocities can be determined either manually (*see* **Note 8**) or through automation (*see* **Note 9**). In both cases, the goal is to determine the displacement, d, of the leukocyte over a period of time, t (*see* **Note 10**). The velocity is then easily calculated by $v = d/t$.

4. Notes

1. Bubble trap: A third port on top of the plate was added in order to facilitate removal of air bubbles that arise during assembly of the chamber.
2. The gasket we use is Duralistic I Sheeting of a thickness of 0.25 mm. This thickness defines the approximate height of the flow chamber. However, the gasket may be compressed slightly when the flow chamber is assembled. To estimate the exact height of the flow field, one can focus on the top surface of the coverslip and subsequently on the underside of the top plate of the flow chamber. The distance the objective moved, which can be determined from the gradation of the focus knob, is an estimate of the exact height of the flow field. From the Duralistic I Sheeting, we cut a gasket with an inner dimension of 5 mm (width) by 80 mm (length). The width of the flow field should be large relative to the height (e.g., 20:1 ratio), so that the flow may be approximated as unidirectional and the fluid velocity is spatially dependent only on the distance from the bottom surface of the flow chamber ***(12,13)***. Data should be acquired in the middle of the flow chamber, away from the edges. With the assumption of unidirectional, laminar flow, the equation for the shear rate at the bottom surface of the flow chamber is given by $\gamma = 6Q/wh^2$, where Q = the volumetric flow rate, w = the width of the flow field, and h = the height of the flow field ***(3,4,12,13)***. The shear stress, τ, is related to the shear rate, γ, by $\tau = \gamma\mu$ where μ is the viscosity of the fluid used in the experiments, typically 0.007 poise for a dilute saline solution at 37°C. Thus, $\tau = 6Q\mu/wh^2$. Any of these parameters could be changed to obtain various shear stresses. Typically, however, the geometry of the chamber is held constant while the volumetric flow rate is altered.

3. Our experiments are performed with the flow buffer and flow chamber at 37°C. When the buffer is preincubated in the 37°C heating bath for an hour or less, bubbles in the flow chamber are more common than when the preincubation is done for several hours.
4. Tightening the screws in a gradual sequential manner avoids cracking the glass coverslip. If this does happen, remove the coverslip, and start again with a new coverslip.
5. The force used to push fluid through the chamber must be a balance: enough to remove the bubbles, but not so strong that the monolayer is disturbed.
6. Adhesion under flow involves a balance between the disruptive force and torque exerted on the leukocyte by the flow of the fluid, and a specific adhesive force and torque mediated by complementary receptor pairs on the surface of the adherent leukocyte and the endothelium ***(14)***. The force and torque exerted by the flow of the fluid, which can be estimated from various theoretical models ***(14,15)***, are related to the shear rate, or shear stress, at the leukocyte–endothelium interface. In addition, several studies have reported in vivo vessel wall shear rates and shear stresses ***(16,17)***. Therefore, in vitro adhesion data are usually reported as a function of the shear stress at the leukocyte–endothelium interface, which is the shear stress at the bottom surface of the flow chamber.
7. The term low velocity refers to the fact that the leukocytes roll at a velocity routinely <10% of the predicted velocity of a noninteracting leukocyte ***(11)***. The theoretical velocity of a noninteracting leukocyte can be estimated from the work of Goldman et al. ***(15)*** and depends on the radius of the leukocyte, the shear rate at the fluid coverslip interface, and the distance the leukocyte is from the surface of the coverslip. If the distance is assumed to be 50 nm, a rough estimate of the theoretical velocity is $^1/_2\ \gamma\alpha$ where γ = the shear rate at the coverslip and a = the radius of the leukocyte. (Strictly, these solutions are only valid if the particle Reynolds number is low, i.e., << 1. The particle Re number is $a^2\gamma\rho/\mu$ where a and γ are defined as above, ρ = the density of the fluid, and μ = the viscosity of the solution. Therefore, typical particle Re numbers are << 1. Also, the solutions assume a uniform shear flow. For the flow chamber discussed in this work, this is a valid assumption owing to the fact that the height of the flow field is much greater than the radius of the leukocytes.) High variance refers to the fact that the leukocytes do not roll with a constant velocity, but rather accelerate and decelerate as they roll across the monolayer surface ***(11)***.
8. In manual mode, the displacement of the leukocyte can be determined either by recording the experiment with a reticle in place or by placing a template over the screen during the analysis. A good template is a clear plastic overhead with calibrated gradations made with a marker. One can simply determine the time required for the leukocytes to roll a given distance and calculate the rolling velocity.
9. Automated image analysis can be done using NIH Image or commercially available software (Optimas, Bioscan Inc., Edmonds, WA).
10. As leukocytes roll over the endothelium, they may detach from the endothelium and then reattach several cell diameters downstream from the point of detach-

ment. These are usually referred to as skipping leukocytes and are routinely not included in the data set used to determine rolling velocities.

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15

Adhesion of Tumor Cells to Endothelium Under Static Conditions

Renato G. S. Chiarivi and Raffaella Giavazzi

1. Introduction

A variety of adhesive interactions take place between tumor cells (TC) and vascular endothelium in tumor progression and metastasis ***(1)***. One of the most important events of the metastatic cascade is the arrest of circulating TC in the capillary bed of secondary organs followed by the interaction with vascular endothelium and subendothelial basement membrane—events that precede TC extravasation and then metastasis formation. Preferential interaction between tumor cells and the microvascular endothelium/extracellular matrix of different areas have been reported to contribute to the homing of metastasis ***(2)***.

The acquisition of techniques able to isolate and culture endothelial cells (EC) **(3)** allows the investigation of the adhesive properties of EC and the molecular mechanisms involved in TC–EC interaction. EC exposed to inflammatory stimuli, such as interleukin-1 (IL-1) and tumor necrosis factor α (TNF) exhibit increased adhesiveness to leukocytes and in analogy to TC ***(4,5)***. In animal models in vivo, these findings have resulted in the augmentation of metastasis ***(6,7)***.

The use of MAbs against adhesion molecules expressed on EC or their ligands on TC allows the investigation of the molecular mechanisms involved in TC–EC interaction. Selected patterns of adhesive molecular mechanisms appear to regulate the adhesion of different types of TC to EC ***(7)***.

Methodologies are described below for investigating the adhesion of TC to resting and cytokine-activated EC. Human umbilical vein endothelial cells, activated by IL-1 and TNF, are proposed as routine substrate, but EC of different origin, modified by a variety of stimuli and conditions, can be used. Antibodies

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against adhesion molecules expressed on EC can then be used to study their role in TC interaction with EC (*see* Chapter 16).

These experiments define the adhesive behavior of TC and investigate the specific role of adhesion molecules expressed/induced on EC. The adhesion pattern of TC of different origin and malignant behavior can also be investigated.

2. Materials

2.1. Cell Cultures

1. Target TC line in routine culture medium.
2. Human umbilical vein endothelial cells (EC) were isolated as described ***(3)***.
3. 96-well plates (tissue-culture-treated) coated with 0.2% gelatin overnight at 37°C.
4. Culture medium for EC: M199 + 10% fetal calf serum (FCS) + 10% newborn calf serum (NBCS) + Heparin (6 U/mL) + ECGF (50 µg/mL) + L-glutamin (2 m*M*)

2.2. Preparation of 5-Iodo-2-Deoxyuridine -[I125]

1. 5-Iodo-2-Deoxyuridine-[I125] ([^{125}I]IUdR) (Amersham, 1 mCi/mL stock).
2. Physiological solution (0.9% NaCl).
3. 0.2-µm sterile filter.
4. γ-Counter and vials compatible with the counter.
5. Lead protection screen (*see* **Note 2**) and Geiger (*see* **Note 3**).

2.3. Preparation of Radiolabeled Tumor Cells

1. Culture medium containing 20% FCS.
2. Minimal essential medium (MEM) with 0.2% bovine serum albumin (BSA) (test medium).
3. Centrifuge and incubator at 37°C and 5% CO_2.
4. [^{125}I]IUdR, 10 µCi/mL stock (*see* **Note 4**).
5. γ-Counter and vials compatible with the counter.
6. Lead protection screen (*see* **Note 2**) and Geiger (*see* **Note 3**).

2.4. Endothelium Activation

1. 96-Well plates with confluent EC cultures.
2. Culture medium for EC (*see* **Subheading 2.1.**).
3. Cytokines in stock solution (*see* **Note 5**).

2.5. Adhesion to Endothelium

1. 96-Well plates with confluent resting or activated EC cultures.
2. MEM with 0.2% BSA (test medium).
3. 0.1 *N* NaOH solution.
4. Cotton swabs.
5. γ-Counter and vials compatible with the counter.
6. Lead protection screen (*see* **Note 2**) and Geiger (*see* **Note 3**).

3. Methods

3.1. Cell Culture

1. Culture the target TC as usual.
2. Plate 4 d prior to the adhesion experiment 5×10^3 EC in each well of a 0.2% gelatin coated 96-well plate (*see* **Note 6**).

3.2. Preparation of 5-Iodo-2-Deoxyuridine-[I125]

1. Dilute 10 µL of [^{125}I]IUdR (Amersham, 1 mCi/mL stock) in 990 µL physiological solution. Final [^{125}I]IUdR stock = 10 µCi/mL.
2. Pass the solution through a 0.2 µm filter, and keep it sterile.
3. Check activity by adding 10 µL in a γ-counter vial, and determine counts per minute (cpm) with a γ-counter (expected count range 100,000–200,000). (*see* **Note 7**).
4. Store refrigerated.

3.3. Preparation of Radiolabeled Tumor cells

1. Two days prior to labeling TC, split cells from a confluent 75-cm^2 tissue-culture flask at a 1:5 dilution (*see* **Note 8**).
2. Label cells by refeeding them with 10 mL fresh medium with 20% FCS, and add 200 µL of [^{125}I]IUdR (stock 10 µCi/mL) to each 75-cm^2 tissue-culture flask (*see* **Note 8**). Incubate at 37°C for 24 h.
3. Twenty-four hours after label addition, wash and refeed flask twice to remove nonincorporated label. Harvest TC as usual. Wash them by centrifugation with culture medium, and then again with test medium (*see* **Note 7**). Count cells by trypan blue exclusion and bring cells to a concentration of 3×10^4 cells in 50 µL of test medium.
4. Determine input counts of labeled cells by adding 50 µl of the labeled cell suspension in γ-counter vials (in triplicate). Place tubes in the γ-counter and determine input cpm by counting 3 min/vial (*see* **Note 9**).

3.4. Activation of Endothelium

1. Dilute the cytokines you want to use in culture medium for EC (*see* **Subheading 2.1.**) at the desired concentration (*see* **Note 5**).
2. Discard the culture medium from the wells with confluent EC, and add 100 µL of the above cytokine solution.
3. Incubate for 4–6 h (routine-time point) in an incubator at 37°C and 5% CO_2 (*see* **Note 5**). EC are now ready for the adhesion assay as in **Subheading 3.5.** (*see* **Note 10**).

3.5. Adhesion to Endothelium

1. Wash the 96-well plates containing the confluent EC twice with test medium, and add 50 µl of test medium in each well (*see* **Notes 11** and **12**).

2. Plate 3×10^4 TC in 50 µL in the EC wells. The total volume per well is now 100 µL (*see* **Note 13**).
3. Leave your adhesion assay plates in an incubator at 37°C for 15–30 min (*see* **Note 14**).
4. Wash the 96-well plates twice with 100 µL test medium in order to eliminate all the nonadhered TC (*see* **Note 12**).
5. Add 100 µL 0.1N NaOH solution in each well, and leave for 15 min. In this way, the cells dissolve.
6. Absorb and clean the wells twice with a cotton swab, and put the swabs from each well in an individual γ-counter vial.
7. Count each vial for 3 min in a γ-counter.
8. Results have to be expressed as percentage of attached cells relative to total added cells: percentage attached cells = (cpm test well / cpm input) × 100% .

4. Notes

1. Alternative types of EC generally used in this assay are bovine aortic EC and mouse microvascular EC; sometimes transformed EC are used. It is critical that EC form a monolayer to be used as adhesive substrate.
2. [^{125}I]IUdR is a γ-transmitter. For your own and the protection of others, always use two pair of latex gloves, and work behind protection screens made of lead.
3. Always control your work space after the experiments with a Geiger, and if necessary, perform a smear-test.
4. Alternative labeling for TC can be used, such as ^{51}Chromium or ^{111}Indium-oxine. Moreover nonradioactive labeling for TC has been proposed, though the sensitivity of the assay might be somewhat lower ***(8)***. TC adhered on EC monolayer can also be estimated with the aid of a microscope, and EC–TC ratio reported ***(4)***.
5. TNF and IL-1 are the cytokines most commonly used to activate EC ***(4)***, alone or in combination with other cytokines; other cytokines can be tested. Preliminary experiments, including dose–response and kinetics of EC response, might be necessary to define their appropriate concentrations and time for EC activation (5).
6. Check carefully on the day of the experiment (day 4) with a microscope if the EC have formed a monolayer. If not, do not perform the experiment, since the TC might adhere to gelatin or extracellular matrix produced by the EC.
7. Pay attention that all your radioactive solid and liquid waste is discarded in a proper way.
8. Target cells should be in log-growth-phase at the time label is added. Each target line grows at a slightly different rate. Adjust dilution at time of split accordingly.
9. Input cpm at 3×10^4 labeled cells should be at least 2250. If labeling is higher than 0.5–1.0 cpm/cell, radiotoxicity might occur. Furthermore, if labeling is >1.0 cpm/cell and cells were labeled in 0.3–0.5 µCi [^{125}I]IUdR/mL culture medium, one should suspect Mycoplasma contamination.
10. If you use blocking MAbs to adhesion molecules, preincubate resting or activated EC with test medium containing the antibodies for additional 30 min at 37°C before the adhesion assay, and leave the antibody during the assay.

11. From here on, it is not necessary to work in pathogen-free conditions.
12. Aspiration and refeeding with test medium must be done carefully. Try to avoid detaching EC with these procedures. Avoid using cold (4°C) test medium that might shock the cells; it is always better to prewarm it before use.
13. Each experimental group has to be performed at least in triplicate.
14. Each target TC line adheres with different speed to EC. Adjust incubation time accordingly.

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16

Adhesion of Tumor Cells Under Flow

Andrea Remuzzi and Raffaella Giavazzi

Introduction

The interaction between circulating tumor cells and the vascular wall is a crucial step in the process of extravasation of these cells and in the subsequent formation of the metastasis. Tumor cells extravasation is believed to be similar to that of leukocites across the vascular wall during inflammation. It has been documented that initial interaction of leukocytes with endothelial surface is characterized by their rolling over the cell surface, which can be followed by firm adhesion of the cells to the vessel wall. It is believed that in some conditions adherent cells can migrate across the endothelial layer and extravasate within the vascular wall ***(1–4)***.

To study the mechanisms responsible for the complex interactions between circulating tumor cells and the vascular wall, in vitro experiments are widely used. The aim of this experimental work is to document the basic steps of these interactions, to investigate which molecules are involved in these processes, and to test the potential effects of blocking the biological activity of these molecules. Since the interaction between circulating tumor cells and the endothelium is strongly influenced by flow conditions induced by blood motion on these cells, in vitro studies are based on a perfusion system that allows mimicking of the fluid dynamics of postcapillary venules. There are several factors physically influencing the flow environment, and there are different cellular and physicochemical components of the fluid suspension that can be investigated. In the following, we will describe experiments aimed at documenting the dynamic interaction of tumor cells with monolayers of cultured endothelial cells. The experimental procedures can be applied to other cell types and to different flow environments using a similar approach.

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2. Materials

2.1. Tumor and Endothelial Cell Preparation

Different types of tumor cells can be used to investigate their interaction with endothelial cells in vitro. We have previously used ***(5)*** cells derived from colon carcinoma, ovarian carcinoma, breast carcinoma, melanomas, and osteosarcoma. Tumor cells are harvested by brief exposure to 0.25% trypsin and 0.02% EDTA, washed twice, and resuspended at a concentration of 10^6 cells/mL in Eagle's Minimal Essential Medium containing 0.1% bovine serum albumin.

Endothelial cells are isolated from human umbilical veins (HUVEC) and grown in culture as previously described ***(6,7)***. Cells are grown in M 199 medium supplemented with 10% fetal calf serum and 10% human serum. HUVEC purity is assessed by indirect immunofluorescence microscopy using rabbit antihuman Factor Vlll antigen. HUVEC at passage 3–4 are plated on 40×22 mm plastic coverslips (Thermanox) coated with gelatin. Cells are used for adhesion assay 2 d after reaching confluence.

2.2. Substrate Preparation

Coverslips with HUVEC monolayers are placed in a standard culture dish and, if required, activated by a 4-h incubation at 37°C with IL-1 at the concentration of 20 U/mL in Ml99 medium with 10% fetal calf serum. Before use for adhesion experiments, untreated and IL-1-treated HUVEC monolayers are washed twice with test medium. Antibodies directed against adhesion proteins are added directly to stimulated or unstimulated HUVEC monolayers at a final concentration of 1:50. Cell monolayers are used for adhesion experiments after 30 min of incubation at 37°C.

2.3. Experimental Apparatus

For adhesion experiments, a parallel plate flow chamber is used. The chamber allows to obtain laminar flow to be obtained over cell surface because of the very small distance (250 μm) between two parallel plates. The perfusion chamber is obtained by the assembly of three parts:

1. A transparent plastic material (machined from polymethacrylate material) containing inlet and outlet conduits;
2. A Silastic® (medical-grade) gasket (254 μm thickness); and
3. A stainless-steel plate with a grove for microscopic field.

The assembly of the chamber is performed with the following procedure. Inlet and outlet port of the chamber are filled with test medium, the gasket is put in place, and the coverslip containing the endothelial monolayer is placed

over the gasket. The metal part of the chamber is then placed over the coverslip, and four screws are used to close the chamber. Gentile and uniform pressure on each screw must be applied in order to avoid cell damage and leak of the perfusion solution. A rectangular adhesion surface of 30 × 10 mm is obtained on the HUVEC monolayer. The inlet and an outlet channel are machined in the chamber with a geometry that allows to distribution of the fluid uniformly along the entrance side of the adhesion surface.

