(a) (b) (c)

This is Daisy, the pig, viewed at different levels and orientations. Her eye and ear (a) in fine detail take on one form of *asymmetry* (top-to-bottom), whereas the nose (minus nose-ring-art) has a **bilateral symmetry**, but is *asymmetrical* into the plane of the photo (b). In (c) we can see her full face from one side, and she *seems* to be asymmetrical in all three planes – but this is because we are looking at a 2D picture. To analyze the 3D structure of any given part to be engineered, this illustrates the importance of reconstructing the *full* structure, based on a number of defined orientations and at representative scales (e.g. from mm and μm to nm).

6 Asymmetry: 3D Complexity and Layer Engineering – Worth the Hassle?

If cells built tissues the same way that men build bridges . . .

If cells built tissues the same way that men build bridges . . .

When analyzing asymmetry and hierarchical complexity, it is important to have a strong sense of scale. Analysis of scale-levels in biofabrication can provide some surprisingly obvious but useful observations. Figure 6.1 shows a simple composite illustration, comparing the proportions of human-scale and cell-scale fabrication. This helps us develop a semi-realistic picture of 'a day in the life' of a tissue construction cell, compared with human construction workers.

If we scale down the main building unit in the human world (i.e. man/woman) to that which we are expecting to build our tissue grafts (i.e.cells), we end up with a ratio in the region of 100,000:1 (10^{-5}). This assumes a nominal spherical cell of 20 μm and 2 m man, both of which are of course debatable but

Extreme Tissue Engineering: Concepts and Strategies for Tissue Fabrication, First Edition. Robert A. Brown.

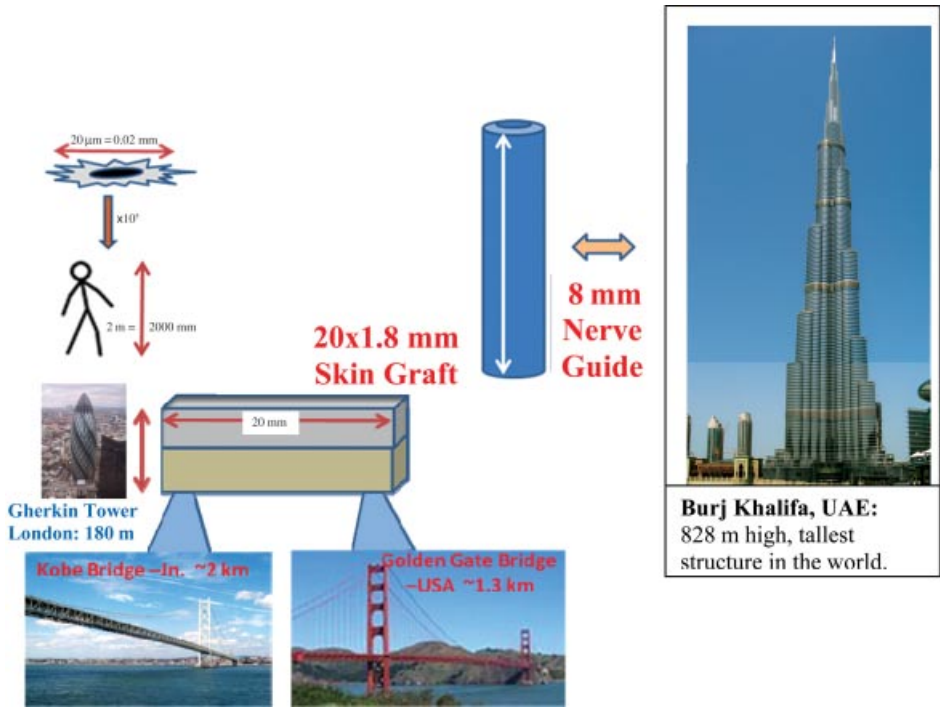


Figure 6.1 More issues of scale: comparing cell and human scales. Both cells and men must still move themselves, and the materials they assemble, across space proportional to their own size. The laws of motion, mass, energy conversion apply to both.

working approximations. This gives us a benchmark for some of the more modest target tissues, such as a small skin graft (say, 20 mm × 20 mm × 2 mm thick) which could at least have cosmetic value (0.0004 m^2 surface area).

If cells were men (in scale), this would be equivalent to them setting out to assemble a structure 2 km long. In length at least, this is the same as the Kobe bridge and 35 per cent longer than the Golden Gate. The width, of course, does not bear comparison in this example. A skin implant, for example, produced at 1.8 mm thick, would be modest for treating serious wounds, but it would still correspond, in human engineering terms, to the height of the Gherkin Tower in London. Again, though, this civil engineering example is only comparable in one plane – its height. The cells would really be aming to make a structure equivalent to the Kobe Bridge in length, one Golden Gate bridge span in width *and* a GerkinTower thick. Quite a tall logistics order!

To add a little bio-medical context to this estimation, a large area of catastrophic skin burns (common targets for tissue engineering) might need graft surface areas in excess of 1 m^2, or 2,500 times larger than our skin graft example. A more common example would be short vascular implants or peripheral nerve graft conduits, which would have major clinical value for a host of post trauma reconstructive surgical procedures – if only we could make them! Even though we could make great use of modest 8 mm long conduits, this would *look*, to its resident cell poplulation, like the Burhj Dubai tower – the largest building humans have yet built.