After assembly, the perfusion chamber is placed on the stage of an inverted phase-contrast microscope equipped with a thermostated hood to maintain 37°C. The microscope is equipped with a video recording system consisting of a CCD video camera, a high-resolution monitor, and a VHS video recorder. Test medium is aspirated from a test tube containing the tumor cell suspension through the chamber using a syringe pump (Harvard Apparatus, Natick, MA). The wall shear stress (τ_w) on the HUVEC surface is calculated as a function of medium flow rate (Q) using Poiseuille's equation:

$$\tau_w = 3\,\mu\,Q / (2\,b^2\,w)$$

where b is the channel half height, (w) is the channel width, and μ is the dynamic viscosity of the cell suspension at 37°C. We have previously measured cell suspension viscosity at 37°C with a glass capillary viscometer and obtained a mean value of 0.8 cP.

3. Methods

3.1. Adhesion Assay

Fresh medium is initially perfused at 3.0 dyn/cm^2 for 5 min for equilibration. Tumor cell suspension is then perfused through the chamber at the same flow rate. To investigate the effect of different shear conditions, different flow rates can be used to obtain shear stress values ranging from 0.3–3.0 dyn/cm^2. At higher values of shear stress, cell adhesion decreases and only a few cells can attach to the endothelial surface. At the beginning of the perfusion, the video recording system is started. During the perfusion, the flow chamber must be kept in a steady position, and the focus should be adjusted if required. After perfusion of the 10 mL of cell suspension, the flow is stopped and test medium without tumor cells is perfused through the chamber for an additional 3 min for washing.

3.2. Image Acquisition and Analysis

All adhesion experiments are videotaped for quantification of adherent and rolling cells. Images are digitized from the videotape recorder using a computer-based image analysis system, consisting of a personal computer, a digitizing board at 256 levels of gray, and general-purpose image analysis software. We use the software NIH.Image (v 1.59). Firmly attached cells are iden-

tified and counted using an averaging technique. Briefly, an image is obtained by averaging screen pixels in four images sequentially digitized within an interval of 4 s. This acquisition is made by the multiframe acquisition option of the digitizing software. Using this procedure, cells not firmly attached to the surface, such as rolling cells and cells flowing in the suspension, are not visible on the image. Only cells remaining in the same position during the acquisition interval contribute to form the image. The number of adherent cells per field is then measured during the perfusion experiment (each minute or more frequently) to estimate the dynamics of the adhesion phenomena.

To measure the number of rolling cells on the endothelial surface and to estimate their mean velocity, the following procedure is adopted. Using multiframe acquisition, a series of 16 images is digitized during an interval of 8 s. The images are then stored in memory, and a postprocessing software developed by us is used to superimpose the image stack. The procedure used is the following. For each pixel in each image of the stack, the highest value is calculated and used to create the resulting image. In the resulting image rolling cells, owing to their movement, draw a wake. The length of the wake is the distance traveled by the rolling cell during the acquisition interval. The number of rolling cells is then counted, and the rolling velocity of tumor cells is calculated by dividing the length of the wake by the acquisition interval. For the calculation of the absolute number of cells per unit surface area and the rolling velocity as μm/s, exact enlargement of the images (μm/pixel) is calculated from screen pixel coordinates using the image of a reference grid (125×125 μm). More than 30 cells in each perfusion experiment are used to calculate mean rolling velocity.

Additional information that can be obtained by the video-microscopy observation is the strength of cell adhesion. Different cells may interact with the endothelium in a different way, and this can result in a different strength in the binding of circulating cells to the endothelial surface. To estimate this strength of adhesion, after perfusion of the cells at low shear stress (0.3 dyn/cm^2) the cell suspension perfusion rate is suddenly increased to 5 dyn/cm^2. Circulating cells do not adhere to or roll on the endothelial surface at high shear, and cells that are initially in contact with the endothelium are suddenly subjected to mechanical forces that tend to detach them from the surface. The changes in number of cells in contact with the surface after the step increase in shear is used as a measure of the strength of the adhesion. If all cells originally adherent remain in the same position and do not detach, this represents a condition of very strong adhesion bounds, whereas, on the contrary, if several cells detach or roll on increase in shear forces, this indicate weak adhesion bounds between tumor cells and the endothelial surface.

3.3. Functional Blockade of Rolling and Adhesion

To study the role of adhesion molecules involved in the processes of rolling and firm adhesion on lL-1-activated endothelium, specific monoclonal antibodies (MAbs) can be used. To this purpose, IL-1-activated HUVEC must be incubated before the perfusion experiment with MAbs against selectins (i.e., E-selectin, P-selectin, and so forth) or integrins (i.e., ICAM-1, VCAM-1, and so on). The effect of these molecules on the process of adhesion and rolling is measured in terms of prevention of rolling and adhesion, respectively. More selective investigations of the role of these molecules in mediating the adhesion process can be obtained using coverslips covered by individual proteins instead of the endothelial monolayer. We have previously shown ***(5)*** that in these conditions, the distinct phases of the adhesion process can be reproduced separately, such as continuous rolling of tumor cells on immobilized selectins and firm adhesion without rolling on integrins.

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17

Platelet Adhesion to the Subendothelium Under Flow

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1. Introduction

The adhesion of cells is normally studied in the absence of flow. In a classic setup, ELISA wells are coated with an adhesive protein and subsequently incubated with a cell suspension. The gravity is responsible for the transport of the cells to the adhesive surface. This setup is not appropriate for blood cells, such as platelets, because platelet adhesion occurs physiologically under flow conditions. The shear stress induced by the flow causes qualitative and quantitative differences in adhesion ***(1)***. Blood platelets have a relatively low density and do not settle easily under gravity. Under flow conditions, convective diffusion of platelets to the vessel wall is responsible for a strong increase of platelet number near the reactive surface, resulting in a strongly enhanced platelet adhesion. A second important effect of flow is mediated by the shear stresses exerted on the platelets. To overcome these stresses, a special protein, the von Willebrand factor, is necessary, which has a very high affinity for the platelet membrane receptor glycoprotein Ib. In the absence of flow, the presence of von Willebrand factor is not necessary for platelet adhesion, but with increasing shear rates, the presence of von Willebrand factor becomes essential. Another disadvantage of studying platelet adhesion under static conditions is that owing to the presence of red blood cells, these experiments must be performed with washed platelets, and the required centrifugation steps cause unwanted platelet activation.

The sequence of events leading to a platelet thrombus includes a reversible attachment of platelets from the circulation to a surface (contact platelets) followed by spreading out of the platelet on the surface. Other platelets will inter-

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act with the spread platelet, forming a platelet aggregate. Platelet adhesion is defined as the sum of contact and spread platelets that make contact with the adhesive surface. Adhesion can be expressed as the number of adherent platelets per unit area or as % of the total surface covered with platelets. Platelets interacting with other platelets already adhered to the surface are not included in this definition. Platelet deposition is defined as the total number of platelets deposited per unit surface, and includes both platelet adhesion and platelet–platelet interaction.

Experiments are described below for the investigation of platelet adhesion to different reactive surfaces under flow conditions. The experiments are based on the use of a perfusion chamber developed in our laboratory *(2)*. Similar results were obtained with other perfusion chambers following the same principles *(3–6)*.

2. Materials

To study platelet adhesion under flow, a specially designed perfusion system is necessary, which creates a laminar, nonturbulent blood flow. Although in the circulation the flow is pulsatile and considerably more complicated, simplifications are necessary to begin to understand the complicated process of platelet–vessel wall interaction. The utilization of laminar flow makes possible the calculation of shear rates and shear stresses, and the comparison of these with those in various parts of the vasculature. The description of the rheologic parameters of the flowing blood is beyond the scope of this chapter, and the reader is referred to an excellent overview by Goldsmith and Turitto *(7)*.

A perfusion system consists of a perfusion chamber and a flow device connected by silastic tubing with an inner diameter of 3.0 mm. If a system with anticoagulated blood is also used, two containers (one to hold the blood and one with washing buffer) are necessary. In the case of recirculating the perfusate a funnel is also introduced. If the blood is drawn directly from the antecubetal vein through the perfusion chamber, a separate container with washing buffer is necessary. This container with washing buffer is also connected with the perfusion chamber. The perfusion system and the container are placed in a water bath at 37°C.

2.1. Perfusion Chambers

A perfusion chamber must fulfill the following criteria:

1. Allow studies at shear rates throughout the physiological range;
2. Allow easy incorporation of surfaces to which platelets adhere; and
3. Allow a relatively easy measurement of the number of platelets interacting with the surface.

The first popular perfusion chamber developed was the annular perfusion chamber of Baumgartner and Haudenschild ***(1)***. In this perfusion chamber made of Plexiglas, a rabbit aorta was everted and mounted on a central rod, which fitted into a cylindric chamber. The blood flowed through the annular space between the surface of the everted vessel and the inner wall of the outer cylinder. By varying the blood flow, a limited range of shear rates could be achieved. For larger variations, the distance between the inner wall of the outer cylinder and the surface of the vessel wall segment had to be varied. Although this original perfusion chamber has taught us the basic principles of platelet adhesion, the technique is very laborious and sensitive to experimental variation. For platelet adhesion studies, the parallel plate perfusion chambers have become popular in recent years.

Several parallel plate perfusion chambers have been developed; our own experience has been with the chamber developed in our laboratory in the early 1980s by Sakariassen et al. ***(2)***. The chamber is shown in **Fig. 1**. The perfusion chamber is made of Plexiglas. The characteristic feature of the chamber is the rectangular cross-section of the flow slit and the presence of two plugs on which a depression is present in which a coverslip fits. The coverslips are exposed to the blood flow when the plug is introduced to the chamber. Adhesive proteins can be adsorbed, or cells can be cultured on the coverslip. The exposed area of a single coverslip is 1.6 cm^2. The height of the slit through the chamber in combination with the flow rate determines the shear rate. The inlet and outlet portals are circular, but taper gradually off to the rectangular dimensions, keeping the flow laminar. The shear rates present in the chamber can be calculated by the formula:

$$\gamma = \frac{6Q}{ab^2}$$

where γ is the shear rate, Q = average flow rate (cm^2/s), a = slid width (cm), and b = slit height (cm).

2.2. Flow Device

Originally the blood was recirculated through the perfusion chamber with the help of a roller pump. It is essential to use a roller pump that is not occlusive to prevent mechanical lysis of red cells. The perfusate is pumped from a container through the perfusion chamber back into the container. A roller pump produces a pulsatile flow. To create a steady flow through the perfusion chamber, a funnel is included between the pump and the perfusion chamber. The height between the funnel and the container determines the flow rate through the perfusion chamber.

A better solution is to use a syringe pump placed distally to the perfusion chamber. The advantage is that the blood is not recirculated, reducing possible

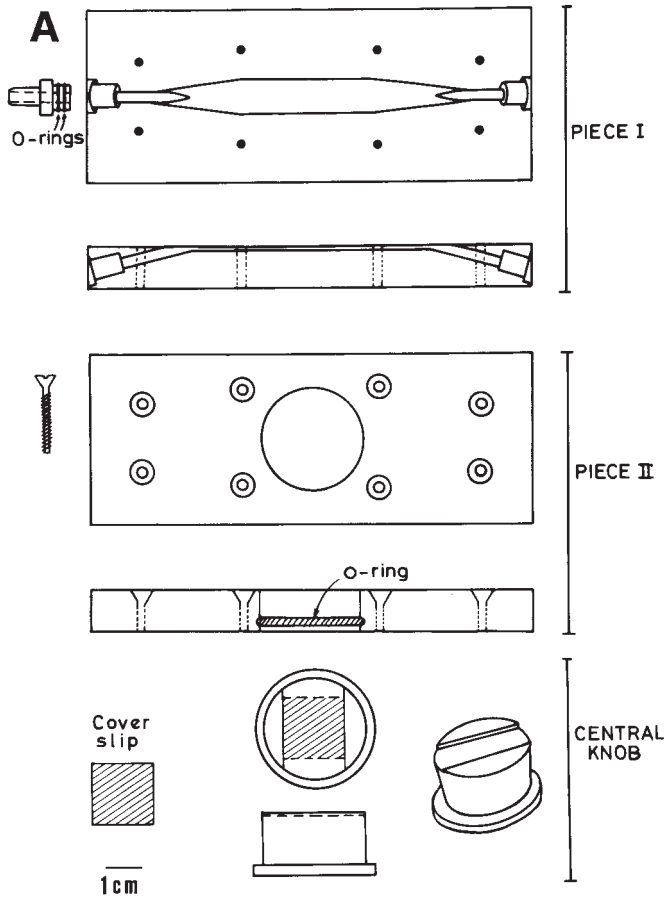

Fig. 1A. Schematic drawing of the parallel plate rectangular perfusion chamber.

systematic platelet activation to a minimum. The disadvantage is that more blood is needed. To reduce the amount of blood, smaller perfusion chamber are presently being developed.

2.3. Blood

The choice of the perfusate depends on the aim of the investigation. If the pathophysiological mechanism of platelet adhesion is studied without the influence of activation of the coagulation cascade, anticoagulated whole blood can be used. The anticoagulant of choice is 20 U/mL low-mol-wt heparin, because platelet adhesion to a number of surfaces, such as collagen types I and III, depends on the presence of Mg^{2+} (thus the use of citrated blood is unsuit-

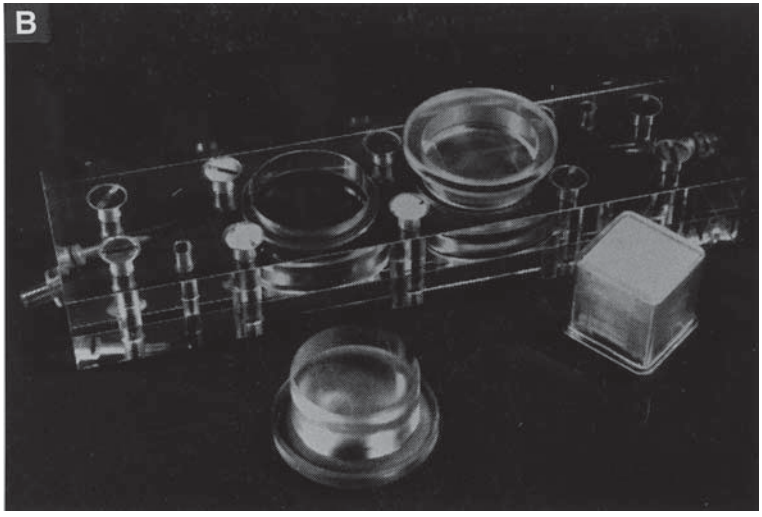

Fig. 1B. Picture of the perfusion chamber. The coverslip is mounted on the plug, introduced in the chamber, and exposed to flow.

able). High-mol-wt heparin might influence platelet function. Citrated blood ($^1/_{10}$th vol of 108 m*M* trisodium citrate) can be used if the extracellular matrix of the endothelial cell surface is used as an adhesive surface. Alternative ways for achieving anticoagulation are the use of hirudin (20 U/mL) or (PPACK) *o*-phenylalanyl-*L*-propyl-*L*-arginine chloromethyl ketone dihydrochloride (40 μ*M* final concentration), but during perfusions, these inhibitors do not completely prevent thrombin formation during the course of the experiment. The advantage of using anticoagulated blood is that the molecular mechanisms responsible for the adhesive process can easily be studied. The disadvantage is that an important part of the hemostatic process, the coagulation cascade, is excluded.

If platelet adhesion is studied in the presence of an active coagulation cascade, nonanticoagulated blood must be drawn directly from the antecubital vein of the donor through the perfusion chamber. A venapuncture is performed with a butterfly infusion set number 19. The disadvantage of this method is that owing to massive fibrin deposition, especially at lower shear rates, platelet adhesion is sometimes difficult to determine.

An attractive method to study the role of plasma components is the use of reconstituted blood. Reconstituted blood consists of washed platelets and washed red cells resuspended in (patient) plasma or buffers containing various purified proteins. Blood anticoagulated with citrate is used to prepare reconsti-

tuted blood. After centrifugation for 10 min at 150*g*, the platelet-rich plasma (PRP) and the packed red cells are separated. The PRP is mixed 1:1 with a Krebs-Ringer buffer, pH 5.0 (4.0 m*M* KCl, 107 m*M* NaCl, 20 m*M* $NaHCO_3$, 2 m*M* Na_2SO_4, 20 m*M* citric acid, 20 m*M* trisodium citrate, and 5 m*M* *d*-glucose) and centrifuged (190*g*) for 10 min. The supernatant is discarded, and the platelet pellet is resuspended in a original volume of Krebs-Ringer buffer again.

The centrifugation step is repeated, and depending on the experimental setup, the platelet pellet is resuspended in Krebs-Ringer buffer (e.g., when the platelet suspension is used for labeling of the platelets), patient plasma, or a human albumin solution (4 m*M* KCl, 107 m*M* NaCl, 20 m*M* $NaHCO_3$, 2 m*M* Na_2SO_4, 2.5 m*M* $CaCl_2$, 20 m*M* trisodiumcitrate, and 4% [w/v] human albumin; dialyze overnight against the same solution without albumin, and filter it to remove impurities; add 5 m*M* *d*-glucose, and adjust the pH to 7.35). The packed red cells are washed twice with a solution containing 150 m*M* NaCl and 5 m*M* *d*-glucose (5 min, 3000*g*) and added to the reconstituted perfusate to give a hematocrit of 40%. The final platelet number in the reconstituted blood is usually adjusted to 2×10^{11}/L.

2.4. Surfaces

To study platelet adhesion to purified proteins, a parallel plate perfusion chamber is used. The protein is coated onto glass or plastic coverslips, which fit in the chamber. Before use, the glass coverslips are washed in chromic acid followed by overnight storage in 80% ethanol. The coverslips are then rinsed with deionized water and dried. Coating of the coverslip with a protein can take place via spraying of a protein solution or via adsorption. The technique of choice depends on the protein (*see* **Table 1**). For spraying, it is necessary to dissolve proteins in a volatile buffer (50 m*M* NH_4-acetate buffer). Collagen is always sprayed onto glass coverslips, because fibrillar collagen is a suspension in buffer. Collagen (1 mg/mL, 90 µL/ coverslip = 3.24 cm^2) is sprayed in multiple runs onto the coverslips by means of a retouching air brush at an N_2 pressure of 1 atm. Between the runs, the finely dispersed collagen droplets are allowed to dry. After spraying, the coverslip is incubated with 1% human albumin solution in PBS (140 m*M* NaCl, 5 m*M* phosphate buffer, pH 7.4) for half an hour. The final collagen concentration is about 30 µg/cm^2. Coated coverslips are stored at room temperature and used within 24 h. The concentrations used for other proteins are mentioned in **Table 1**.

Adhesive proteins are adsorbed to coverslips by incubating the coverslip with 100 µL of the protein solution for 1 h. After washing, the coverslip is incubated for 30 min with 1% albumin solution in PBS. Coverslips coated with adhesive proteins are used the same day. The optimal protein concentrations for the different proteins are mentioned in **Table 1**.

Table 1
Coating Glass Coverslips with Purified Proteins

Protein	Spraying conc. on coverslip	Adsorption	
		Conc. of solution	Conc. on coverslip
Collagen types I and III	30 µg/cm^2	300 µg/mL	Unknown
Collagen type IV	30 µg/cm^2	300 µg/mL	Unknown
von Willebrand factor	3 µg/cm^2	10 µg/mL	60 ng/cm^2
Fibronectin	5 µg/cm^2	[a]	
Fibrinogen	6 µg/cm^2	100 µg/mL	Unknown
Fibrin[b]	6 µg/cm^2	Not possible	
Laminin	No information	100 µg/mL	350 ng/cm^2
Thrombospondin	15 µg/cm^2	200 µg/mL[c]	Unknown

[a]Coverslips with adsorbed fibronectin resulted in a low and inhomogeneous platelet adhesion.
[b]To coat fibrin, 0.4 NIH U/mL thrombin was added to 100 µg/mL fibrinogen just before the start of the spraying procedure.
[c]Dissolve thrombospondin in a buffer containing 0.5 m*M* Mg^{2+} and 2 m*M* Ca^{2+}.

The subendothelium is defined as the vessel wall component localized between the internal elastic lamina and the endothelium. To study the subendothelium *ex vivo*, only the original Baumgartner perfusion chamber can be used. Sources of subendothelium are human umbilical artery or rabbit aorta. For the preparation of these surfaces, the reader is referred to other articles ***(8)***.

The extracellular matrix produced by cultured endothelial cells is a good alternative for the subendothelium. It is a highly reactive surface for platelets, and the protein composition of the matrix is comparable with the composition of the subendothelium. The characteristics of the extracellular matrix in relation to platelet adhesion are extensively described elsewhere ***(9)***. Basically, endothelial cells or other vascular cells are cultured on glass or plastic coverslips, and after having reached confluence, the cells are removed, leaving the extracellular matrix attached to the coverslip. There are a number of different techniques available to remove the cells. The cells can be incubated with 0.1 *M* NH_4OH (10 min), 0.1% Triton X-100 (10 min), 2% EDTA (30 min), or 2 *M* urea (30 min), followed by washing, after which the matrices are ready for experimentation. The method of choice depends on the experimental setup. We normally use 0.1 *M* NH_4OH (fast) or urea (highest von Willebrand factor concentration in the matrix). Some residual membrane fragments may remain incorporated to the extracellular matrix. When using Triton X-100, remnants of the cytoskeleton also remain attached to the matrix. Matrices can be stored at 4°C for at least 1 wk without losing reactivity.

An interesting application of the parallel plate perfusion chamber is to mount a cryosat cross-section of blood vessels onto the coverslip. This enables the study of platelet adhesion to various layers of the vessel wall during one single perfusion run. Such an approach is particularly useful for the study of the thrombogenicity of atherosclerotic lesions ***(10)***.

Another application of the perfusion system is using it to study the short-term interaction of blood and artificial surfaces, such as polyethylene-teraphtalate (Dacron) and polytetrafluorethylene (PTFE, Goretex, Flagstaff, AZ). It is necessary to process the artificial surfaces onto coverslips that match the dimensions of the depression of the knob of the perfusion chamber.

3. Methods

3.1. Preparation of a Perfusion Experiment

The perfusion procedures with the different perfusion chambers are similar in essence. The different components of the system, perfusion chamber, flow device, funnel (if necessary), and container(s) are connected by means of the tubing. The chamber and containers are placed in a water bath that is kept at 37°C. Before the first experiment, the system is extensively washed, preferably by a preperfusion with plasma for 10 min to coat the whole system with proteins. This is necessary to have the same results in the first and second perfusions. The system is checked on leakage. The roller pump is adjusted to the proper speed for the desired shear rate. Simultaneously, the first perfusate is prewarmed at 37°C. The adhesive surface is introduced in the chamber, the container with the perfusate is connected, and the perfusion starts. The time measurement is started. The second perfusate is prewarmed at 37°C.

3.2. The Actual Perfusion Experiment

The start of the perfusion time is defined as the moment when the pump starts to draw blood from the container. During the experiments, it should be determined that no air bubbles are present in the perfusate.

3.3. The Termination of a Perfusion Experiment

Perfusion experiments with recirculating perfusates are finished by stopping the pump. The container with blood is replaced by a container with washing buffer (10 m*M* HEPES, 140 m*M* NaCl), the pump is started again, and the chamber is perfused with 10 mL washing buffer. After washing, the knobs with the coverslips are removed from the chamber as quickly as possible. The coverslips are removed with a pair of tweezers, washed again with a Pasteur pipet on both sides of the coverslip with the same washing buffer, and immersed in fresh fixative (normally 0.5% glutaraldehyde).

Perfusion experiments performed with a syringe are stopped by turning a valve switching from blood to washing buffer.

3.4. Between Two Perfusion Experiments

Between two perfusions, the total system is extensively washed with washing buffer. The shear rate must be checked.

3.5. Evaluation of Platelet Adhesion

A large number of morphometric and chemical methods have been developed to measure the number of platelets adhering to the surface. The technique of choice depends on the information desired. Morphometric evaluations have important advantages:

1. One actually sees what has happened, and irregular adhesion is immediately noticed.
2. In rectangular perfusion chambers, only a part of the adhesive surface is exposed to flow. With chemical methods, it is difficult to discriminate between exposed surfaces and nonexposed surfaces.
3. It is easy to discriminate between platelet adhesion and platelet deposition.

Morphometric evaluation of en face preparations starts with a 5-min fixation of the coverslip with methanol followed by 4 min of staining with May-Grünwald and 15–20 min Giemsa, respectively. The staining is performed at room temperature. The coverslips are mounted on glass slides and examined by microscopy. Originally, the surface was evaluated with a 10×10 μm grid eyepiece micrometer at a total magnification of 1000×, and the number of platelets per unit area were counted. Nowadays automatic densitometers coupled to the microscope, "image analyzers," are available to measure the staining intensity or gray levels. After correction for the background, the percentage of the surface with platelets is given. It should be noted that the surface covered by a contact platelet is less than the surface covered by a spread platelet. Thus, the surface coverage is not exactly the same as the number of platelets per unit area, but if one type of adhesive surface is used, % platelet coverage is a useful measure for adhesion. For endothelial cell matrix, a 100% coverage corresponds to about 10^7 platelets/cm^2.

To improve the sensitivity and the specificity, an alternative technique may be to stain the platelets with an antibody. After perfusion, the coverslips are treated for 0.5 h with 2% paraformaldehyde as fixative, incubated 0.5 h with 0.1 *M* glycine, and subsequently incubated with a monoclonal antibody (MAb) directed against a specific platelet antigen, e.g., glycoprotein Ib. After washing with PBS, the coverslips are incubated with antibody against mouse IgG coupled to peroxidase. After washing, the coverslips are stained for 10 min with a DAB solution. (DAB =3,3-diaminobenzidine tetrachloride. DAB-solution: 50 mg DAB in 100 mL 0.1 *M* Na-acetate, pH 6.0; add just before use, 33 μL 33% H_2O_2. To improve the staining, 0.1 g $CoCl_2$ in 10 mL phosphate

buffer and 0.1 g $[NH_4]_2Ni[SO_4]_2$ in phosphate buffer can be added.) The coverslips are then evaluated with an image analyzer. To improve the staining, 0.1 g $CoCl_2$ in 10 mL phosphate buffer and 0.1 g $(NH_4)_2Ni(SO_4)_2$ in phosphate buffer can be added).

For examination of the surfaces with scanning electron microscopy, the samples are fixed with 2% glutaraldehyde for 1 h followed by a postfixation with 1% osmiumtetroxide for 1 hour. After dehydration through increasing concentrations of ethanol (50–100%), the samples are critically point-dried and sputtered with gold. The samples are ready for examination.

Evaluation of semithin sections is the technique used to measure platelet deposition (*see* ref. ***8***) for technical information). For platelet adhesion measurements, the technique is not very practical. It should be noted that en face evaluation may underestimate platelet adhesion, because platelets sometimes are spread so thinly that the staining is not sufficiently different from the background to detect with an image analyzer. However, to measure platelet adhesion, the technique is too labor-intensive for the limited extra information on adhesion that can be obtained.

To allow transmission electron microscopy, the coverslips must be separated from the adhered platelets to allow ultrathin sectioning. Glass coverslips must first be covered with a melamine foil. This foil is inert toward platelets, sticks well to coverslips, and can be removed from the coverslip after fixation together with the adherent platelets. Another approach is the use of Thermanox^R coverslips instead of glass coverslips. Thermanox can be removed by thermoshock. To do so, after fixation of the sample with 2% glutaraldehyde, the samples are fixed again with 0.5% osmiumtetroxide in 0.1 *M* phosphate buffer, pH 7.4, for 15 min. After washing three times with PBS, the sample is dehydrated through incubating with increasing concentrations of ethanol ($2 \times 75\%$, $2 \times 85\%$, $2 \times 95\%$, $2 \times 99.8\%$) for at least 3 min per dehydration step. The sample is incubated for 3 min with pure propylene oxide, and put upside down in a mixture of one part propylene oxide and two parts epon (composition: 8.6 mL epon-816; 8.8 mL dodecenylsuccinic anhydride [DDSA]; 2.8 mL methyl nadic anhydride [MNA], and 0.3 mL DMP-30 [dimethylaminomethylphenol] for 1 h). The sample is placed in normal position on an inert material, and one or two drops of pure epon are put on the coverslip. Then it is incubated for 6 h at 65°C (or overnight at 50°C). To remove the Thermanox from the polymerized epon, the sample is placed on a warm plate (about 70°C) followed by a submersion in liquid N_2. The Thermanox coverslip is separated from the epon. The epon is used to prepare thin sections of 1 μm. These sections can be stained for evaluation or used for electron microscopy studies.

Labeling of platelets can take place with radioisotopes or with fluorescent labels. Radiolabeling of platelets is performed after the first wash step during

the preparation of washed platelet (*see* page 6). The best results are obtained with ^{111}In-troponolate. The platelet pellet is resuspended in 1 mL PBS and 10 MBq ^{111}In-troponolate is added under gentle agitation and incubate for about 10 min at 37°C. Forty milliliters of Krebs-Ringer buffer are added, and the platelets are collected after centrifugation for 15 min at 1000*g*. The platelets are ready for suspension in a reconstituted perfusate. To prevent platelet activation, aspirin (10μ*M*) can be added simultaneously with the label.

To visualize platelets with a video system, the platelet can be labeled by adding the fluorescent dye mepacrine (10 μ*M*, quinacrine dihydrochloride) to whole blood. Although this dye also labels leukocytes, these cells could easily be distinguished from platelets by their size. Mepacrine accumulates in the dense granules of the platelets and has no effect on platelet function in an aggregometer.

If platelets are labeled with a fluorescent dye, the actual process of platelet adhesion can be recorded with the help of video imaging systems. During the perfusion, the flow chamber is mounted on an epifluorescence microscope stage equipped with a video camara coupled to a video recorder. Perfusion experiments are recorded on a videotape and the video images are evaluated.

4. Notes

There are a number of factors that influence the outcome of a perfusion experiment. It is therefore important to control the experimental conditions, especially when different experiments are compared. Lowering the temperature below 37°C decreases platelet adhesion. Platelet adhesion strongly depends on the hematocrit. Increasing the hematocrit increases platelet adhesion, but decreasing the hematocrit decreases platelet adhesion. Platelet adhesion depends on the platelet number in the perfusate up to a platelet count of about 1.2×10^{11}/L. The influence of platelet count on platelet adhesion depends on the shear rates used. The higher the shear rate, the lower the platelet count necessary for optimal adhesion. Another problem is that compounds, such as antibodies, added to the perfusate often activate platelets and cause platelet clumping. A substantial decrease in platelet number during perfusion may influence the number of platelets adhering. It is important to check the platelet number after perfusion. This can be done by taking a sample of 100 μL blood before and after the perfusion from the perfusate and mixing this with 900 μL 0.5% glutaraldehyde solution in PBS, followed by counting the platelet number in a cell counter. The ratio between platelet number after perfusion divided by platelet number before perfusion × 100 is the percentage platelet disappearance by clumping. A platelet disappearance of <20% is acceptable.

The perfusion chambers currently in use have the disadvantage that they require relatively large amounts of blood: 10 to 15 mL of blood for a single run

of a perfusion experiment with recirculating blood and up to 50 mL for an ex vivo perfusion. There is an absolute need for the development of smaller perfusion chambers, which use less blood. Special perfusion chambers have recently been devised in which an obstruction is placed in order to mimic a vessel stenosis ***(11,12)***.

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18

Leukocyte Transmigration Through Vascular Endothelium

An In Vitro Method

Paola Allavena and Aldo Del Maschio

1. Introduction

The endothelial lining of blood vessels forms an intact monolayer throughout the vascular system, and constitutes a critical barrier between the blood flow and the tissues beneath. Endothelial cells (EC) have a prominent role in the regulation of hemostasis, immunity, and inflammation; in particular, EC are active participants in regulating the traffic of cells and substances from blood to tissues and vice-versa ***(1,2)***.

The extravasation of leukocytes requires the adhesion and subsequent transmigration through the vascular endothelium. These processes are mediated by the interaction of different adhesion molecules on leukocytes with their respective endothelial ligands ***(3,4)***. In normal conditions, resting EC have relatively low adhesive properties for leukocytes, which in turn circulate in a non-adhesive state. However, in some circumstances, high levels of cytokines and chemotactic factors modulate the expression of adhesion receptors and/or increase the avidity for their respective ligands on EC. This leads to a consequent increase of leukocyte adhesion and transmigration.

This chapter will describe an in vitro method of transendothelial migration. This method employs EC grown on polycarbonate filters with defined pore size. The passage of leukocytes from the upper compartment, through the EC monolayer, to the lower compartment is measured. This approach has been successfully used to characterize the transmigratory ability of various leukocyte subpopulations and to identify the role played by adhesive molecules dur-

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ing this process. Various methods for the study of transendothelial migration have been reported in the literature. In some cases, penetration through collagen matrix and the use of digestive enzymes has been used ***(5)***. Other methods employed staining of leukocytes and a visual assessment by light microscopy ***(6,7)***.

The method described here employs radioisotope-labeled leukocytes, and has the advantange of a rapid and unbiased readout. Two variants of the assay will be described: the first employs disposable, commercially available, Transwell polycarbonate membrane inserts, which are easy to use, but quite expensive; the second employs polycarbonate filters mounted in Boyden chambers for chemotaxis. Although somewhat cumbersome to perform (it is not a microassay), this variant has the advantage of being inexpensive.

2. Materials

2.1. Isolation and Culture of Endothelial Cells

1. Phosphate-buffered saline (PBS) with and without Ca^{2+}.
2. EC Growth Supplement (ECGS) (Collaborative Res) dissolved in PBS (without Ca^{2+}) at 5 mg/mL.
3. Heparin sodium salt (grade I-A from porcine intestinal mucosa) (Sigma, St. Louis, MO) dissolved in PBS (without Ca^{2+}) at 10 mg/mL.
4. Medium 199 (M199), newborn calf serum (NCS), and fetal bovine serum (FBS) (Gibco).
5. Complete M199 for EC culture on Transwell membrane inserts is defined as: M199 supplemented with 20% NCS + ECGS and heparin, at the final concentrations of 0.05 and 0.1 mg/mL. For culture on polyvinylpirrolidone-(PVP) free polycarbonate filters, complete M199 is supplemented with 10% FBS, instead of NCS.
6. Fibronectin (from human plasma), gelatin (type B: from bovine skin), and trypsin(Sigma).
7. Human recombinant tumor necrosis factor (TNFα) (Genzyme).
8. Transwell polycarbonate membrane inserts (24.5 mm diameter; 8.0 μm pore size), PVP-free polycarbonate filters and nitrocellulose filters, (13 mm diameter; 5.0 μm pore size) from (Costar).
9. Cell incubator (37°C in a 5% CO_2 humidified atmosphere).
10. Six- and 24-well tissue-culture plates (Falcon).
11. Boyden chemotactic chambers (Neuroprobe).

2.2. Isolation and Radiolabeling of Leukocytes

1. Lymphoprep (Nycomed Pharma AS).
2. Percoll and Dextran T500 (Pharmacia-Upjohn).
3. RPMI 1640 medium screened for being endotoxin-free (Gibco).
4. $Na_2{}^{51}CrO_4$ (37 MBq; 1 mCi) (Amersham).

3. Methods

3.1. Culture of Endothelial Cells on Transwell Membrane Inserts

1. Prepare cultured EC purified from human umbilical vein to confluence as described elsewhere *(8)*.
2. Coat Transwell membrane inserts with 10 µg/mL of fibronectin (final volume 1 mL) for 2 h at 37°C. At the end of coating, membrane inserts are washed with M199 (NCS-free).
3. Detach EC by brief exposure to trypsin (0.25%) EDTA (0.022%) and then resuspend with complete M199 at a concentration of $1.5–2 \times 10^5$/mL.
4. Layer cell-culture inserts with 1.5 mL EC suspension and place into six-well tissue culture plates filled with 2.6 mL of complete M199.
5. Wash both compartments and refill daily with complete M199.
6. EC confluence is usually reached at the fifth day of culture (*see* **Note 1**).

3.2. Culture of Endothelial Cells on PVP-Free Polycarbonate Filters

1. Coat PVP-free polycarbonate filters with 0.1% gelatin (final volume 1 mL in 24-well plate) for 2 h at 37°C, and then wash filters with M199 (NCS-free).
2. EC are obtained as described *(see* **Subheading 2.1.**) and then layered onto the filters at a concentration of $8–10 \times 10^4$/well. EC confluence is usually reached at the fifth day of culture (*see* **Note 1**).
3. Mount filters with confluent EC monolayers in Boyden chemotactic chambers, the lower compartment contains 0.2 mL of complete M199, overlayered by an uncoated nitrocellulose filter. The upper compartment should contain 0.15 mL of the same medium. Usually three chambers are prepared for each group under study.

3.3. Leukocyte Preparation and Radiolabeling

1. Different subpopulations of leukocytes are separated from buffy coats of normal blood donors as described for neutrophils *(9)*, monocytes *(10)*, NK cells and T-lymphocytes *(11)*.
2. For labeling, resuspend leukocytes in RPMI + 10% heat-inactivated FBS at $2\text{-}4 \times 10^7$/mL and label with of $Na_2{}^{51}CrO_4$ (100 µCi for $2–4 \times 10^7$ cells), for 1 h at 37°C (*see* **Note 2**).
3. At the end of incubation, wash cells twice with 15 mL of PBS and then resuspend in RPMI + 10% heat-inactivated FBS at the concentrations detailed below.