Although these comparisons may at first glance look fatuous, it is how these structures 'look' to the

resident cells that we need to consider. The logistics of mass (materials) transport and cell/worker mobility apply at both scales and, despite the many differences between the cell and human worlds, both operate under the same basic rules of physics.

6.1 Degrees of tissue asymmetry

Like so many parts of human self-perception and psychology, perception of our own (a)symmetry can be described either as a paradox (academic critique) or 'up the wall' (colloquialism).

The funny thing about our own bodies (*no*, not that!) is that they are, like most vertebrates, profoundly asymmetrical in two of our three planes, but surprisingly symmetrical in the third (Figure 6.2a). If you slice us through the waist, we (happily) have a serious head-to-bum mismatch. Similarly, bisecting us top-to-bottom in a line between our ears and our hips leaves all facial features in one half (but none in the other); spine and buttocks in one (but belly and chin in the other); palms in one (but wrinkly backs-of-hands in the other). But slice us between the eyes and legs and the halves are remarkably similar.

We have a high uniaxial (left-right, lateral) symmetry with almost identical leg/arm length and facial sense organ symmetry – eyes, ears, nostrils*.

* The main exceptions are found in some of our internal organs, e.g. heart, stomach, liver.

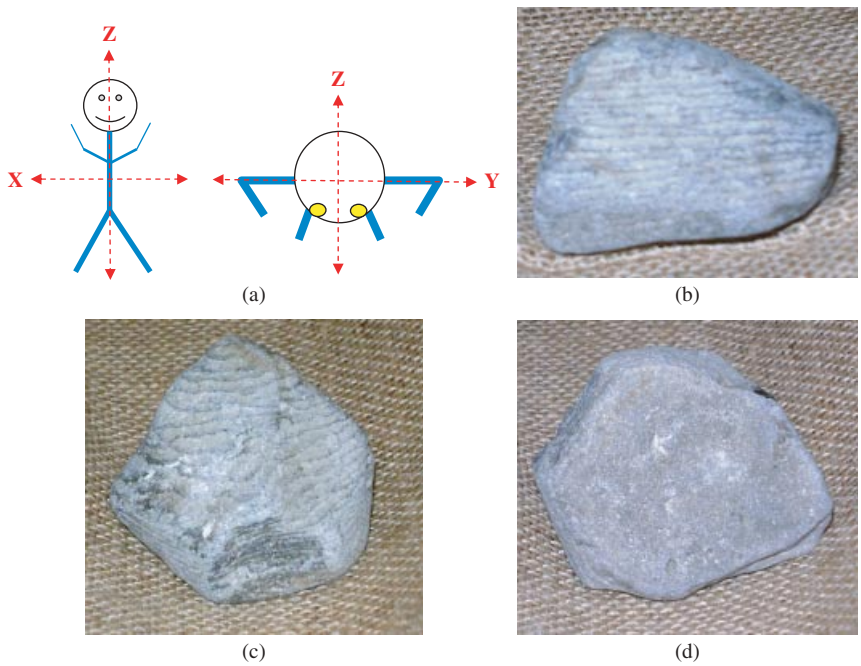


Figure 6.2 (a) Planes of human asymmetry can be seen if we imagine being sliced: front-to-back top ('Y'); left-to-right ('Z'); top-to-bottom ('X'). In general, we are 'mostly' asymmetrical: we are asymmetrical in planes X and Y but largely symmetrical in the Z plane. (b–d) The three pictures here are of the same fossil, viewed in three separate planes (arbitrarily, *x*, *y* and *z*, shown in (b), (c), (d)). It is a single fragment of a fossilized tree, with its growth rings running through. (b) is in the *x*-axis cross-section, (c) is the *y*-axis (showing growth rings eroded at an angle to the plane). But (c) shows the *z*-plane, parallel to a single growth ring (boring!).

Yet, despite our profound asymmetry in two of the three possible planes, we are completely obsessed with the *slightest* asymmetry in our symmetrical plane. We get seriously upset by the smallest difference in leg or arm length. Tiny differences between left and right ears or mouth shape are a personal (cosmetic) disaster, sometimes ending in fashion surgery. The small left-right differences in foot or breast size are common, but mean an instant visit to the airbrush studios in Hollywood. Yet we are profoundly asymmetrical beings, and this has considerable selective/functional benefits.

Asymmetry of our gross structure is not as irrelevant as it might seem to our considerations here, of tissue μ-structures. In fact, *gross* anatomical asymmetry makes it almost inevitable that the fine structures of the tissues in these body parts are also profoundly asymmetrical. After all, it means that they are made of essentially anisotropic material structures.

For example, arms, legs, toes and fingers inevitably have two quite distinct ends: one attached and one non-attached. This is complete mechanical polarity. Although we have a nice symmetrical pair of eyes, each has one end buried in the brain and the other end looks at the outside world (indeed, this page) through a transparent window – your cornea. The result is that all the building blocks and structural components of the tissues have a stark polarity (distal-proximal, in anatomical terms). This is a major consideration when we want to manufacture replacement tissue parts but, being so familiar/obvious to us, it is also easily overlooked. It is a good idea, then, to reproduce those native asymmetries.