3.4. Leukocyte Transmigration with Transwell Inserts

1. Activate confluent EC by replacing medium of the upper compartment with complete M199 containing either 100 U/mL TNF or 50 ng/mL interleukin-1 (IL-1) for 4–24 h, depending on the type of adhesive molecules under study (*see* **Note 3**).
2. Transmigration may alternatively be induced by adding 100 n*M* fMLP or other chemoattractants, e.g., C5a, phorbol 12-myristate 13-acetate (PMA), and chemokines, in the lower compartment at the time of leukocyte layering.

3. Layer radiolabeled leukocytes (1.8×10^6/mL in a final volume of 1.5 mL) onto the EC monolayer and incubate for 60 min at 37°C.
4. After incubation, gently mix the medium in the upper compartment in order to resuspend the nonadherent leukocytes. Collect medium and count in a γ-counter for its leukocyte-associated radioactivity. This is the fraction of nonengaged leukocytes.
5. Scrape the bottom of the filter with a cotton bud, and count together with the medium of the lower compartment. This is the migrated leukocyte fraction.
6. Radioactivity that remains associated to the filter represents the fraction of adherent leukocytes.
7. The absolute number of transmigrated leukocytes is calculated from the following formula:

$$\text{no transmigrated leukocytes} = \frac{\text{total no of leukocytes} \times \text{cpm of transmigrated leukocytes}}{\text{cpm of total leukocytes}}$$

8. The same formula can be applied to calculate the number of adherent leukocytes ***(12)***.

3.5. Leukocyte Transmigration in Boyden Chambers

1. Stimulate confluent EC in the conditions described above (*see* **Subheading 3.4., Step 1**).
2. Layer radiolabeled leukocytes ($2–4 \times 10^6$/mL in a final volume of 0.15 mL) onto the EC monolayer, and incubate for 60 min at 37°C (*see* **Note 4**).
3. After incubation, collect the medium with nonadherent cells, and gently wash the monolayer with 0.5 mL of complete M199 at 37°C. These two fractions are pooled and counted for their leukocyte-associated radioactivity. This is the fraction of nonengaged leukocytes.
4. Collect the EC monolayer with adherent leukocytes with cotton buds. This is the fraction of adherent leukocytes.
5. Transfer the double-filter system to vials together with the medium of the lower compartment (migrated leukocyte fraction).
6. The absolute numbers of transmigrated leukocytes is calculated as described in **Subheading 3.4., Step 7** ***(13)***.

3.6. Identification of Adhesion Molecules

The identification of adhesion molecules involved in leukocyte–EC interaction is performed by the addition of blocking MAbs—most of which are commercially available—specific for the adhesion structures expressed by leukocytes or EC (*see* **Note 5**).

Studies with specific MAb have demonstrated that two main pathways are involved in leukocyte transmigration: the interaction of leukocyte β2 integrins with their endothelial counterreceptors: intercellular adhesion molecule (ICAM-1 and ICAM-2) and the leukocyte β1 integrin very late antigen (VLA-4) with endothelial vascular cell adhesion molecule (VCAM-1).

Although monocytes and lymphoid cells use both these pathways, neutrophils use only the first one. In addition, platelet/endothelial adhesion molecule (PECAM-1) expressed in both leukocytes and EC, plays an important role during transmigration ***(3,4,14)***.

Below advice regarding the use of these MAb is briefly considered.

1. Dispense radiolabeled leukocytes, resuspended in complete medium (10^6/mL), in small polypropilene tubes. Each tube corresponds to a group of MAb treatment and should contain the total number of cells for all the necessary replicates (usually three). The final volume should be the lowest possible (0.2–0.4 mL) in order to save as much MAbs as possible (*see* **Note 6**).
2. Incubate leukocytes for 10–20 min at room temperature, then dilute with complete medium at the desired concentration, and layer onto the EC monolayer for the transmigration assay.
3. If MAb against endothelial adhesive molecules are used, add them to EC in a final volume that should not exceed 0.5 and 0.1 mL for transmigration assay on Transwell inserts and Boyden chambers, respectively.
4. After 15–30 min of incubation at room temperature, layer leukocytes and continue the transmigration assay as described above (*see* **Note 7**).

4. Notes

1. It is of major importance to check the integrity of the endothelial monolayer. Prepare few spare EC-coated filters or Transwell inserts, stain with Diff-Quick, and observe EC integrity at light microscopy.
2. Leukocytes may alternatively be labeled with 5 µCi/10^7 cells of ^{111}Indium-oxide (Amersham) for 15 min at room temperature. Leukocytes are then washed twice and used as above described.
3. Expression of adhesion molecules on TNF- or IL1-activated EC depends on the time of exposure. E-selectin is expressed within 4–6 h after cytokine stimulation and declines after 12 h of incubation. ICAM-1 and VCAM-1 are poorly expressed before 4 h and remain in plateau for 24–48 h ***(3,4)***.
4. When the transmigration assay is performed with activated NK cells, the incubation should be no longer than 30–60 min. Activated NK cells are highly cytotoxic, and EC are a sensitive target ***(13)***.
5. Not all MAbs directed against adhesive molecules are functionally blocking. Be sure that the MAb under study is effectively blocking the adhesion.
6. Use mAb at the lowest optimal concentration. Usually ascites work when diluted up to 1:50–100, whereas hybridoma supernantants should not be diluted more than 1:5–10. A preliminary dose–response experiment is advisable.
7. Under the conditions described here, all MAb inhibiting adhesion will of course inhibit migration, since migration is dependent on adhesion. To identify putative molecules involved in migration without the interference of adhesion, mAbs may be added 30 min after the onset of the assay, after having washed away (gently) the non adherent cells. Thereafter, the assay may continue for 60 min.

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19

Cell Migration into a Wounded Area In Vitro

Maria Grazia Lampugnani

1. Introduction

In vitro wound healing is a complex phenomenon in which cells mechanically released from tight confluence migrate in an oriented fashion to an area made free of cells ***(1)***. Moreover, beyond migrating, cells at the advancing border often re-enter the cell cycle ***(2–4)***. In the perspective of adhesion molecules, the process involves remodeling of the cellular adhesive interactions both with the adjoining cells and with the extracellular matrix. Therefore, aspects of cell physiology related to the molecules controlling adhesion to neighboring cells ***(5–7)*** and to substrate ***(8)***, to components of the cytoskeleton ***(9–12)***, as well as to extracellular matrix remodeling ***(13–15)*** can be studied during the transition from the resting to the migratory/proliferative phenotype.

The response is further complicated by the action of growth factors, often with motogenic activity ***(16,17)***, which are present in the culture medium ***(18)*** and sometimes are released by the scratched cells ***(19–22)***, and is often modulated by proteolytic enzymes (e.g., metalloproteinases ***[23,24]***) and plasminogen activators ***(25)*** and relative inhibitors produced by the cells.

As one can realize from the following sections, this experimental model is technically relatively simple. It requires cell biology sensitivity and skill. On the other hand, it is very versatile, and it allows analysis not only at the cell population level, but also at the local level (applying immunofluorescence microscopy and/or *in situ* hybridization; *see* Chapters 8 and 10), through the comparison of cells in different states (migrating at the wounded front, activated at the near back of the front, resting in the regions far from the front) in the same culture and at different time-points.

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2. Materials

1. Cells to be tested, seeded in either 24- or 6-well plates or 10-cm diameter Petri dishes depending on the analysis, which will be applied (**Note 1**). Cell seeding concentration depends on cell type. Final point of culture is a highly dense cell layer.
2. Glass coverslips (13 mm-diameter) to be set in each well of a 24-well plates are needed for immunofluorescence microscopy (*see* Chapter 8 of the present vol.).
3. Wounding instruments: Sterile plastic tips (1–200 µL, 200–1000 µL) for automatic pipets (**Note 2**).
4. Fast Green FCF 0.02% in methanol, for fixation.
5. Crystal violet 0.5% in methanol/water, 20/80 (v/v), for staining.
6. Manual analysis: ocular micrometer (10 mm micrograduated scale with 100-µm graduation, e.g., Nikon) to be adapted in the ocular of an inverted microscope.
7. Computerized analysis: microscope either inverted or upright connected to a computer for image analysis. If only an upright microscope is connected to the computer, cells can be cultured on glass coverslips, as for immunofluorescence, stained with crystal violet mounted as for immunofluorescence microscopy (*see* Chapter 8 of the present vol.), and observed with the upright microscope for image acquisition and analysis. Time-lapse video microscopy can also be applied to follow the kinetics of the process in living cells.
8. Immunofluorescence microscopy: Materials and methods for this technique are described in Chapter 8 of the present vol.
9. *In situ* hybridization for identification of mRNA: For a description of the materials and methods required for this technique see Chapter 10 of the present vol.
10. Western blot and/or immunoprecipitation of proteins. Northern blot and/or PCR. For detailed description of materials and methods, *see* refs. ***26*** and ***27***.

3. Methods

1. Under sterile conditions, remove the excess of culture medium, but leave enough liquid to avoid cell drying during the wounding procedure.
   a. Twenty-four-well plates, mostly used for quantitation of the wound healing rate and immunofluorescence microscopy: Wearing gloves, take a sterile tip (200–1000 µL, fixed or not to the automatic pipet, as you feel more comfortable). For specific aims, mostly for short-time incubation (up to few hours) or for cells migrating very slowly (a narrower wound can be produced using 1–200 µL tips) and keeping the tip perpendicular to the culture surface, produce a wound along one diameter of the well. Start the wound from one edge and proceed gently and firmly to the other (**Note 3**).
   b. Six-well plates and 10-cm Petri dishes, mostly used for biochemical detection of proteins and nucleic acids: In this case, multiple wounds are required to induce migration in most of the culture. This can be achieved producing parallel wounds (e.g., around 50 wounds in 10-cm diameter Petri dish), then rotating the plate through 90° and producing another set of parallel wounds (*see* **Notes 2** and **4**).

c. After wounding each well or dish, add culture medium with serum, mix the culture vessels vigorously, and aspirate medium to remove the pieces of cell layer, which float as clumps and that being composed of living cells, can reattach randomly to the cell layer during the following incubation. Wash once more the cell layer with culture medium containing serum (*see* **Note 5**) and incubate with culture medium with serum (**Note 6**). Incubate for the length of time required by the specific experimental protocol. A time-course analysis can often be advisable.

2. Stop the incubation according to the test to be performed:
   a. For quantitation: Quickly wash the cell layer once with PBS containing calcium and magnesium. Fix the cells with Fast Green in methanol (for 3 min) followed by crystal violet (for 5 min). Wash twice with distilled water, and let the cell layer dry before quantitation. Starting from a very dense cell layer, mostly if the cells tend to pile one on the other, the migrating front, constituted by the cells in contact with the substrate, is very obvious and appears as a lightly stained band extending from the original position of the wound in comparison to the bulk of the culture, which is heavily stained ***(5)***. The depth of this lighter band can be quantified manually or by image analysis (*see* **Note 7**).

      Measures obtained in parallel wells at each time-point are pooled to calculate a mean value.
   b. For immunofluorescence microscopy: Fix the cells according to the protocol described in Chapter 8 of the present vol.
   c. For *in situ* hybridization: fix the cells according to the specific protocol described in Chapter 10 of the present vol.
   d. For biochemical analysis of proteins and nucleic acids, extract cell proteins or nucleic acids according to the specific protocols ***(26,27)***.

4. Notes

1. Coating of the culture surface with proteins (for example, fibronectin or gelatin) depends both on cell type and on the specific aim of the experiment.
2. Various devices, often homemade, have been reported to produce wounds in cell layers, e.g., razor or scalpel blades, Pasteur pipet tip smoothened by flaming, rubber policemen, or rubber or steel combs (for multiple wounds). A razor blade offers the advantage that the position of the initial front is labeled ***(28)***. This is particularly required for quantitation of the migration rate, and using cell cultures (e.g., endothelial cells) that forming of a monolayer at confluence do not present a migration front clearly distinct from the line of the original wound.

   In our experience, plastic tips for automatic pipets (200–1000 µL) can be used for many purposes to produce wounds reproducibily of at least 1-mm width.
3. For quantitation, two or three diameters of a 24-well plate can be removed. At the relatively low magnification required (for most purposes, ×100), it is rather easy to understand where the front edge is localized.

   For immunofluorescence, just one diameter wound is mostly advisable. It can indeed be very useful to be able to analyze in the same culture the cell layer at

different densities, i.e., far from the front and at the front near the back (two or three cell rows of cells inside the front) beyond the front itself, and too many fronts make the analysis confusing mostly at the high magnification (X1000) required for detailed morphological observation ***(6,29,30–32)***.

4. Multiple wounds, mostly for 10-cm Petri dishes, can also be obtained using comb devices (*see* **Note 2**). In this case, concentrical scrapes are produced **(33)**.
5. The use of serum during washing and incubation can be omitted for specific experimental purposes and replaced by 0.1% bovine serum albumin in culture medium.
6. Always control the edges of the wound produced under an inverted microscope. Regular wound edges are particularly wanted for quantification and immunofluorescence analysis. Following incubation, examine the cultures from time to time under the inverted microscope to check the progression of cell migration from the wound edges directly.

   Preliminary experiments are as usually required to optimize the conditions of both wound production and healing for a particular cell type and for a specific experimental purpose, e.g., the time requested to cover the wound produced by a specific "scraper."
7. For cells that do not overgrow (e.g., human endothelial cells from umbilical cord vein), quantitation of the migrated path is more complex and requires that the initial site of wound is labeled to be easily recognized following incubation. This can be done using a razor blade as described (*see also* **Note 2**; ***19,25,28***).

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20

Determination of the Endothelial Barrier Function In Vitro

Geerten P. Van Nieuw Amerongen and Victor W. M. Van Hinsbergh

1. Introduction

Endothelial cells forming the inner lining of all blood vessels are actively involved in the regulation of the extravasation of fluid, macromolecules, hormones, and leukocytes. Only a few decades ago, it was thought that the endothelium functioned as a passive semipermeable barrier, but nowadays it is generally recognized that the endothelial cells actively and semiselectively play a pivotal role in the regulation of the passage of nutrients, hormones, and macromolecules. Over the past several years, the concept has emerged that the increase in endothelial permeability induced by vasoactive compounds, such as histamine and thrombin, is owing to intercellular gap formation. On the one hand, this is caused by contractile forces generated in the cell margins by actin–nonmuscle myosin interaction *(1,2)*. On the other hand, alteration in cell–cell and cell–matrix interactions affect the endothelial barrier function *(3)*.

An in vitro model in which endothelial cells are grown on porous filters has proven to be a valuable tool for the evaluation of the endothelial barrier function. In this chapter, a commonly used model of human endothelial cells cultured on polycarbonate filters placed in a adapted Boyden chamber is described (**Fig. 1**). In **Subheading 3.1.**, a protocol is given for seeding and culturing endothelial cells on porous filters. In **Subheading 3.2.**, the use of these monolayers in assaying the passage of a specific tracer, horseradish peroxidase (HRP), is described, and examples are given regarding how to influence the barrier function. For the passage of leukocytes, the reader is refered to Chapter 18 in this vol.

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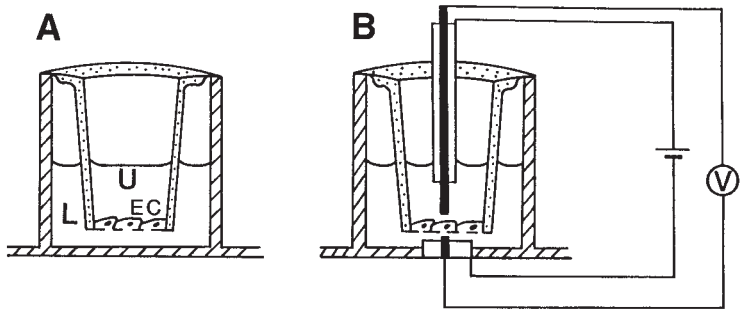

Fig. 1. **(A)** Schematic view of the assay system for measuring the diffusion of macromolecules through monolayers of endothelial cells. Endothelial cells are seeded in high density and cultured for 4–7 d to form a tight endothelial cell monolayer. During experiments, a known amount of HRP is added to the upper compartment (U); at several time-points, a sample is taken from the lower compartment (L). **(B)** The transendothelial electrical resistance is measured in a special chamber; *see* **Note 6**. An alternating current (50 µA, 1 pulse/min) passes the monolayer by two source electrodes (2-cm distance). Two other electrodes detect the potential difference across the monolayer (3-mm distance).

2. Materials

2.1. Cell-Culture Media

1. Human serum is prepared from freshly collected blood obtained from healthy donors. The sera of 15–25 subjects are pooled and stored at 4°C (for up to 3 mo) or at –80°C. Before use the sera are filtered through a 0.45-µm Acrodisc (bottle-top) filter at room temperature. Human serum can be heat-inactivated by incubating for 30 min at 56°C (i.e., the bottle contents should be at 56°C for 30 min).
2. Newborn calf serum (NBCS) is purchased from Gibco-BRL (Grand Island, NY) or another commercial supplier. It is stored at –20°C. NBCS has to be heat-inactivated (30 min at 56°C) before use.
3. A crude preparation of endothelial cell growth factor (ECGF) can be made according to the method of Maciag et al. ***(4)***. The lyophilized preparation can be stored at 4°C for 6 mo. It is dissolved, for example, in M199 medium supplemented with 20 m*M* HEPES and 5 U/mL heparin (the latter is optional, but improves the stability of the growth factor), and sterilized by filtration (0.22-µm filter—only once to prevent loss of material). Instead of a crude preparation of ECGF, human recombinant basic fibroblast growth factor (bFGF, 10 ng/mL) can be used. The bFGF can be purchased from various suppliers, e.g. Intergen (New York). Heparin (5000 IU/mL) can be purchased from Leo Pharmaceutical Products (Ballerup, Denmark) or other commercial suppliers.

4. Complete medium: M199 medium supplemented with 20 m*M* HEPES (pH 7.4), 10% human serum (heat-inactivated), 10% NBCS (heat-inactivated), 150 µg/mL crude ECGF, 5 U/mL heparin, 100 IU/mL penicillin, and 100 µg/mL streptomycin.
5. M199 medium/1% HSA (the medium that is used in the permeability assay): M199 medium supplemented with 20 m*M* HEPES, 1% pyrogen-free human serum albumin (HSA), 100 IU/mL penicillin, and 100 µg/mL streptomycin (Boehringer, Mannheim, Germany). A 20% (w/v) HSA stock solution can be purchased from the Central Laboratory of the Blood Transfusion Service (Amsterdam, The Netherlands).

2.2. Materials for Culture of Human Endothelial Cells on Porous Filters

1. Fibronectin can be purchased from commercial suppliers or can be prepared from the cryoprecipitate of human plasma obtained from a local blood transfusion service. To that end, fibronectin is purified by gelatin-Sepharose chromatography according to the method of Vuento and Vaheri ***(5)***, which yields a mixture of fibronectin and vitronectin. It is dialyzed against 10 m*M* CAPS buffer (pH 11.0) supplemented with 1 m*M* $CaCl_2$ and 150 m*M* NaCl, and subsequently stored in 1-mg aliquots. For experimental use, a 10 µg/mL human fibronectin solution is diluted at 37°C in M199 medium and sterilized by filtration through a 0.2-µm filter. Instead of fibronectin, a 1% gelatin solution can be used.
2. Trypsin/EDTA: 0.05% (w/v) trypsin, 137 m*M* NaCl, 5.4 m*M* KCl, 4.3 m*M* $NaHCO_3$, 5 m*M* *D*-glucose, and 0.67 m*M* EDTA (pH 7.3).
3. HRP (EC 1.11.1.7) type I, Sigma Chemical Company (St. Louis, MO).
4. Transwell system: Transwell polycarbonate filters (pore size 3 µm, filter area 0.33 cm^2) in multiwell dishes (Costar, Cambridge, MA).

3. Methods

3.1. Culture of Human Endothelial Cells on Porous Filters According to the Method of Langeler and Van Hinsbergh (6,7)

The culture of endothelial cells must be performed in a sterile environment, e.g., a down-flow laminar hood. Use sterile gloves and a laboratory coat to prevent infection of the cultures and to protect yourself.

Routinely, human umbilical vein endothelial cells (HUVEC) are used in permeability assays. These cells are isolated according to the method of Jaffe et al. ***(8)***; for alternative endothelial cell types, *see* **Note 3**.

1. Coat the Transwell filters with a thin layer of 10 µg/mL fibronectin (30 min at room temperature) or with 1% gelatin.
2. Aspirate the fibronectin solution from the filter immediately before seeding the cells.

3. Detach confluent cells with trypsin/EDTA solution.
4. Mix the suspension of detached cells with the volume of culture medium that is necessary to obtain 1 mL of cell suspension per filter. In order to obtain a high density culture, seed the cells on the filters at a density that is equal to that of the confluent primary culture. Add 0.2 mL of the cell suspension to the upper compartment. Place a small amount of complete medium under the filter so that the suspension is sucked through. Add the rest of suspensed cells in small portions to the upper compartment. This is important in obtaining a tight monolayer. Furthermore, if too much medium is added at once, cells may float in the lower compartment and colonize the bottom of the dish.
5. Put the cells into an incubator at 37°C with an atmosphere of 5% CO_2/95% air.
6. Aspirate the nonattached cells the next day, and renew the culture medium every other day (1 mL/filter). Always add the medium to the upper compartment until it is full before adding the residual medium to the lower compartment. This avoids hydrostatic pressure underneath the cells.
7. Use the cells 4–7 days after seeding for experiments.

3.2. Determination of the Permeability of Human Endothelial Cell Monolayers on Porous Filters

1. Aspirate culture medium from lower and upper compartment, and wash the cells once with M199 medium/1% HSA to remove the serum. Add 150 µL of M199 medium/1% HSA to the upper and 700 µL of M199 medium/1% HSA to the lower compartment, and incubate for 1 h. When preincubation of the drugs under investigation is desired, these drugs can be added in this stage. Immediately replace the Transwell system in the incubator at 37°C.
2. Keep 600 µL of M199 medium/1% HSA (+drugs)/filter for volume replenishments.
3. Prepare 150 µL/filter of M199 medium/1% HSA/5 µg/mL HRP with drugs.
4. Take cells out of the incubator. Start HRP passage, after the 60 min preincubation period (as indicated in **step 1**), by replacement of the upper compartment media with the HRP-containing solutions as indicated in **step 3**. Put the cells back into the incubator.
5. At time points 0.25, 0.5, 1, 2, 3, and 4 h, take 100-µL aliquots from the lower compartments and transfer the samples into a 96-well dish. Replace the missing medium by adding (to the lower compartments) 100 µL of M199 medium/1% HSA (+drugs) as indicated in **step 2**. Immediately after each sampling, replace the cells into the incubator. Cover the samples in the 96-well dish with Saran wrap.
6. After 4 h, take 50 µL samples from the upper compartment. These samples can be used to determine the HRP amount that was not transported through the filters and, in combination with the other samples, the total amount of HRP added to each well.
7. Store the samples frozen at °20°C until required for HRP assaying.

Assays for HRP can be performed using various techniques, e.g., the concentration of HRP can be derived from the HRP activity in each sample with

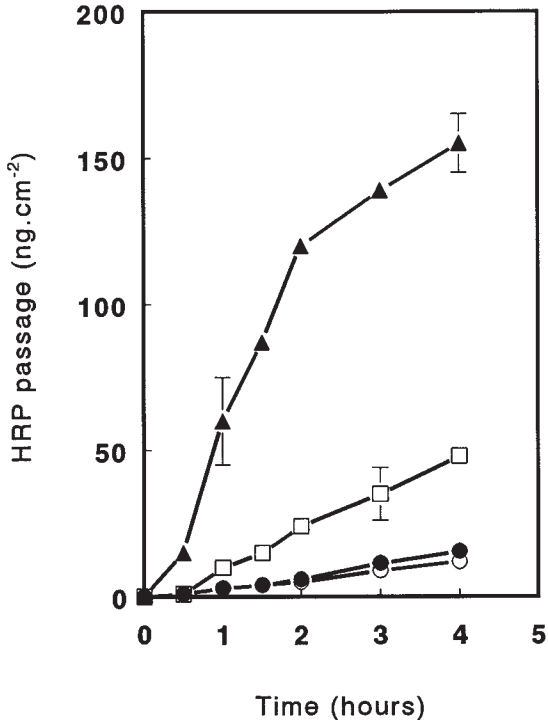

Fig. 2. Passage of HRP through an endothelial monolayer. The time-courses of HRP passage through HUVEC monolayers are presented under basal conditions (open squares), after incubation with 10 μ*M* forskolin (open circles), after stimulation with 1 U/mL thrombin (closed triangles), or after stimulation with thrombin and preincubation of forskolin (closed circles).

peroxide and tetramethyl benzidine (TMB) as substrate and expressed as ng passed/cm^2 in a certain time interval. A typical result is shown in **Fig. 2**.

4. Notes

1. Alternative tracers include iodonated lipoproteins (LDL, VLDL, and so forth), albumin, FITC/RITC-labeled dextrans (mol-wt range of 4–2000 kDa), tritium-labeled sugars (e.g., sucrose/galactose), and ^{22}Na (or other radioactive ions, such as Ca and Rb).
2. Time sampling interval can be shortened by gently stirring the lower compartment with a stirring bone at maximally 100 rpm. If not compatible with the incubator equipment, cells can be kept under normal air, but at 37°C. In that case, M199 medium has to be replaced by M199 medium modified with Hanks' salts.

3. Other human endothelial cell types that have been used in this permeability system are foreskin microvascular, iliac vein/artery, umbilical vein/artery, pulmonary artery, and aorta endothelial cells. Isolation and characterization have been described previous ***(9–12)***. Rabbit, pig, and bovine endothelial cells can also be used ***(13–15)***.
4. In calculating the passage of peroxidase through the cell monolayer, a correction has to be made for the dilution of the medium in the lower compartment by the compensatory medium replenishment.
5. The permeability coefficient (PC) can be derived from Fick's law of diffusion and is determined by:

$$PC = \text{mass flux peroxidase}/[(\text{peroxidase})_{UC} - (\text{peroxidase})_{LC}]$$

where UC is the upper compartment and LC is the lower compartment. The mass flux of HRP is expressed in $ng \cdot cm^{-2} \cdot h^{-1}$. Especially in the case of passage of small molecules, such as ions, the PC has to be corrected for the contribution of the filter membrane:

$$1/PC_{EC} = 1/PC_{EC\text{-}F} - 1/PC_{F}$$

where PC_{EC} represents the PC of the endothelial monolayer, PC_F the PC of the empty filter, and $PC_{EC\text{-}F}$ the PC determined for the filter and monolayer together.
6. The transendothelial electrical resistance (TEER) can be determined in a special chamber ***(6)***. A four-electrode system is required because of the relatively low value of TEER. We have used a device with circular electrodes to improve reproducibility. In the device (**Fig. 1B**), an alternating current (50 μA, 1 pulse/min) is passed across the monolayer by two source electrodes (distance 2 cm), and the potential difference across the monolayer is measured by two detecting electrodes (distance 3 mm). The electrical resistance is calculated by Ohm's law and expressed in $Ohm \cdot cm^2$. Electrical resistance of the filter without an endothelial monolayer is subtracted from all measured values.

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21

Induction of Intracellular Signals Through Binding of Adhesive Molecules

Activation of p125FAK Tyrosine Phosphorylation

Mascia Venturino, Cristina Olivo, Guido Tarone, and Paola Defilippi

1. Introduction

p125FAK tyrosine phosphorylation is a primary response to integrin stimulation. The first evidence that integrin occupancy by ligands induces tyrosine phosphorylation came from studies on the fibrinogen receptor αIIbβ3 in platelets ***(1)***. Further work showed that both in normal and transformed cells several different integrin receptors can trigger tyrosine phosphorylation of intracellular proteins following interactions with matrix proteins or integrin receptor clustering by specific antibodies ***(2)***.

Adhesion-dependent tyrosine phosphorylation of proteins of apparent molecular weight in the range 100–130 kDa and of 70 kDa was observed in a number of different cells types as the major phosphorylated molecules in response to adhesion. Among these components. several defined proteins have been identified. such as the p125FAK kinase ***(3,4)***, the tyrosine kinase p72syk ***(5)***, the p130Cas ***(6–8)***, and the cytoskeletal protein paxillin ***(4)***. The p125FAK is a tyrosine kinase specifically localized in focal contacts ***(9,10)***. Integrin-dependent tyrosine phosphorylation of p125FAK is involved in signaling events leading to cytoskeleton organization and cell proliferation ***(2)***.

In this chapter, we will outline the procedure used to determine integrin-induced p125FAK tyrosine phosphorylation. The same approaches may be used to study tyrosine phosphorylation of other kinases involved in adhesive receptor- or growth factor receptor-dependent signaling pathways.

To analyze p125FAK tyrosine phosphorylation triggered by integrins, two main strategies may be followed. Cells may be allowed to adhere on culture

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dishes coated with matrix proteins or anti integrin monoclonal antibodies (MAb). Alternatively, integrins can be clustered by means of specific antibodies on the cell surface of suspended cells. In the first case. following integrin–ligand binding, cells organize cytoskeletal structures and undergo spreading on the substratum. Thus, processes occurring after integrin–ligand interactions may further affect tyrosine phosphorylation events. Following adhesion or integrin clustering, cells are detergent-extracted and p125FAK is immunoprecipitated from cytoplasmic cell extracts with specific antibodies. The immunocomplexes are subjected to SDS-PAGE, transferred to nitrocellulose, and analyzed with antiphosphotyrosine antibodies.

2. Materials

2.1. Adhesion-Induced Tyrosine Phosphorylation

1. 10-cm diameter polystirene tissue-culture dishes.
2. Coating solution: 10 ug/mL matrix proteins (fibronectin. vitronectin, laminin. collagens), poly-L-lysine (Sigma), or purified MAbs to integrin subunits diluted in PBS (*see* **Notes 1, 2,** and **3**).
3. Blocking solution: 1% bovine serum albumin (BSA;Sigma) in PBS.
4. Cycloheximide (Sigma) dissolved 20 m*M* in water and monensin (Sigma) dissolved 1 m*M* in isopropanol to prevent synthesis and protein secretion (*see* **Note 4**).
5. Cell-detaching solution: 5 m*M* EDTA in PBS.
6. Washing solution: PBS containing 1 m*M* $MgCI_2$ and 1 m*M* $CaCl_2$.
7. 10–50 mL polystyrene tubes to collect detached cells.
8. Refrigerated centrifuge with swing-out rotor capable of centrifuging 10–50 mL tubes at 1000 rpm (100*g*).
9. Plating medium: serum-free Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 20 m*M* HEPES, 20 m*M* cycloheximide, and 1 m*M* monensin.
10. CO_2 incubator at 37°C to allow cell adhesion.
11. Stop solution: 5 m*M* EDTA, 10 m*M* NaF, 10 m*M* $Na_4P_2O_7$, and 0.4 m*M* Na_3VO_4.
12. Lysis buffer: 1% NP-40, 150 m*M* NaCl, 50 m*M* Tris-HCl, pH 8.0, 5 m*M* EDTA, 10 m*M* NaF, 10 m*M* $Na_4P_20_7$, 1 m*M* Na_3VO_4 10 µg/mL leupeptin, 4 µg/mL pepstatin, and 0.1 trypsin inhibitory U/mL aprotinin (all lrom Sigma) (*see* **Notes 7** and **8**).
13. Refrigerated fixed-angle centrifuge capable of centrifuging 1.5 mL Eppendorf tubes at 23,700*g* to separate nuclei from cytoplasmic extracts.

2.2. Tyrosine Phosphorylation Following Integrins Clustering in Nonadherent Cells

1. Cycloheximide (Sigma) dissolved 20 m*M* in water and monensin (Sigma) dissolved 1 m*M* in isopropanol to prevent synthesis and protein secretion.
2. Cell detaching solution: 0.25% trypsin in PBS.
3. Washing solution: DMEM containing 20 m*M* HEPES and 2% FCS to block trypsin effect.
4. 10–50 mL polystyrene tubes and 1.5 mL Eppendorf tubes.

5. Refrigerated centrifuge with fixed-angle or swing-out rotors capable of centrifuging 15–50 mL tube at 1000 rpm (100*g*).
6. Clustering solution 1: serum-free DMEM. 20 m*M* HEPES, 20 m*M* cycloheximide, and 1 m*M* monensin, containing 10 µg/mL purified anti-integrin primary antibodies (*see* **Notes 2** and **3**).
7. Clustering solution 2: serum-free DMEM, 20 m*M* HEPES, 20 m*M* cycloheximide, and 1 m*M* monensin, containing 25 µg/mL purified secondary antibody.
8. Termostatic bath at 37°C.
9. Stop solution: 5 m*M* EDTA, 10 m*M* NaF, 10 m*M* $Na_4P_2O_7$, and 0.4 m*M* Na_3VO_4.
10. Lysis buffer: 1% NP-40, 150 m*M* NaCl, 50 m*M* Tris-HCl, pH 8.0, 5 m*M* EDTA, 10 m*M* NaF, 10 m*M* $Na_4P_2O_7$, 1 m*M* Na_3VO_4, 10 µg/mL leupeptin, 4 µg/mL pepstatin, and 0.1 trypsin inhibitory U/mL aprotinin (*see* **Notes 7** and **8**).
11. Refrigerated fixed-angle centrifuge capable of centrifuging 1.5 mL Eppendorf tubes at 14,000 rpm (23,700*g*) to separate nuclei from cytoplasmic extracts.

2.3. Immunoprecipitation of p125FAK

1. System to quantify protein concentration in cell extracts (BioRad Protein Assay. BioRad; BCA protein assay, Pierce) (*see* **Note 9**).
2. 1.5 mL Eppendorf tube.
3. Protein-A-Sepharose beads (Pharmacia Biotechnology. Uppsala. Sweden) swelled in TBS (150 m*M* NaCl, 20 m*M* Tris-HCl, pH 7.4), 0.5% Triton.
4. Specific MAb or polyclonal antibodies anti p125FAK (UBI, Santa Cruz, Transduction Laboratories, Chemicon).
5. Immunoprecipitation washing buffer 1: TBS (150 m*M* NaCl, 20 m*M* Tris-HCl, pH 7.4), 0.5% Triton.
6. Immunoprecipitation washing buffer 2: TBS, 0.5% Triton, and 0.1% sodium dodecyl sulfate (SDS).
7. Laemmli sample buffer: 2% SDS, 10% glycerol, 60 m*M* Tris, pH 6.8, 200 m*M* , β-mercaptoethanol, 0.001% bromophenol blue.

2.4. Analysis of p125FAK Tyrosine Phosphorylation

1. Apparatus for SDS-PAGE electrophoretic separation of proteins.
2. Semidry or liquid apparatus to transfer proteins.
3. Nitrocellulose (Amersham).
4. Ponceau S solution: 0.2% Ponceau S in 6% trichloroacetic acid.
5. Western blot washing buffer: TBS plus 0.3% Tween (TBS-T).
6. Blocking solution: 5% BSA in TBS-T.
7. Incubation solution: primary antiphosphotyrosine antibody (PT66, Sigma; PY20, Transduction Laboratories; 1G2, Amersham; 4G10, UBI) diluted in 1% BSA in TBS-T at the final concentration suggested by the suppliers.
8. Secondary antibody anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase (Sigma) diluted in 1% BSA in TBS-T. Chemiluminescent peroxidase substrate (ECL, Amersham).
9. Stripping solution (62.5 m*M* Tris-HCl, pH 6.8, 2% SDS, 100 m*M* β-mercaptoethanol).

3. Methods

3.1. Adhesion-Induced Tyrosine Phosphorylation

1. Dilute matrix proteins, MAbs to integrins, or poly-L-lysine at 10 µg/mL in PBS, and coat 10-cm diameter tissue-culture dishes overnight at 4°C: use 3 mL for each dish (*see* **Note 1, 2,** and **3**). Wash the dishes twice with 5 mL PBS, and postcoat with 2 mL/dish of blocking solution at 37°C for 1 h. Wash tissue-culture dishes four times with 5 mL PBS to eliminate excess BSA.
2. Pretreat cells at confluence for 2 h with 20 m*M* cycloheximide in culture medium to block protein synthesis (*see* **Note 4**). Wash the cells twice with 5 mL PBS at 37°C. Detach cells with 4 mL of cell-detachinq solution (5 m*M* EDTA in PBS) for 10 min at 37°C collect the cells by pipeting, and centrifuge at 1000 rpm (100*g*) for 5 min at 4°C. Wash pellets twice with 10 mL PBS, 1 m*M* $CaCl_2$, and 1 m*M* $MgCl_2$ to block EDTA.
3. Resuspend cells in 3 mL plating medium (serum-free DMEM supplemented with 20 m*M* HEPES, 20 m*M* cycloheximide, and 1 m*M* monensin). Plate the cells on 10-cm diameter coated dishes for 30–45 min at 37°C (*see* **Note 5**).
4. Wash twice with 2 mL cold stop solution, and extract the cells with 500 µL lysis buffer for 15 min at 4°C. Lysis buffer containing Nonidet P-40 (NP-40) leaves cell nuclei intact. Precipitate nuclei by centrifuging for 10 min at 14,000 rpm (23,700*g*) at 4°C, collect supernatants, and proceed to the analysis of phosphorylated proteins (*see* **Subheading 3.3.**).