In practical terms, and especially at the μm-scale, asymmetry in nature is the result of a series of layers, zones, hollows, channels and voids. Not surprisingly, then, the idea of reproducing and fabricating long-range asymmetries in structure by 'layering' has appeared as a fabrication strategy for both tissues and conventional goods[9]. Very crudely, the way in which layering can be biomimetic is indicated in Figure 6.2(b–d) using fossil structure. Interestingly, this is grossly symmetrical in two planes and asymmetrical in one plane, just as we are.

[9]To be specific, we can consider 'layering' as the linear or radial deposition of many thin sheets (similar or dissimilar), in sequence.

6.2 Making simple anisotropic/asymmetrical structures

On the whole, even small solid-tissue elements are rarely random or isotropic in structure. In fact, our lab has a small competition running to identify exceptions, firstly in mammals, then in animals generally. This is not a trivial point as, in most cases, 3D structure and spatial complexity seems to be essential for basic tissue function. As a result, designs for engineered tissues which *start* with the aim of spatial uniformity or randomness, particularly at the nano-micro scale, need to be viewed with caution.

However, even with this seemingly sensible and basic proposition, we rapidly find ourselves coming up against one of the widest held tenets of tissue engineering – *homogeneous, random porosity is good*. This is particularly obvious in the design of biomaterial cell supports. These commonly claim to have random (sponge-like) interconnecting perforations and channels, with a close range of diameters, as a *major merit*. It appears, then, that we have stumbled on another of the paradoxes of tissue engineering. Perhaps we might learn something useful by looking beneath the supporting assumption, or rather the dilemma between these two basic and opposing assumptions.

The first assumption is likely to encounter very little discussion. This assumption is that it is good for us to aim at engineering *simplified* tissues, at least in the first instance. It comes with the further assumption that the resident cells will later have the capacity/information to provide all necessary spatial complexity. It is illuminating, though, that the ambiguity arises when we come to the assumption of what specifically we mean by 'simplified', and again we may see a hint of inter-disciplinary bias.

Clearly, our aim is essentially to assemble the 'bio' **components** into a 3D **structure** which **functions**, 'eventually'. Our simplification question reduces down to: 'In the early stages of assembly, is it better to simplify the composition or the spatial organisation of the construct, to achieve function?' Notice that the 'timing' caveats here are becoming ever more important to analyze (Text Box 6.1).

The big question now rationalizes down to: which one of these two biological complexities – composition or 3D structure – can we reduce down to its bare essentials *without* losing the ability to reach our goal of tissue function *in a reasonable timescale*? In this chapter, we explore the possibilities of reducing composition complexity at time-zero to a minimum but *at the same time* building up spatial complexity, by layer engineering.

In considering the case for initial simplicity of composition, we shall return to analogy – in this case is the construction of a particularly beautiful building, the Blue Mosque in Istanbul (Figure 6.3a). We could start by assembling a very few key supporting components – stones, wood and concrete – to give the skeletal shape and basic template of this complex domed, fluted and colonnaded edifice. The strategy here is to produce a structure which resembles what we know we need in size, shape and proportions. Then we invite the sculptors, carpenters, goldsmiths, painters and plasterers (over period of time) to embellish and build on the initial skeletal structure, creating the beautiful mosque we expect.

Conversely, we might collect all of the hardwoods, fine carpets, paintings, gold leaf and ornate windows we would expect would contribute to the beauty of the mosque. But in this case, the multiplicity of components is stacked into simple piles or a single great mound, because there is little or nothing to support any spatial complexity. In this strategy, there is an attempt to assemble the compositional complexity *before* fabrication of any supporting structures.

We can see from this that *sequence* is an essential component of the construction, just as important as the choice of roof and floor tiles. Without the template of the 3D structure, there is nowhere for the wood, coloured glasses and gold to be worked in. In fact, the complexity they bring (out of sequence) might actually degrade the construction process through confusion. It is hard to imagine, in the latter case, how the exquisite form and function of the Blue Mosque could rise out of a design which brings all the necessary diversity of components but no structural template around which to hang them.