3.2. Tyrosine Phosphorylation Following Integrins Clustering in Nonadherent Cells

1. Pretreat cells at confluence for 2 h with 20 m*M* ciclohexymide to block protein synthesis (*see* **Note 4**). Wash the cells twice with 5 mL PBS at 37°C. Detach cells with 1 mL 0.25% trypsin in PBS for 5 min at 37°C. Collect cells by washing once with 10 mL cold DMEM. 20 m*M* HEPES, 2% FCS, which blocks trypsin and centrifuge for 5 min at 1000 rpm (100*g*) in the cold (4°C). Wash two times with 10 mL cold DMEM, 20 m*M* HEPES and centrifuge at 4°C (*see* **Notes 6** and **10**).
2. Resuspend cells in cold DMEM, 20 m*M* HEPES, aliquot in Eppendorf tubes, and centrifuge at 4°C for 30" at 23,700*g*. Resuspend the cells in 300 µL of primary antibody diluted 10 µg/mL in cold clustering solution 1, and incubate the cells for 50 min at 4°C in the cold under rotation (*see* **Notes 2** and **3**). Wash the cells twice with 500 µL cold DMEM, and 20 m*M* HEPES.
3. Resuspend cells in 300 µL of secondary antibody diluted at 25 µg/mL in clustering solution 2, and incubate for 10 min at 37°C under shacking to induce clustering.
4. Wash twice with 1 mL cold stop solution, and extract the cells with lysis buffer as described above (*see* **Subheading 3.1., step 4**) before proceeding to the analysis of phosphorylated proteins.

3.3. Immunoprecipitation of p125FAK

1. Measure protein concentration in eac:h cell extract by the Bio-Rad protein assay method based on Bradford dye-binding procedure (BIO-RAD, GmbH) or any other protein assay (for example, BCA, Pierce) (*see* **Note 9**).

2. Incubate cell extracts for 1 h at 4°C with 50 µL protein-A-Sepharose beads without adding antibodies. This preclearing step is necessary to remove proteins that bind nonspecifically to the affinity matrix.
3. To immunoprecipitate p125FAK, add 2 µg MAb or 5 µL polyclonal antibodies anti-p125FAK to aliquots of extracts containing a total amount of 500 µg of proteins in Eppendorf tubes. Incubate for 1–3 h at 4°C under rotation to allow the formation of the antibody–antigen complexes.
4. To recover the immunocomplexes. add 100 µL Protein-A-Sepharose beads (50% vol/vol in TBS, 0.5% Triton), and incubate for 1 h at 4°C. Since protein-A-Sepharose has low affinity for mouse immunoglobulins. when anti-p125FAK MAbs are used, add 2 µg rabbit antimouse immunoglobulins to the immunoprecipitation mixture.
5. Collect the immunoprecipitates bound to the protein-A-Sepharose beads by centrifugation at 14,000 rpm for 15 s at 4°C, and wash three times the immunocomplexes with washing buffer 1 (TBS 0.5% Triton) and once with washing buffer 2 (TBS 0.5% Triton, 0.1% SDS).
6. Remove the fluid of the final wash as much as possible. Add 50 µL Laemmli sample buffer, boil for 3 min to release the bound antigen, and collect the supernatants for SDS-PAGE analysis.

3.4. Immunodetection of Tyrosine Phosphorylated p125FAK

1. Separate samples on 6% polyacrylamide gel electrophoresis (SDS-PAGE), and transfer proteins to nitrocellulose using a semidry apparatus (Novablot, Pharmacia) according to manufacturer's instructions.
2. Immerse the nitrocellulose sheet in Ponceau S solution for 2 min at room temperature to visualize protein transfer, and rinse with several changes of distilled water. Mark position of mol-wt markers. Destain by washing in TBS-T for 5 min at room temperature. Incubate the blot for 45 min at 42°C in blocking solution (5% BSA in TBS-T), and wash the nitrocellulose with TBS-T with two changes of 5 min each at room temperature.
3. Incubate the blot in antiphosphotyrosine antibodies diluted in TBS 1% BSA. overnight at 4°C, or for 1 h at room temperature. Discard the primary antibody, and wash the blot with TBS-T with two changes of 15 min each (*see* **Notes 11** and **12**).
4. Incubate for 2 h at room temperature with peroxidase-conjugated anti-mouse or anti-rabbit IgG in TBS-T,1% BSA, and wash twice with TBS-T with two changes of 30 min each (*see* **Note 11**).
5. Visualize p125FAK tyrosine phosphorylation by the chemiluminescent detection method ECL (Amersham. UK). Place blot in chemiluminescent substrate for 1 min, and expose in the dark for 1–10 min. E xposure times have to be set in order to obtain a linear response.
6. Following detection of phosphotyrosine levels, the p125FAK protein may be quantified on the same blot. Strip nitrocellulose blot by incubating for 45 min at 42°C with the stripping solution (62.5 m*M* Tris-HCl, pH 6.8, 2% SDS, 100 m*M* β-mercaptoethanol), and wash three times with TBS-T with changes of 15 min each.

7. Saturate blot in blocking solution as indicated in **Step 2**, wash, and incubate with antip125FAK as primary antibody as described in **Step 3** and secondary horseradish peroxidase-conjugated antibody as clescribed in **Step 4**. Visualize by the peroxidase chemilumiscent substrate.
8. See also **Notes 13** and **14**.

4. Notes

1. Before performing adhesion assays described in **Subheading 3.1.**, it is important to assess the adhesive properties of the cells. This allows the discovery of good adhesive substrates for a given cell and the optimal concentration for coating dishes. The best way to test adhesive substrates is to perform an ,adhesion assay on 96-well dishes (for protocols, *see* Chapter 12). Fibronectin, laminin, collagen IV and vitronectin are the most common adhesive substrates. to which the majority of cell lines adhere. The different substrates should be tested in a range of concentrations within 1 and 50 µg/mL.
2. When adhesion assays are performed on dishes coated with MAbs to specific integrin subunits, it is important that cell-surface expression of these molecules has been previously assessed. This can be easily done by fluorescence-activated cell-sorting analysis or by immunoprecipitating the integrin subunits from surface labeled cells (follow the protocol described in ref. ***11*** for cell surface labeling with I^{125} and in **Subheading 3.3.** for immunoprecipitation).
3. The antibodies used to coat dishes or to perform clustering experiments are affinity-purified on protein-A-Sepharose as described in ref. ***(12)***. After purification, 2 µg of the antibodies are run on SDS-PAGE to test the purity.
4. Cycloheximide blocks protein synthesis, and monensin prevents secretion. These two drugs are routinely added during the adhesive assays, since they prevent the cells from producing and rapidly secreting endogenous extracellular matrix proteins. They are particularly useful when cells are plated on MAbs for a specific integrin subunit, since they avoid nonspecific adhesion.
5. The number of cells to be plated in the adhesion assay varies according to the size of different cells. It is better to star. from an high cell number, since cells are lost during detachment and washes. We normally plate the cells obtained from one confluent 15-cm diameter dish on each 10-cm diameter dish used in the experiments.
6. In integrin-clustering experiments, we noted that cells have to be kept strictly at 4°C to avoid nonspecific phosphorylation. The better way to perform clustering experiments is to work in the cold room, and if the cold room is not available. after detachment, always manipulate cells in ice.
7. In lysis buffer, sodium orthovanadate (Na_3VO_4) (Sigma) is used to prevent phosphotyrosine phosphatases activity. A 50 m*M* stock solution is prepared by dissolving solid Na_3VO_4 in water. The pH of the stock solution is approx 10.0; and at this pH, vanadate is predominantly monomeric as HVO_4^{2-}. Immediately before each experiment, an aliquot of the stock solution is diluted in the culture medium of confluent cell dishes.
8. As an alternative to nonionic detergent (Nonidet P-40), extraction of cells can also be performed with anionic detergents. such as SDS or sodium deoxycholate

(DOC). RIPA buffer is a widely used lysis buffer containing anionic detergents: 5C mM Tris pH 7.5, 150mM NaCl, 5mM EDTA. 1% Triton X-100. 0.1% SDS, 1% DOC, 10 µg/mL leupeptin. 4 ug/mL pepstatin, and 0.1 trypsin inhibitory U/mL aprotinin. Nonionic detergents do not denaturate proteins. while anionic detergents do. Thus it is better to test whether antibodies used to immunoprecipitate a specific protein still recognizes denaturated proteins.

9. Detergents also influences the quantitative protein assays by affecting the development of dye color by chemical-protein and/or chemical-dye interactions. It is thus better to read carefully the protein assay instruction manuals, which normally indicate the detergents compatible with the procedure.
10. In clustering experiments, we found that EDTA detachment interferes with the ability of cells to clusterize. Thus. since integrins are trypsin-resistant receptors, we used trypsin to detach cells. However trypsin may hydrolyze other cell-surface receptors.
11. To avoid background during the immunodetection procedure. wash extensively in each step. We have found that addition of 0.3% Tween to TBS (TBS-T) during washes decreases the background ieve!.
12. Primary antibodies may be reused. We found that diluitions of antiphosphotyrosine antibodies PY20 (1:3500) may be utilized three times. Diluitions are stored at 4°C with sodium azide preservative.
13. An alternative method to analyze p125FAK tyrosine phosphorylation is to immunoprecipitate phosphorylated p125FAK with anti phosphotyrosine antibodies (PY20, 1G2, 4G10; *see* **Subheading 2.4.7.**) and stain the blot with anti p125FAK antibody. By this mean only the phosphorylated p125FAK will be immunoprecipitated and revealed.
14. The procedures described in this chapter can be applied to the analysis of every protein whose phosphorylation is regulated by signalling pathways.

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22

Induction of Ca^{2+} Flux by Adhesion Molecules in Lymphocytes

Ruggero De Maria, Gabriella Palmieri, and Angela Santoni

1. Introduction

Integrins participate in numerous complex biological processes that include cell growth, differentiation, and migration, tissue organization and remodeling, inflammation, and immune response ***(1)***. Signals contributed by integrin-mediated cell adhesion cooperate with classical activatory receptors in initiating cellular activities, such as mitosis, secretion, or gene expression ***(2)***; by these means, cellular responses take into account all aspects of their environment.

Ca^{2+} is an ubiquitous intracellular signaling molecule controlling a wide array of cellular processes, including exocytosis, cytoskeletal rearrangement, and cell proliferation; in particular, a rise in $[Ca^{2+}]_i$ is an essential triggering signal for many of the events associated with lymphocyte activation, function, and proliferation ***(3)***. A variety of protein kinases, phosphatases, and other enzymes are regulated in their activity by $[Ca^{2+}]_i$ ***(4,5)***.

Although integrin engagement has been shown to increase the concentration of intracellular free calcium in various cell systems, the exact response appears to be specific to the integrin, ligand, and cell type. In some instances, it involves phospholipase C-mediated, $Ins(1,4,5)P_3$-induced calcium mobilization from the endoplasmic reticulum; in other cases, it is mediated by transport of extracellular calcium through plasma membrane channels ***(6–8)***.

Various methods exist to monitor $[Ca^{2+}]_i$ variations, both at single-cell or population level. We will describe a method to measure kinetic variations of $[Ca^{2+}]_i$ in heterogeneous cell populations, or in specific cell subpopulations, which can be gated by surface staining with subset-specific antibodies (Abs). The method is based on the use of the Fluo-3 fluorochrome, a fluorescein derivative that increases its emission intensity on Ca^{2+} binding ***(9)***. Fluo-3 has

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the same emission/excitation spectra of fluorescein, and does not display significant wavelength shifts after Ca^{2+} binding, being particularly suitable for the use with conventional argon laser flow cytometers ***(10)***. The fluorescence emission can therefore be registered with a cytofluorimeter, on a gated population. A calibration procedure allows the fluorescence values to be converted in absolute cytoplasmic Ca^{2+} concentration ***(9)***.

Among the other methods, confocal microscopy allows the analysis of $[Ca^{2+}]_i$ fluxes in subcellular compartments ***(11)***. However, this approach requires a more sophisticated, expensive, and less available apparatus, as well as considerable expertise by the operator, thus rendering this approach more cumbersome.

2. Materials

2.1. Preparation of Ab coupled beads

1. 2.5-µm diameter polystyrene latex beads (IDC, Portland, OR).
2. Carbonate buffer: 30 m*M* Na_2CO_3, 70 m*M* $NaHCO_3$, pH 9.5.
3. Purified MAb or adhesive proteins (such as extracellular matrix components, or their active proteolytic fragments) in carbonate buffer or in phosphate-buffered saline (PBS). Avoid the use of any buffer or medium containing serum or bovine serum albumin (BSA), since they interfere with the binding procedures.
4. Formaldehyde 0.01% in PBS.
5. Isotype-specific fluorescent (fluorescein [FITC]- or phycoerythrin-[PE] coupled) anti-mouse Ab. These reagents are used when monitoring what amount of MAb bound to the beads is required (e.g., in the case of simultaneous crosslinking of two or more receptors).

2.2. Cell Labeling

1. Pluronic F-127 (Molecular Probes, Inc., Europe BV, ND) dissolved in dimethylsulfoxide (DMSO) (250 µg/mL). Store at –20°C.
2. Fluo-3/AM (acetoxymethyl ester) (Molecular Probes): dissolve in Pluronic/DMSO at 1-m*M* concentration; light-sensitive; store at –20°C.
3. Different Abs, labeled with one ore more fluorochromes not overlapping with fluorescein (e.g., PerCP [Becton Dickinson, San Jose, CA] or Cy-Chrome [PharMingen, San Diego, CA]), specific for the cell subsets to be studied.

2.3. Ca^{2+} Determination

1. Flow cytometry apparatus, able to excite at 488 nm and to analyze an emission range of ~525–650 nm. A software allowing a continuous analysis of the data will simplify the acquisition procedures.
2. Microfuge with good acceleration/deceleration performances.
3. Ca chloride and Mn chloride.
4. Nonfluorescent Ca ionophore (Ionomycin or bromo-A23187).

3. Methods

3.1. Preparation of Ab- or Protein-Coupled Beads

1. Wash 200 µL of polystyrene latex beads twice with the carbonate buffer, and incubate the beads with 50–200 µg of purified MAb or proteins, in 300–500 µL of carbonate buffer, for 30–60 min at room temperature under continuous agitation (*see* **Note 1**). Washing of the beads can be easily performed in a microfuge, in the pulse operation mode (by allowing it to achieve top speed and stopping after a few seconds).
2. Wash beads three times with PBS and resuspend them in 2 mL of PBS without calcium and magnesium ions. Ab-bound beads can be sterilized and stored for up to 1 yr at 4°C. To this purpose, conjugated beads are incubated for 30 min at 37°C in 0.01% formaldehyde (1 mL) and washed extensively in sterile PBS. Natural ligands, such as extracellular matrix components, are preferably used shortly after conjugation to the beads.

3.2. Cell Labeling

Cells are first washed in RPMI, Hank's balanced salt solution (HBSS), or equivalent medium containing a low amount of fetal calf serum (i.e., 1%), which is used throughout the procedure, and then incubated (up to 10×10^6/mL) with 4 µ*M* Fluo-3, dissolved in DMSO containing 1 µg/mL Pluronic F-127, for 30–45 min at 37°C in the dark, with gentle shaking every 10 min. After Fluo-3 loading, cells are washed three times with warm medium, and stored at 4°C until stimulation and flow cytometry analysis are performed (*see* **Note 2**). If a particular subset of the whole-cell population has to be analyzed, cells can be stained with any fluorescent MAb being excited at 488 nm, and having a fluorescence emission over 600 nm (*see* **Subheading 2.2.**).

3.3. Ca^{2+} Determination

After the labeling procedure, cells are aliquoted in different tubes, so that each tube will contain ~10^6 cells in 0.5 mL, kept at 37°C for 10 min, and run on the flow cytometer for the determination of basal fluorescence relative to the amount of intracellular free calcium in unstimulated cells. Cells are then mixed with 50 µL of stimulating beads, transferred in an Eppendorf tube, spun for 10 s at 8000*g*, and immediately acquired on the flow cytometer. The acquisition can be either continuous or discontinuous, depending on the software available. If the acquisition procedure is discontinuous, the acquisition intervals should not be longer than 20 s, and the number of events to be acquired should be around 2000–3000. The kinetics may vary considerably from experiment to experiment; however, a good evaluation is usually obtained by monitoring the $[Ca^{2+}]_i$ levels for at least 6 min. The evaluation of fluorescence changes on the ~525 channel will allow a measurement of $[Ca^{2+}]_i$ in arbitrary fluorescence units.

3.4. Calibration Procedure

To convert fluorescence values into absolute $[Ca^{2+}]_i$, a calibration procedure is carried out at the end of each experiment ***(9)***. $[Ca^{2+}]_i$ is calculated using the equation:

$$[Ca^{2+}]_i = Kd \times \frac{(F - F_{min})}{(F_{max} - F)}$$

where the dissociation constant (*Kd*) of Fluo-3 is 400 n*M* at vertebrate ionic strength, F is the sample mean fluorescence, and F_{max} is obtained by exposing the cells in 1 m*M* Ca^{2+} ($[Ca^{2+}]_e$ can be easily adjusted by adding calcium chloride to the medium) to 5 µg/mL ionomycin, which equilibrates the $[Ca^{2+}]_i$ to the concentration present in the medium (*see* **Note 3**). To obtain F_{min}, ionomycin-treated cells are exposed to 2 m*M* manganese chloride. Mn^{2+} displaces Ca^{2+} from Fluo-3, forming a complex eightfold more fluorescent than the metal-free dye, but five times lower than the Ca^{2+}/ Fluo-3 complex. Therefore, F_{min} can be calculated as follows:

$$F_{min} = 1.25 \times F_{MnCl2} - 0.25 \times F_{max}$$

(*See* **Note 4**.)