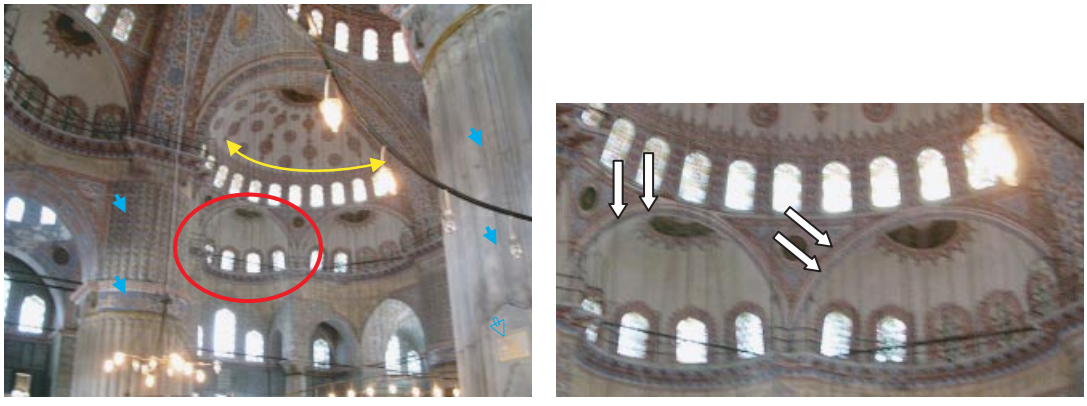
In fact, this analogy is a surprisingly close reflection of the development of structures in the vertebrate embryo. Tissues start as 'simple' templates, with a few basic components, and are sequentially remodelled to produce more and more complexity. With that increased spatial complexity, of course, comes subtlety of function. The vertebrate skeleton, for example, first condenses as a simple, cartilage-only replica of the final edifice. Gradually it is vascularized, calcified and articulated to form a fully functional, working skeleton, *though it still retains its original pattern*.

So, our first working assumption in this section is that design of early-stage tissue constructs needs to start with a short, simple component list. These

Text Box 6.1 Timing caveats

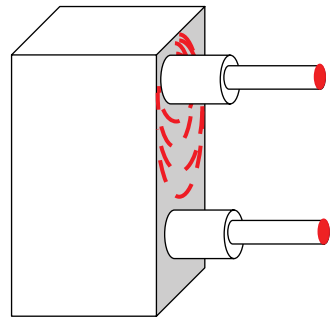
Timing caveats are important and become more so as we go on. We are particularly interested here in the simplifications that we *must* make at the start of the fabrication process, in terms of how they will impair function downstream. How long we can sustain the tension between making the 'simplification' and needing the 'function' depends on what we are trying to make, but the *time lag* is inevitably a component in our strategy. For example, it is common to assume that early-stage limitations of tissue function (e.g. in mechanical strength) can be improved by gradual *addition* of structural complexity, commonly through resident cell remodelling or growth. This time lag is key to the process design.

(a)



(b)

Figure 6.3 (a) The Blue Mosque – and the materials its walls, roof and floors are made from. (b) Inside the Blue Mosque, the apparently endless complexity turns out to be made of hierarchies of similar structures, repeated at the different length scales. Firstly, the roof mass is supported on huge columns and arches (left top, arrowheads). Between these, are rows of half domes, set within larger bays (ringed and arrowed in the lower view). Within each of these levels there are repeating half-rings of arches (curved arrow, top), each arch carrying its own repeated embellishment. It can be difficult to show the comparable 3D repeating structure of tissues in this way, except as diagrams, which can be found in any good histology text. This is because of how we slice and image solid structures (see Figure 6.2) and so have to reconstruct the third dimension. The inset diagram (right) represents a small repeating structural unit of bone. This comprises blood capillaries and nerves (red tube), running through Haversian canals (larger tube), surrounded by rings of bone matrix, bone cells and their own micro-scale connecting channels, known as lamellae (or layers – dotted red). Spot the similar repeating of layers across scales.



components should be carefully chosen as the basics needed for construction – even if the eventual structures are to be complex 3D shapes. This applies at all scale levels. It will form and then act as the template onto which further layers of complexity can be added, in sequence. In the case of biological structure mimicry, the term 'non-random complexity' should be translated as containing:

- asymmetrical layers (planar and radial);
- repeating zones;
- ridges and channels; or
- anisotropic fibrous sheets.

We shall shortly learn what these mean in more detail, but at this stage it is important to understand that they are useful terms and concepts for our task of producing reductionist structure designs. Importantly, these structures must cross many hierarchies, from the nano to the metre scale.

6.3 Thinking asymmetrically

The complex, repeating asymmetry of many tissue structures is so familiar to us (the owners) that we often do not notice it. Neither do we lay awake pondering *how* and *why* our eyelids, tongue, earlobes or fingertips are built up of so many repeating layers and zones. The layers are mainly asymmetrical, sometimes in more than one plane. They are interlaced with repeating dense/less dense zones and fibre-based anisotropies. These layers and asymmetries and structural repeats have a direction and go through a sequence of patterns.

At first glance, this complexity looks daunting, but then again so does the interior space of the Blue Mosque. Look at Figure 6.3b and notice how the (asymmetrical) arches in the roof are organized in strings through space, following the curved outline of the domes. Sometimes there are sub-bulges (with their arches) pressed out of the *natural arc* of that dome (white arrows in Figure 6.3b). In other aspects, different types of arch, with a higher aspect ratio and greater asymmetry, are arranged *in sequence* in the curved space (red circle in Figure 6.3b).

The arch is only one many of asymmetrical structures present when we look carefully enough at the mosque. Eventually, you should find that the 3D patterns from which the structure is made become more and more evident. Interestingly, as your eye starts to see this 'detail' of the structure, so the construction possibilities of how it might be put together also become clear. In effect, our eyes are starting to recognize the directions in which the various shapes and components have been built up. It ceases to look like a single, rather daunting mass of complex shapes, and it becomes a rational, repetitive sequence of layers and zones. This is when we recall that there is nothing supernatural in this architecture – it was fabricated by people (however inspired)! In other words, it is quite possible to do this again.

What is most helpful is to realize that it is made up of relatively simple building units (3D asymmetries) repeated in patterns and variants. With a little more practice, one can also identify those few basic, often *support* elements which are used in the first phases of construction to carry the weight and maintain the general shape of the building – in other words, the template structure.

In fact, these tend to be the foundations, walls, pillars (Figure 6.3b) and buttresses, which are often hidden or cut away in later stages. These *carry* or *support* the many smaller components to give the final complexity. The same can be true for biological tissues, as we shall see later. Interestingly enough, it is often those users and scholars of such structures – those closest to them – who are least likely to perceive them in this reductionist manner. But it is the role of anatomists and histologists on the one hand, or followers of religions who use such buildings on the other, to deduce meaning from the *overall* finished structure, not to reduce and simplify it again. In building tissues, that job falls to other tribes – and in this case, us.

In the interests of scale, clarity and indulgence (not to mention accessibility), we now switch analogies from the massive and drop-dead gorgeous, to confection! The familiarity of this example helps us to get right up and close to the questions of:

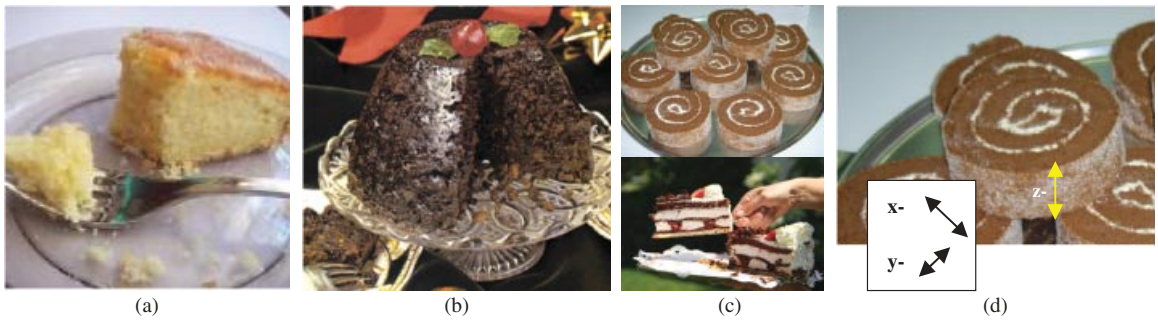


Figure 6.4 (a) Victoria sponge cake, at a gross scale, is compositionally *and* structurally simple. (b) Christmas pudding is compositionally complex but structurally simple (i.e. random). (c) Swiss roll (upper) and Black Forest gateau (lower panel) are both spatially complex, though only the Black Forest gateau is both spatially *and* compositionally complex (see text). (d) Swiss roll planes: *x*, *y* and *z*.

- How 'simple' can simple *really* get (and still be useful)? and
- Where does complexity start to take off as a problem?

Cake-world examples here start with the Victoria sponge, Swiss roll and Black Forest gateau, and lead on to the British Christmas, or plum, pudding (Figure 6.4).

At the scale we are familiar with, Figure 6.4a shows how the Victoria sponge is structurally homogeneous, especially inside. We shall come back to the 'scale-caveat' later (Text Box 6.2). In contrast, we find examples of 'non-homogeneous' cakes in 'layered desserts' such as the Swiss roll and Black Forest gateau in Figure 6.4c). These are inhomogeneous partly in terms of the very different substances which make up their mass (*compositional inhomogeneity*) and also in the way they are spatially organized is into distinct *zones* and *layers*, with separating *interfaces*. In other words, they are made up of different components, such as cake, cream, icing and fruit, arranged (asymmetrically) in *3D space* into layers, depots, tracks or zones.

Notice also the tendency for *structural repetition*, which is an important and extremely useful feature of non-random structures. This is useful for us to understand, as it is highly biomimetic but relatively simple to fabricate. Black Forest gateau (Figure 6.4c, lower panel), illustrates another important feature for biomimetic fabrication in its obvious top-bottom polarization. In other words, its layers have a distinct *sequence* of shapes and compositions. Once we are aware of these layers and sequences, we also see their interfaces!

The '**interfaces**' are, in effect, lines of rapid change in composition, density or phase between layers or zones. Though easily overlooked, interfaces can also be key spatial factors:

- for producing biological function; and
- to assist fabrication and processing (more of this later).

It is often useful to consider an interface as just another, very thin, layer in the structure, which either fixes to or glides between its neighbours. It is important, though, that interfaces should not be seen as being *only* present between planar surfaces. Radial or non-linear interfaces are illustrated by the Swiss roll (cross-section in Figure 6.4c, top) and around the zones lying *within* the planar layers of the Black Forest gateau (lower panel).

Such radial interfaces are most commonly seen in natural tissues in the form of blood vessels, nerves and ducts, which can comprise 1–5 radial layers in cross-section (depending on the size scale). Each one represents an interface. Since nerves and blood vessels are effectively tube-like in structure, their interfaces are strictly 'radial-planar' in 3D. Those around spherical glands might best be described as 'simple radial' (as in the white cream zones in the Black Forest gateaux).