4. Notes

1. The exact incubation time and amount of Ab or protein needed for optimal bead conjugation can be varied between the limits cited in **Subheading 3**. The amount of Ab bound to the beads can be monitored by fluorochrome-conjugated secondary Ab staining and flow cytometry analysis. This procedure is required to compare the amount of different MAb bound to the beads. To this purpose, both control and Ab-coated beads are kept for 15 min in 2% BSA and then incubated for other 15 min with a fluorescent secondary Ab. The use of isotype-specific secondary Ab may be useful in determining the amount of different MAb simultaneously bound to the same beads.
2. After the cell labeling, cells can be stored at 4°C for up to 3 h before the Ca^{2+} measurement is performed. However, the best results are obtained by immediately analyzing the samples.
3. Different from fluorescein, Fluo-3 displays a very low fluorescence when it is not bound to calcium. After ionomycin treatment, the enhancement of cellular fluorescence is usually greater than five-to sixfold the basal Ca^{2+} levels of resting cells. A ionomycin-induced fluorescence increase lower than fourfold in the presence of 1 m*M* Ca^{2+} in the medium would suggest a low efficiency of the loading procedure, which could affect the measurement sensitivity. In preliminary experiments, a range of Fluo-3 doses (1–5 m*M*) and incubation times (25–45 min) should be used to ensure that optimal loading is reached.

4. If cells are kept at 37°C after the loading, a time-dependent loss of cellular Fluo-3 will be observed. This may affect the calibration procedures if F_{min} and F_{max} are not evaluated in each sample at the end of the kinetic experiment.
5. Flow cytometry analysis of $[Ca^{2+}]_i$ offers several advantages over spectrofluorimetry, the traditional method used for this measurement. Cellular debris can be easily gated out by physical and fluorescence parameters. Moreover, the use of fluorochrome-conjugated mAbs, directed against surface molecules expressed on different contaminant cells, is particularly useful to analyze distinct cell populations, thus avoiding complex purification steps.
6. $[Ca^{2+}]_i$ in resting hematopoietic cells is around 100 n*M*. The entity of Ca^{2+} fluxes may vary considerably depending on both signal quality and cross-linking extension. Owing to the nature of their ligands, most of the adhesion receptors require extensive crosslinking to induce Ca^{2+} fluxes. The use of a secondary Ab may be sometimes helpful. However, many receptors require a more intense stimulation to propagate the signal. Ab- or ligand-coupled polystyrene latex beads have been successfully employed to induce early signaling generation through β1 integrins and CD44 ***(12,13)***.
7. Fluo-3 is cell-permeable in its acetoxymethyl (AM) ester form. Unlike other Ca^{2+} indicators, Fluo-3 AM is nonfluorescent, even in the presence of Ca^{2+}, until hydrolyzed inside the cells. It is therefore necessary to keep the cells for 15–20 min at room temperature, after the loading, so that the Fluo-3 AM can be hydrolyzed and converted to its functional form. Pluronic F-127 is a weak detergent, which has been shown to increase the Fluo-3 loading efficiency ***(10)***.
8. The contribution of the intracellular Ca^{2+} stores in Ca^{2+} fluxes induced by receptor stimulation can be analyzed either by using an extracellular Ca^{2+} concentration of ~80 n*M* or pretreating cells with nanomolar concentrations of thapsigargin, a cell-permeable sesquiterpene lactone, which depletes the intracellular Ca^{2+} stores via the inhibition of the endoplasmic reticulum Ca^{2+}-ATPase **(14)**.

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23

Integrin-Mediated Stimulation of Tyrosine Phosphorylation in Lymphoid Cells

Angela Gismondi and Angela Santoni

1. Introduction

Engagement of integrins by natural ligands or specific antibodies initiates biochemical signaling events within the cell, important for regulating different cell functions, such as migration, adhesion, proliferation, differentiation, apoptosis, and specific gene expression ***(1–3)***. Integrin-mediated cell adhesion has been shown to be accompanied by tyrosine phosphorylation of several proteins in a variety of cell types including lymphoid cells ***(4–7)***.

The identification of the substrates of integrin-activated tyrosine kinases has become one of the most active areas in the study of signaling pathways initiated by integrin receptor crosslinking. Some of the methods used to approach this issue include Western blot, immunoaffinity purification, and immunocomplex kinase assay ***(7,8)***.

It is possible to identify inducible changes in the spectrum of tyrosine phosphorylated proteins by immunoblotting whole-cell lysate from integrin-stimulated vs unstimulated cells with an anti-phosphotyrosine (pTyr) monoclonal antibody (MAb). This often leads to educated guesses regarding the identity of tyrosine phosphorylated proteins of a specific molecular mass. The precise identity of tyrosine phosphorylated proteins can be evaluated by immunoaffinity purification of tyrosine phosphorylated proteins with an anti-pTyr MAb followed by immunoblotting with an antibody directed against a specific protein or vice versa using an antibody directed against a specific protein for the immunoaffinity purification and the anti-pTyr MAb for the immunoblotting analysis. Using the immunocomplex kinase assay, it is possible to evaluate whether a specific tyrosine phosphorylated protein has tyrosine kinase activity.

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2. Materials

2.1. Cell Stimulation and Lysate Preparation

1. RPMI-1640 medium.
2. Antibodies against α- and/or β-integrin subunits.
3. Extracellular matrix (ECM) or vascular cell adhesion molecule-1 (VCAM-1) proteins (1–10 µg/mL in PBS).
4. 10-cm petri dishes or 24-well plates.
5. Washing buffer (0.4 m*M* EDTA-Na_2, 10 m*M* $Na_4P_2O_7$, 10 m*M* NaF, 0.1 m*M* Na_3VO_4 in PBS, pH 7.4).
6. Lysis buffer (1% vol/vol Triton X-100, 1 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 0.1% NaN_3 (**caution:** sodium azide is poisonous), 1 m*M* phenylmethylsulfonyl fluoride (PMSF), 2 µg/mL aprotinin, 2 µg/mL leupeptin, 10 m*M* NaF, 150 m*M* NaCl, 10 m*M* iodoacetamide (*see* **Note 1**).

2.2. Immunoprecipitation

1. Cell lysate.
2. Protein A Sepharose CL-4B (Pharmacia BioTech AB, Uppsala, Sweden).
3. Anti-immunoglobulin rabbit serum.
4. Incubation buffer (0.25% Triton X-100, 50 m*M* Tris, 150 m*M* NaCl, 5 m*M* EDTA-Na_2, 2% bovine serum albumin [BSA], pH 7.4).
5. RIPA washing buffer without proteins (0.25% Triton X-100, 50 m*M* Tris, 150 m*M* NaCl, 5 m*M* EDTA-Na_2, 0.1% SDS, 0.1% Nadeoxycholate, pH 7.4).
6. RIPA washing buffer with proteins (0.25% Triton X-100, 50 m*M* Tris, 150 m*M* NaCl, 5 m*M* EDTA-Na_2, 0.1% SDS, 0.1% Nadeoxycholate, 0.1% BSA, pH 7.4).
7. SDS sample buffer (60 m*M* Tris, 2% SDS, 20% glycerol, 0.05% of a bromophenol blue sature solution, 2% β-mercaptoethanol, pH 6.8).
8. Additional reagents and equipment for SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

2.3. Western Blotting

1. Transfer buffer (25 m*M* Tris, 192 m*M* glycine, 20% methanol).
2. 0.45 µm Nitrocellulose membrane filter (Schleicher & Schull, Dassel, Germany).
3. Ponceau S solution (0.5% Ponceau S/1% acetic acid in H_2O).
4. Blocking solution (5% nonfat dry milk in PBS or 3% BSA in PBS).
5. Enhanced chemiluminescence (ECL) kit (Amersham Int., Amersham, UK).
6. Photographic equipment.

2.4. Kinase Assay

1. Kinase buffer (30 m*M* Tris, pH 7.5, 10 m*M* $MgCl_2$, and 1 m*M* $MnCl_2$).
2. 10 m*M* ATP.
3. ^{32}PγATP (SA > 5000 Ci/mmol).
4. Protein kinase substrate.
5. 2X concentrated SDS sample buffer.
6. Staining solution (0.25 % Coomassie blue in 45% methanol, 10% acetic acid).
7. Destaining solution (40% methanol, 10% acetic acid).

3. Methods

3.1. Cell Stimulation and Lysate Preparation

Cell stimulation by integrin crosslinking with antibodies:

1. Resuspend the cells in RPMI 1640 serum-free medium (1×10^8 cells/mL), and incubate with saturating doses of the antibody directed against α- or β- integrin subunits for 30 min at 4°C.
2. Wash off the unbound antibody using ice-cold PBS, resuspend the cells in prewarmed RPMI 1640 serum-free medium, add the secondary antibody (100 µg/mL × 10^8 cells/mL) and allow incubation to proceed for a desired time period (from 1–45 min) at 37°C.
3. Add ice-cold washing buffer to stop the stimulation and centrifuge the cells for 5 min at 500*g*.
4. Decant washing buffer, add 1 mL of ice-cold lysis buffer, and incubate for 30 min at 4°C.
5. At the end of the incubation period, remove insoluble material by centrifuging cell lysates at 15,000*g* for 15 min at 4°C in a microcentrifuge.

Cell stimulation by integrin crosslinking with natural ligands:

1. Resuspend ECM components (laminin, fibronectin, collagen) or VCAM-1 in PBS (1–20 µg/mL), add 5 mL of this solution to a 10-cm diameter dish, and incubate overnight at 4°C.
2. Wash the dish three times with PBS, add 3 mL of the cell suspension (1×10^7 cells/mL) and allow cells to adhere to the immobilized substrate for the desired time period at 37°C (*see* **Note 2**).
3. Add 5 mL/dish of ice-cold washing buffer to stop the stimulation.
4. Recover nonadherent cells by centrifuging the supernatant for 5 min at 500*g* at 4°C, and adherent cells by adding 1 mL of lysis buffer to the dish.
5. Combine adherent and non adherent cells and lyse as above.

3.2. Immunoprecipitation

Conjugation of antibody to protein A-Sepharose CL-4B beads:

1. Add 100 µL of 20% protein A-Sepharose CL-4B beads suspension to a 1.5-mL Eppendorf tube, wash the beads by adding 800 mL of RIPA washing buffer without proteins, centrifuge for 30 s in an Eppendorf microcentrifuge, and remove the buffer by aspiration.
2. Add to the washed beads 300 µL of RIPA incubation buffer and 5 µg of affinity-purified rabbit polyclonal antibody directed against mouse or rat immunoglobulins, and incubate with agitation for at least 1 h at 4°C (*see* **Note 3**).
3. Wash three times the beads, with RIPA washing buffer with proteins by centrifuging them for 30 s in an Eppendorf microcentrifuge, and removing the buffer by aspiration.

4. Add to the washed beads 300 µL of RIPA incubation buffer, and a saturating concentration of the desired mouse or rat antibody, and incubate with agitation for at least 2 h at 4°C.
5. Wash the beads three times as described in **step 3**.

Cell lysate preclearing and immunoprecipitation:

1. Add 100 µL of 20% protein A-Sepharose CL-4B beads suspension to a 1.5-mL Eppendorf tube, and wash the beads by adding 800 µL of RIPA washing buffer without proteins.
2. Add 300 µL of cell lysate ($30–50 \times 10^6$ cell equivalent) to the washed beads, and incubate with agitation for 2 h at 4°C (*see* **Note 4**).
3. At the end of incubation period, spin and recover the precleared cell lysate, combine it with the antibody-conjugated beads, and incubate for at least 2 h with agitation at 4°C (*see* **Note 5**).
4. Wash the immunocomplexes five times with RIPA washing buffer with proteins, three times with RIPA washing buffer without proteins, then resuspend them in 40 µL of SDS sample buffer, boil for 5 min, and then centrifuge for 2 min (*see* **Note 6**).
5. Load the supernatant into a gel lane, and separate proteins by SDS-PAGE.

3.3. Western Blotting

1. Following electrophoresis, equilibrate the gel in transfer buffer for 10 min at room temperature (*see* **Note 7**).
2. Transfer the proteins from SDS-PAGE gel to nitrocellulose membranes at 50 mA for 2–3 h or at 15 mA overnight at 4°C (*see* **Note 8**).
3. The transferred proteins can be visualized by staining the membrane for 5 min with Ponceau S solution (*see* **Notes 9** and **10**).
4. Rinse excess stain with PBS-T (PBS plus 0.05% Tween 20), and place the blot into blocking solution (5% nonfat milk or 1–3% BSA in PBS-T). Allow the blot to block for 1 h at room temperature or overnight at 4°C with agitation (*see* **Note 11**).
5. Decant the blocking solution, and rinse the membrane using two changes of washing buffer (PBS-T). Then wash once for 10 min and three times for 5 min with fresh changes of the washing buffer at room temperature with agitation.
6. Add the primary antibody appropriately diluted in PBS-T plus 1% BSA (i.e., 4G10 anti-pTyr [Upstate BioTechnology, Lake Placid, NY] mouse MAb can be used at 1 µg/mL). Incubate with agitation for at least 1 h at room temperature or overnight at 4°C (*see* **Note 12**).
7. Decant the primary antibody solution, and wash the membrane as detailed in **step 5**.
8. Decant the washing buffer, and add the enzyme- (i.e., horseradish peroxidase) conjugated anti-mouse IgG, appropriately diluted in PBS-T plus 1% BSA. Incubate with agitation for 45 min or 1 h at room temperature (*see* **Note 13**).
9. Decant the secondary antibody solution, and wash the membrane as indicated in **step 5**.
10. Detect immunoreactivity using an enhanced chemiluminescence kit (i.e., Amersham International plc, Amersham, UK) according to the instructions (*see* **Note 14**).

11. The blot can be stripped with 62.5 m*M* Tris-HCl (pH 6.8), 2% SDS, and 100 m*M* β-mercaptoethanol at 50°C for 30 min, washed three times with large amount of PBS-T, blocked, and reprobed (*see* **Note 15**).

3.4. Protein Kinase (ERK) Assay with an Immunocomplex (See Note 16)

1. After immunoprecipitation, wash the immunocomplexes three times at 4°C with 1 mL of lysis buffer and once with kinase buffer. The supernatant is then removed by aspiration.
2. Incubate the immunocomplexes for 30 min at 30°C with 25 µL of the kinase assay buffer containing 10 µ*M* ATP, 5–10 µCi ^{32}PγATP, and 7.5 µg of the appropriate protein kinase substrate myelin basic protein (MBP).
3. Stop the reaction by adding 25 µL of 2X concentrated electrophoresis sample buffer, and boiling for 5 min.
4. Centrifuge the samples for 2 min at 4°C in a microcentrifuge, and run the soluble fraction on SDS-PAGE.
5. After electrophoresis, the gel can be fixed and stained with 0.25% Coomassie blue (in 45% methanol, 10% acetic acid), destained (40% methanol, 10% acetic acid), dried, and exposed to X-ray film at –70°C. Kinase activity will be indicated by the presence of phosphorylated protein substrate.

4. Notes

1. PMSF should be freshly added to lysis buffer; a 100-m*M* PMSF stock solution in isopropanol can be stored at –20°C).
2. Because lymphocytes poorly adhere to ECM components or to VCAM-1, this binding may be enhanced by pretreating cells with PMA or stimulatory Ab directed against integrin receptors before plating cells on ECM- or VCAM-1-precoated plastic dishes ***(7,8)***.
3. An affinity-purified rabbit polyclonal antibody directed against mouse or rat immunoglobuline is used when the primary antibody is a mouse or rat MAb, which does not directly bind to protein A.
4. Preclearing removes nonspecifically absorbing material; it can also be performed on protein A-Sepharose CL-4B precoated with an antibody directed against an unrelated protein, and not crossreacting with the protein of interest.
5. Reaction between antigen and antibody can be extended overnight, but this may increase the background when using antibody bound to Sepharose.
6. Immunocomplex should not be left overnight in the washing buffer, since significant dissociation may occur.
7. Use gloves when manipulating filter papers, gels, and nitrocellulose. Oil from hands blocks the transfer to the nitrocellulose paper.
8. Transfer time is dependent on the thickness of the gel and the size of the protein being transferred. In general, proteins are transferred within 1–6 h, but high-mol-wt molecules may take longer. Overnight transfer is reliable and convenient.
9. If no transfer of protein has occurred, check that correct orientation of filter and gel relative to the anode and cathode electrodes was used.

10. If air bubbles are trapped between the filter and the gel, they will appear as clear white spots on the filter after blotting and staining. Take extra care to ensure that all bubbles are removed.
11. For anti-pTyr blot, use 1–3% BSA in PBS-T as blocking solution.
12. Dilution of primary antibody required to give optimal results will vary and should be determined for each antibody used.
13. Do not include sodium azide in any solution containing horseradish peroxidase-labeled reagents in that sodium azide can induce an irreversibile inactivation of horseradish peroxidase.
14. The presence of multiple bands in the blot may be related to excessive amount of lysate, protein degradation, or nonspecific binding by the primary or secondary antibody. Excessive or diffuse signal may be related to much protein on the gel, excess of primary antibody, or to overexposure. No signal or weak signal may be owing to inadequate transfer of proteins, horseradish peroxidase inactivation, or low amount of protein.
15. Following stripping, incubate the membrane with ECL detection reagents and expose to film to ensure removal of antibody.
16. Different reagents and conditions have to be employed depending on the kinase activity to be assayed. Materials and method described concern the evaluation of ERK activity.

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24

The Identification of Signaling Molecules by the Yeast Two-Hybrid System

Stefano Biffo and Pier Carlo Marchisio

1. Introduction

The yeast two-hybrid system is a sensitive method to detect intracellular interactions between proteins. It has been successfully employed in the identification of novel interactors of several proteins, including adhesion molecules (for a general review on the method, *see* **refs.** ***1*** and ***2***). In general, the high sensitivity of the yeast two-hybrid system allows the detection of weak and transient interactions that escape biochemical analysis. Howewer, in some limited cases, interactions found with biochemical methods have not been reproduced in the yeast assay, possibly because of the lack of some posttranslational modifications. More generally, the high sensitivity of the yeast interaction assay may lead to the disclosure of false-positive protein–protein interactions. For this reason, it is always necessary to confirm the yeast interaction data using other methods of study for protein binding.

The rationale of the yeast two-hybrid system relies on the physical separation of the DNA binding activity from the transcriptional activity of transcription factors. In its simple form, the protein of interest is fused to a DNA binding protein (bait). Once transfected into yeast cells, this fusion protein would ideally translocate into the nucleus and bind its target DNA sequence that is artificially located upstream of a reporter gene. However, in the absence of a proper activating domain, transcription from the reporter gene does not occur. In the following step, yeast cells are transfected with a plasmid cDNA library where the cDNA proteins are fused with a transcriptional activation domain. In the ideal case, when a protein encoded by the cDNA library binds to the bait, the DNA binding and the transcriptional activities are rejoined and transcription from the reporter gene is started. The reporter gene is normally either a *lac*Z (conferring blue color), an enzyme (for amino acid syntesis), or both.