Our first conclusion, then, is that Repetitions, Interfaces and Polarities (RIP) are our most

important tools for the design and fabrication of biomimetic structures.

Another useful distinction is illustrated by the Christmas pudding example (Figure 6.4b). This is the opposite extreme combination to the Swiss roll, being, perhaps, the most non-homogeneous material of the series in its composition. For those of you who have not experienced such a dessert, the visible lumps within its mass are composed of a huge variety of different edible materials. In fact, it has a very pronounced *compositional* heterogeneity: it is the essence of complexity in terms of what it contains. However, there is absolutely no structural organisation of these elements; they are randomly distributed in such a way that, when averaged over a significant volume, the 3D structure is homogeneous*.

* Ironic comment: as if to emphasize this spatial homogeneity, a classical Christmas pudding is a sphere (tri-axial symmetry – beat that!).

Christmas pudding, then, is *spatially homogeneous* but, at the same time, compositionally complex. Perhaps the fact this is rarely a desirable template for natural tissues (i.e. poorly biomimetic) is a good lesson for us to take away as aspirant fabricators of tissues. Actually, it is a combination which is closer to that of adult mammalian repair tissues, i.e. scars (lots of good tissue components with poor 3D organisation – see Chapter 1). However, the body is all too good at making scar tissues, at all sites, without our help.

The second take-home-message, then, is that the far end of the 'success spectrum' is not the *absence* of a tissue, but the engineering of damaging scar tissue – Christmas pudding mimicry.

To delve deeper into our cake-related analysis of organizing spatial factors, it turns out that cake can teach us a great deal about different forms of symmetry, asymmetry and layering. Figure 6.4c illustrates two further levels of non-homogeneity which are easy to miss. At our comfortable visual scale (≈1 mm and above), Swiss rolls are made of only two components – flavoured cream and chocolate cake – but these are rolled up into the characteristic spiral. Swiss roll, then, is 'compositionally biphasic' (i.e. simple), but spatially non-homogeneous/complex. We can measure this either in terms of the rate of change of structure/composition or as the number of interfaces encountered across the cross-sectional diameter of a single slice (Figure 6.4d, x and y planes). This cake is both simple and complex.

In contrast, Black forest gateau contains numerous substances arranged into spatially distinct layers and zones, in a number of planes throughout the cake. This is the most tissue-like but the hardest to fabricate, particularly as it does not yet hint at the way biological complexity must extend down to the cell-critical micro- and nano-scales.

Could it be, then, that the Swiss roll embodies what we are looking for as the initial tissue template – a practical compromise between complexity and simplicity? Ideally, we are looking for the complexity that is essential for bio-mimicry, but it needs to be simple enough to fabricate. So if this is our spatial paradigm, it must be important to understand the secret of the Swiss roll and its clever compromise. **Why is its complexity so simple to generate?**

The clue lies in the number of interfaces crossed in the Swiss roll when tracking in the x–y plane, across its cross-section (Figure 6.4d shows x–y–z planes). There are approximately 12 interfaces in the x plane across the diameter this cake slice (excluding the cake/air edges (arrowed)). This would equal or even exceed the same measure in a Black Forest gateau. However, the key here lies in the z plane, where the Swiss roll scores a stark *zero* interfaces crossed (i.e. the tracking plane is parallel to the layers).

In conclusion, we can propose that the design and fabrication of spatially complex; hopefully, biomimetic tissue templates should be possible by repetitive assembly of many (compositionally) simple layers. **In effect, this is *layer engineering*.**

Perhaps, then, our detailed analysis of cake structure, in search of fabrication strategies, has not been such a self-indulgent exercise. It has provided a framework to enable us more easily to identify current mismatches between our targets and approaches, in particular where we again may be 'aiming low and still missing'. The high target which was originally set, to make tissues with complex

structure and composition, has unwittingly forced us to rely on the 'relatively low aim' approach of persuading cells to make them for us. The modest success of this approach has tended to lower the aim still further, such that engineered tissues are considered successful if they achieve two or, exceptionally, three layers with distinct structures.

However, the message from the cake analysis is that spatial complexity *can* be achieved if standard engineering concepts are adopted to simplify composition. This is based on the use of repetition and polarity to generate many interfaces (spatial complexity) through layering. Repetitious assembly of simple components is a field that we humans excel in (at scales from mobile phones (nano-micro) to trucks and ships, and from microns to kilometres.

Furthermore, layer engineering carries the potential for evolving greater and greater complexity at larger and smaller size scales, as fabrication of the layers becomes more sophisticated. After all, unlike Swiss rolls, the layers **do not all have to be the same**. Increasing the options for the types of layer, layer sequence and changing layer thickness (i.e. more and thinner layers) will inevitably permit exponential increases in structural complexity, but with the same simple process.

Our next task must be to understand just how this layer engineering idea can be applied in practice to assembling *living* tissue. After all, making elegant, but non-living multi-layered structures, and then expecting to get cells into the right place, is not a good sequence. The first step is to take a much closer look under a stone which we have repeatedly skimmed past – **the scale caveat** (Text Box 6.2). In short, the scale caveat qualifies what we aim to make in terms of the size scale it must work at.

The size caveat, then, highlights something of a weak spot in our understanding of cell control needed for tissue engineering. Although it is well known that cells use and respond to substrate shape, structure and mechanical properties, it is much less certain how to *use* the language of these cues. In particular, how cells respond to different features on their top and bottom surfaces (e.g. non-2D, multi-polar) is not understood.

It is difficult, then, to imagine them being applied in the near future for the practical fabrication of tissue detail. In many ways, this is comparable to the problem of growth factors in tissue engineering. It is clear that they convey potent messages to cell systems, but we do not know the language. On the other hand, the opposite approach of *physically fabricating* a man-made, complex structural hierarchy (i.e. not reliant on cell synthesis and assembly) is seriously daunting, particularly in biomedical sciences.

We shall return to this later, as it may represent less of a real blockage and more a result of limited cross-disciplinary, between-tribe thinking. After all, the engineers and designers responsible for mobile phones can fabricate and assemble parts within the same size scales. However, unlike tissue engineers, mobile phone engineers cannot be tempted by even the most remote dream of finding a cell which will make a competitor to the iPhone – they have no choice! Paradoxically, there are no cells in a cell-phone.

6.4 How do we know which scale to engineer first?

The cake analogy teaches us that it could be a good idea to engineer small sections, or layers of the structure and *then* to create structural complexity through a subsequent assembly step. But which 'small-scale, simple component(s)' should we begin with?

Although it is possible to simplify our thinking on which target structures need to be assembled, it remains of huge practical importance to establish how we might assemble such 3D structures, with all that this implies for final shape, symmetry and anisotropy. First, many of the most common target tissues (and certainly the connective tissues) have key structural features which operate at all levels, from the gross (mm to metres) to the meso-scales (μm- to nm). In other words, it is now well understood that the non-cell (extracellular matrix) bulk of tissues is built up of hierarchies of repeating structures, aggregated all the way from the nano up

Text Box 6.2 The 'scale' caveat

The crucial thing about cakes is that we can only really do the analysis at the gross (visible) scale. Effectively, this is on the mm to cm range (or metres if you do party catering or gluttony). In clinical terms, this is the scale which most interests both the surgeon and the patient, as mostly this is where they measure success. However, in fact, it is really sub-millimetre and, indeed down at the μm and nm scales, where most of the critical cell-based business is done – and where success or failure lies.

The heart of this effect is easier to understand if we consider cells as tiny (20 μm) globular factories. Their business is to take incoming shipments of small, simple molecules, nutrients, oxygen, etc., sized in the (sub-) nano range, and assemble these into ever larger, more complex biological building blocks (proteins, fats, polysaccharides). Once synthesized, more and more of these building blocks undergo continuous, repetitious aggregation and assemble to form more cells, cell layers or cell masses on the one hand, and complex structural materials on the other (extracellular matrix: ECM).

Eventually, these aggregated structures come to our scale of attention as visible parts of our bodies, like pimples, fingernails or that welcome new lump of skin filling in the hole where the salad knife slipped. These personally important structures are 'simply' millions and millions of fibre-forming proteins (ECM building units at 10–100 nm scale) aggregated up to the mm-cm scale. The cell sits in the middle of this scale hierarchy like a pump motor, sucking up sub-nm matter and pushing it out into sub-metre body parts by endless repetition.

In effect, then, it is the size of cells and their repetitious assembly habits which generates the long-scale structural hierarchies we see in tissues. Each smaller level of structure is integrated with the next level of structure up, and so on. Thus it is often difficult to divide 'the function' into clear size scales. This is particularly clear in the load-carrying connective tissues, where structures such as the fibrils in tendons get larger by addition of more and more of the same small parts. In the case of tendon, collagen microfibrils (nm) aggregate into fibrils and then into fibres, eventually fibre bundles and mm-scale tendon fascicles. Each of these adds a little more to the functional (tensile) load-carrying capacity total as the fibre structures get thicker.

In this respect, a tissue would be much more like a high-rise hotel block than a holiday camp full of chalets; tissues tend to come as an 'integrated whole' rather than a loose federation of independent units (or huts). Remodelling the accommodation in the hotel is trickier than the chalet site, due to the interdependence of the hierarchy layers. It is the complete scale hierarchy which functions – either altogether, or nothing at all. So, to be truly biomimetic in our engineering, especially of mechanical tissues, we may need to build up by integrating small (cell-scale) units through continuous repetition.

Clues as to the 'important' scale, where we need to provide cell controls, come from research into the size and types of surface 'texture' to which cells respond. Features such as ridges, channels, pits and lumps (generically termed topography) change cell behaviour. At the lowest level, surface structures of less than 20 nm can trigger a few cell responses, while at the 'big' end, ridges or fibres of 10–100 μm diameter will cause many cells to align or move in specific directions.