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Table 1
Main Features of the System Commercialized by Clontech and Distributed by Roger Brent's Lab

System	DNA binding domain	Activating domain	Reporter genes
GALG	GAL4	GAL4	His3/LacZ
LexA	LexA	BII2	Leu3/LacZ

At the present, there are two main two-hybrid system variants: one is commercialized by Clontech (Palo Alto, CA). In the second, which will be referred here as the LexA system, the reagents are distributed by Roger Brent's lab and/or can be bought from Invitrogen. **Table 1** shows the main technical features of the two systems. Both methods have been successfully used in order to isolate interactors of adhesion molecules; for instance, the interactor cytohesin-1 has been isolated by the Roger Brent system ***(3)***, and the LIF-1 interactor by the Clontech ***(4)***. The decision between the two systems relies on several factors, the most important being, to our knowledge, the choice of the library. Complete protocols for the Clontech system are available through the Internet server of the company (http: //www.clontech.com/clontech/Catalog/MATCHMAKER/MMintro. html). Very good protocols and information for the LexA system can be obtained through the Internet (http://xanadu.mgh.harvard.edu/brentlabweb/interactiontrap.html).

Since many annotated protocols for the interaction hunt are widely available through the Internet, in this chapter, we will simply give a bench-oriented outline of the method, together with the description of the basic techniques necessary to implement successfully an interaction hunt, and some useful advice that we have found to be important in our experiments.

The method is based on three points:

1. Preparation of the bait and test of its suitability;
2. Actual screening of the library (interaction hunt); and
3. Preliminary characterization of clones.

Many techniques may be required: for instance, subcloning and sequencing, PCR and Southern blot, protein analysis. These will be not described in detail here.

Basic knowledge of yeast techniques is also required ***(5)***. The final part of the chapter will describe a protocol to transform yeast cells, a technique that is the basis for each step of a two-hybrid screen. A shaking platform and a cell incubator set at 30°C are needed. Yeast cells are better handled under sterile conditions.

2. Materials and Preparative Work

1. Yeast Media are supplied by DIFCO (Detriot, MI).
2. Large plates for library screening are obtained by Nunc (Naperville, IL) (square 24 × 24) .
3. Common suppliers for interaction hunt-related reagents are: Clontech, and Invitrogen.
4. We prepared DNA for transfection using Qiagen (Germany) columns.
5. All other reagents can be obtained through several commercial suppliers.

We have obtained yeast strains and plasmids through the lab of Roger Brent, who has freely distributed the reagents to more than 2000 laboratories. However, when this chapter was written, the reagents were distributed through commercial suppliers. Informations on the distribution policy can be found by contacting *LaurenHa@molbio.mgh.harvard.edu*. **Table 2** contains the "minimal" plasmids and yeast strain that are needed in order to perform an interaction hunt.

Carrier DNA for transfection must be prepared in advance. The use of carrier DNA dramatically improves transfection efficiency. It is important that carrier DNA is of high molecular weight (around 7 kb), since shorter fragments of DNA can inhibit transfection. Protocols for carrier DNA preparations are in **ref. *6***.

Plates and media should be prepared and used fresh. We limit the storage of plates to 1 wk at 4°C.

3. Method

In the LexA system, the protein of interest is fused to a LexA binding protein in the plasmid pEG202 and transfected in EGY48 yeast cells. A plasmid cDNA library fusion protein containing an activation domain is cotransfected (plasmid pJG4-5). When the cDNA-encoded protein binds the bait, the activation domain starts transcription from the LexA operator. The reporter genes are Leu2 (leucine metabolism, stably inserted in yeast cells) and *lac*Z (β-galactosidase, inserted as a reporter plasmid).

All the plasmids that are used in yeast contain a marker for bacterial amplification (Amp), and a marker for yeast selection (various enzymes for metabolic pathways).

3.1. Bait Testing

1. Prepare a fusion plasmid containing the protein of interest into the expression vector pEG 202 (**Note 1**).
2. Check for lethality of the bait by rapid yeast cotransformation of the EGY48 strain with the pEG202 bait and plasmid pSH18-34 (protocol 1). Plate onto Ura–, His–, Leu+, Trp+, Glu plates. Incubate 48 h at 30°C (**Notes 2** and **3**).

Table 2
Plasmids and Yeast Strain Necessary for an Interaction Hunt

Reagent	Use	Features	Notes
Yeast strain EGY 48	Yeast strain for the interaction hunt	It does not grow in Leu–, Leu2 gene is controlled by LexA promoter	Alternative strains differing in the LexA promoter strength are available
Plasmid pEG202	Used for preparing the bait	LexA binding protein in frame with protein of study	HIS3 selection
Plasmid pJK101	Reporter plasmid for repression assay	GAL-inducible promoter followed by LexA binding sites and *lac*Z	URA 3 selection
Plasmid pSH18-34	Reporter plasmid for transcriptional activation	LexA operators in front of *lac*Z gene	URA 3 selection
Plasmid pJG4-5	Plasmid for the cDNA library	GAL promoter, nuclear localization signal and activation domain, HA tag, polylinker	TRPI selection

[a]Note that several other variant plasmids have been developed, but are not included here. Positive and negative control plasmids for transcription and repression are also needed (originally pRFHM1 and pSH17-4). We may also provide some.

3. If yeast colonies are visible, take three colonies for each bait (plus positive and negative controls) with a sterile tip, put them in 20 µL of sterile Tris-EDTA buffer, and dot them as follows:
   a. 5 mL onto Ura–, His–, Leu–, Trp+, Glu plates (self-activation test 1). The expected result is absence of growth;
   b. 5 mL onto Ura–, His–, Leu+, Trp+, Glu, X-GAL plates (self-activation test 2). The expected result is growth of yeast colonies that remain white.
4. If yeast colonies do not grow in the self-activation test 1, and do not turn blue in the self-activation test 2, it means that the bait does not activate transcription *per se*. It must be now checked that the bait goes into the nucleus and recognizes its

promoter. This is achieved by the repressor test. In order to do so, cotransfect EGY48 cells with plasmids pJK 101 and the bait. Include a positive and a negative control. After selection in Ura-, His-, Leu+, Trp+, Glu plates, take three colonies with a sterile tip, mix them in 20 mL of sterile Tris-EDTA buffer, and dot:

b. 5 µL onto Ura–, His–, Leu+, Trp+, Gal, X-GAL plates. If the colonies are light blue or white, proceed to the next step (**Note 4**).

3.2. Interaction Hunt

1. Amplify library for tranformation. Around 200 µg of library must be prepared in order to perform an interaction hunt. Amplification is achieved through transformation in *Escherichia Coli* followed by plating and scraping of colonies. DNA is then prepared by conventional Qiagen (Quiagen, Germany) columns or cesium chloride gradients (**Note 5**).
2. The interaction hunt now starts! Prepare 31 large plates of Ura–, His–, Leu+, Trp–, Glu plates. Dry them well (**Note 6**).
3. Make 30 transformations of EGY48 yeast cells containing the bait and the reporter plasmid pSH18-34 by adding for each tube 1–5 µg of library DNA and 100–200 µg of carrier DNA. Plate on Ura–, His–, Leu+, Trp–, Glu plates to select for plasmid DNA. Plate one tube of yeast cells without transformation. Incubate at 30°C for 48 h (**Note 7**).
4. Quantify the number of colonies. At least 1 million primary transformants must be obtained.
5. Scrape plates with yeast clones using a sterile slide. Perform the work under an hood. Do not remove any agarose, since it contains traces of glucose that will inhibit galactose induction of the fusion protein. Collect the yeast slurry in a sterile Erlenmeyer. Add an equal volume of TE and mix well (**Note 8**).
6. Spin at 1400*g* for 10 min. Pour off the supernatant, and scrape the top layer that contains agarose fragments. Resuspend in an equal volume of TE, mix, and spin again. Pour off the supernatant and resuspend in storage buffer. Freeze at –80°C in aliquots (**Note 9**).
7. Take a 50-µL aliquot and inoculate a 5-mL culture of YPD-galactose to induce the expression of the library. Incubate for 4–5 h shaking at 250 rpm.
8. Plate out 4 mL and 400 µL of the YPD-galactose induced yeasts on Ura–, His–, Leu–, Trp–, Gal plates. Also plate 400 µL on Ura–, His–, Leu–, Trp–, Glu plates to check for the background growth (none should grow). Also plate 1:100 and 1:10,000 dilutions in Ura–, His–, Leu+, Trp–, Glu plates to count viable cells (**Note 10**).

3.3. Preliminary Analysis (Note 11)

1. Pick the first colonies growing up (your interactors). Dot all colonies again onto:
   a. Ura–, His–, Leu–, Trp–, Gal plates (they must grow);
   b. Ura–, His–, Leu–, Trp–, Glu plates (they should not grow);
   c. Ura–, His–, Leu+, Trp–, Gal, X-GAL plates (they should turn blue, *see* **Note 12**).

2. Isolate yeast DNA from clones (see **Note 13**).
3. Electroporate on KC8 bacteria, and recover the plasmid cDNA (see **Notes 14** and **15**).
4. Perform analysis in order to identify unique inserts. Make large-scale DNA preparation in order to retransform the EGY48 strain containing your bait and pSH18-34. Replate onto the plates as in Step 1: the results should be reproduced.
5. Sequence the cDNAs.

4. Notes

1. In general, it is necessary to prepare several constructs and test their suitability in the following assays. There are two type of tests that must be performed. The first one is to check that the bait is unable *per se* to activate transcription or is lethal. The second is to assess whether the bait can enter the nucleus and bind its target sequence. Indeed, some constructs may prove either to self-activate or to be unable to enter the nucleus. Hydrophobic regions are sometimes unable to be translocated in the nucleus. The size of the protein to be inserted is not a dramatic limit. However, constructs encompassing more than 300–400 amino acids are not used. Our smallest working construct had 23 amino acid, the biggest being 303. Cloning must be performed keeping the protein of interest in frame. If the DNA fragments to be subcloned are prepared by PCR, it is necessary to sequence the plasmids.
2. All these protocols and the following rely on optimal yeast transfections (*see* **protocol 1**). Following transfections, the yeasts are always plated on selective media (*see* **protocol 2** for recipes) and grown at 30°C. Colonies should be seen after 2–3 d. When yeast cells are dotted from a single colony to a new plate, growth should be allowed for 48 h.
3. Also included in this experiment are a positive and a negative control for self-activation. The positive control will turn blue in X-GAL plates and grow in Leu–, whereas the negative control will neither turn blue nor grow in Leu–. These two plasmids can be obtained either commercially or, if the LexA system is used, by contacting our lab. The Leu- test is more sensitive than the X-GAL test. It can be commonly observed that transfected yeasts do not turn blue, but grow in Leu–. Since the Leu– test is the first to be used in the actual screening, in case yeast cells grow in Leu–, one has to decide whether the generated background is too high. If possible, baits that present even a minimal growth in Leu– should be discarded. Alternatively, yeast strains that are less responsive can be also used (informations on these strains is available through the Internet).
4. In the repression test, EGY48 cells are cotransfected with the bait and with plasmid pJK101 (the plasmid can be obtained through Clontech). pJK101 has Ura3 as selectable marker. Following cotransfection, yeasts are therefore grown in Ura–, His–. pJK101 contains binding sites for LexA that are downstream of a GAL promoter and upstream of a *Lac*Z gene. In the presence of galactose, *Lac*Z is transcribed (and yeast cells turn blue), unless the bait sits on its target sequences and inhibits transcription. In this test, light blue colonies are the expected result

for a good bait. Very rarely, white colonies are seen. The test is performed with positive and negative controls that can be obtained as above. If the bait does not repress, it normally means that it does not enter the nucleus. It is possible to perform Western blot analysis in order to check for protein integrity.

5. Libraries are normally obtained commercially or through other labs. The libraries for the two systems (GAL4 and LexA) are unfortunately not compatible. Library amplification should be always performed on plates in order to avoid loss of representation.
6. Plates should be dried under a hood. Be careful that the agar solidifies without waves on the surface, since this will make the subsequent scraping without taking agarose clumps very difficult.
7. The first step is done only to select for the cDNA plasmid. The selection for interactors is better performed as a second separate step. To perform this step, yeast cells are grown overnight, diluted, and made competent in the morning. Transfection is more efficient if performed in small volumes. It is important to work under sterility conditions. Plating should be done using glass beads. Colonies should be visible within 48 h. It is important also to plate a batch of nontransfected yeasts in order to check the background.
8. Two precautions have to be taken in these two steps:
   a. Keep sterility; and
   b. Mix the yeast slurry very well. The second precaution is necessary in order to maintain the representativity of the library. In order to have a chance that rare clones are picked up with the interaction hunt, 10-fold excess of clones are plated in the selection step (if 1 million clones are obtained in the primary transformation, 10 millions are plated in the second).
9. The yeast cells can be frozen for long periods at –80°C. For the library, we normally freeze 500-microliter aliquots.
10. In this step, a simplified procedure is presented. In theory, one should titer the library and plate the right number of colonies. However, the procedure outlined allows a faster screening.
11. Colonies can be picked up starting from the second day. It is advisable to collect colonies that grow at the second, at the third, and at the fourth day separately. Often colonies arising on the same day represent similar clones.
12. If a yeast does not grow in glucose, but grows and turns blue in galactose, it means that upon induction of the protein of the library, transcription from LexA operator is started. From this point, the logical steps are to isolate the plasmid cDNAs, and retransfect yeasts in order to be sure that the effects are mediated by the plasmid. One common problem at this stage, is to have too many clones to analyse. The possible strategies are:

    a. analyse only the strong interactors (turning blue in X-GAL);
    b. PCR from yeast and do restriction analysis to pool similar clones;
    c. Blot yeast colonies and screen with probes to eliminate known proteins.

In our screenings, we proceed as following:

a. Calculate the minimal number of clones to be analysed in order to detect rare RNAs;
b. Dot them and select the ones that have no growth in glucose;
c. Extract DNA and perform PCR;
d. Dot blot PCR products, and crosshybridize them with ECL labelled probes;
e. Pool duplicate clones;
f. Transfect in E. Coli the selected clones.

13. There are several protocols for plasmid DNA extraction from yeasts. The one we favor is the classical method of Holm et al. ***(1)***. Faster protocols that make use of glass beads are described in Kaiser et al. ***(5)***. Plasmid DNA yield from yeasts is normally low and does not allow direct restriction analysis.
14. It is necessary to use and transform KC8 cells and plate them on tryptophane minus media in order to rescue only the library plasmid, and not also the reporter and the bait plasmids. A protocol for KC8 bacteria handling is available at: http://xanadu.mgh.harvard.edu/brentlabweb/interactiontrap.html)
15. DNA from KC8 is generally good for transfection, but less for sequencing. We have routinely sequenced KC8 recovered DNA with automated sequencing using the upstream primer BCO1 (*see* again http://xanadu.mgh.harvard.edu/brentlabweb/interactiontrap.html). Following sequencing, several artifacts may be found. Of these, the most common are heat-shock proteins and ribosomal proteins. To eliminate gross artifacts, we have analyzed all our interactors for their ability to bind mutated versions of our bait. If this strategy is not available, biochemical work is required.

5. Protocols

5.1. Rapid Yeast DNA Transformation

1. Start from 50 mL of overnight yeast culture: dilute to 300 mL (10–15 transformations); grow to OD_{600} of O.8;
2. Pellet 2500 rpm, 5 min.;
3. Rinse with 20 mL of sterile water;
4. Rinse one time with TE-LiAc (0.1 *M* lithium acetate, pH 7.2; 0.1 *M* Tris, pH 7.5; 50 m*M* EDTA) and pool tubes;
5. Incubate for 5 min. at 30°C, inverting every min;
6. Spin and resuspend by flipping 1:1 in TE-LiAc;
7. Incubate at 30°C for 10 min;
8. Take 100 µL yeast suspension; mix with 1–5 µg DNA (plus 100 µg carrier DNA), and pipet up and down one time;
9. Add 600 µL of a solution of 40% PEG 4000 inTE-LiAc. Slowly pipet until it is homogenous;
10. Incubate at 30°C for 30–45 and invert every 5 min;
11. Heat-shock for 15 min at 42°C
12. Spin for 2 min at 1600 rpm; remove 600 µL of PEG;

13. Add TE (400 µL for big plates) and spread with beads (all in big 24 × 24 plates, 40 µL in small ones);
14. Incubate for 48 h at 30°C.

5.2. Preparation of Yeast Solutions

1. YPD (full medium): 2% peptone; 1% yeast extract. Autoclave and add the appropriate sugar source to 2%.
2. YPD plates: Add to the above medium one NaOH pellet/L and 2% Bacto agar. Autoclave and add the appropriate sugar source to 2%.
3. Yeast minimal media: They are prepared adding a nitrogen source, the amino acid mixture, a sugar, plus the specific amino acids to be used in the selection. Agar plates are prepared from liquid media by adding Bacto agar to 2% and autoclaving.
    a. 20% Glucose, 20% galactose: Prepare a 20% solution in distilled water (10×) and sterile-filter. Store at room temperature and open only under a hood.
    b. 10× YNB (yeast nitrogen base): Dissolve 6.7 g of YNB (DIFCO, 0919-15-3) in 100 mL of distilled water. Sterile-filter, and store as above.
4. 20× Amino acid mixture: Weight out: Adenine (0.9 g), arginine (0.432 g), methionine (0.432 g), tyrosine (0.648 g), isoleucine (0.648 g), lysine (O.648 g), phenylalanine (1.08 g), glutamate (2.16 g), aspartate (2.16 g), threonine (4.32 g), serine (7.92 g), valine (3.24 g) in 900 mL distilled water. Autoclave for 17 min. Store at room temperature.
5. 200X leucin, 200X histidine, 200X tryptophane: Weight 4.8 mg/mL and autoclave. Store 200X Tryptophane in the dark.
6. 100X Uracil: Weight 2.4 mg/mL and autoclave.
7. YNB base, liquid: 1X YNB, 1X amino acid mixture, 2% sugar. Add the appropriate amino acids for selection.
8. YNB plate: Prepare directly a 1X YNB solution containing 2% Bacto agar, and an NaOH pellet (1/L). Autoclave. Add the amino acids and the sugar. Pour the plates.

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