This gives us a guide to the range of scales where the behaviour of cells can be influenced by their substrate. The lower end of the range seems to be governed by the dimensions at which cell membrane integrins can be clustered (at the points where cell membranes attach) to generate enzyme-based signals inside the cell. The upper end (100–200 μm fibre diameter) seems to be the level where the cell itself (20–60 μm when spread) is unable to tell the difference between a very gentle curve and a flat surface (just as we struggle to *perceive* the planet's curve).

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to the gross level, with almost no discernable lines of functional segregation. The most obvious, and in this case most relevant, example is the hierarchy of collagen organisation.

Collagen is a fibrous protein in which the 1.5 nm diameter, rope-like monomer forms the basic building block and aggregates in a semi-crystalline manner to form long fibrils. These range from 30–500 nm diameter, depending on age and tissue. The fibrils can be packed or woven into larger laterally packed aggregates called fibres and fibre bundles (≈500 nm–500 μm) and, in a few tissues, to discrete, outlined fascicles (≈mm: see Chapter 1, Figure 1.1). As in the case of the fibres of cotton or wool that make up the more familiar fibres of our clothes, collagen fibrils are of indeterminate length. This allows them to be packed, knitted or woven together in a regular manner, or randomly intertwined for long-range load transmission. The density and overall 3D architecture of this fibril network is what gives each connective tissue its characteristic mechanical properties.

Unlike man-made fibres, though, collagen fibril networks would typically enclose resident tissue cells. In contrast to the relatively fixed size and proportion of the collagen component, these cells are complex, constantly moving and shape-dynamic. Let us assume for now that they are roughly spherical (Text Box 6.3) and call them nominally 20 μm diameter.

As always, it is important here to get a clear understanding of the scale of this cell-fibril relationship. If we want to illustrate this relationship, it also needs to be based on the spatial relationship in native tissues, where **the collagen comes out of the cell**. For the purposes of this analogy, then, let us imagine we could reduce a sheep to be the size of a cell. This would make the strands of its fleece roughly proportional to the diameter of collagen fibrils as they aggregate around the cell. Mathematically speaking, fleece-hairs = collagen-fibrils: sheep = cell, as in Figure 6.5. This emphasizes the idea that hairs/fibrils are very much the product of, and so spatially associated with, the sheep (or the cell).

It also allows us to look at the next size scale up; what happens where lots of sheep get together, like the cells in a tissue? When considering a flock of sheep, it is reasonable to expect air spaces between the sheep in a flock. Clearly, this is not going to be true for cells in a connective tissue, which are separated only by collagen-rich ECM, with a few vascular and nerve channels. It is as if each sheep's fleece could grow to an enormous thickness. The woolly fibres become completely entangled, even bonded together in the inter-sheep spaces. This is exactly what happens to cells as their collagen 'fleece' becomes increasingly thick and cross-linked.

An interesting corollary of this sheep analogy starts to be very important to tissue function. If the fleeces of sheep in contact really did interlink and

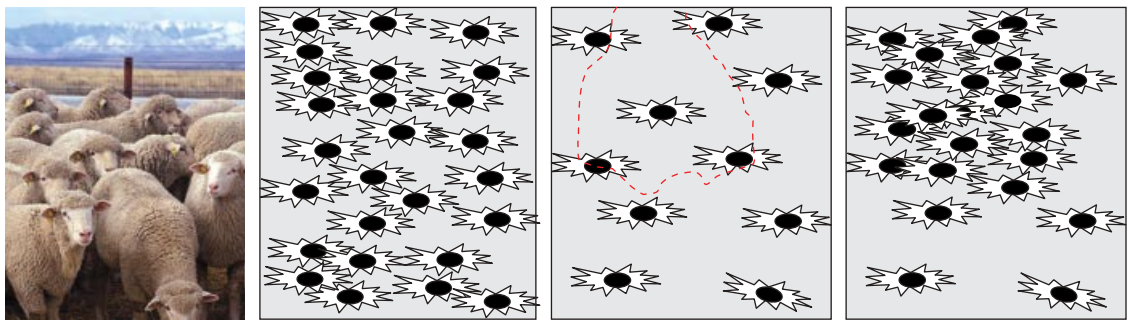
Text Box 6.3 Cell shape and size

In systems such as this, where we are getting right under the skin of the '3D question', we can quickly get into difficulties over the changing shape of cells. In suspension, and with minimal attachment to a matrix or tissue culture plastic, most cells tend to become spherical, commonly in the order of 15–20 μm in diameter. A classic exception to this generality is the mammalian red blood cell, which is a bi-concave disc of 6–8 μm diameter and ≈2 μm thick, with absolutely no attachment and no nucleus.

Dramatically, things happened to cell shape when they attached down to a flat plastic culture dish. They spread out and move, generating complex and bizarre shapes. These resemble fried eggs in monolayer, as the soft cell cytoplasm is flattened and extended over the flat plastic, leaving the stiff nucleus standing high in the centre. In 3D culture, and in natural tissues, these flattened structures are rare. In culture, this spreading, sometimes to as much as 80 or 100 μm across, gives the wrong impression of large cell size and it makes discussion of 3D cell size and shape confusing. For simplicity here, we consider cells as non-suspended and spherical (at the time of seeding). How, when and why they change their shape from then on may be the target of future work.

(a) (b)

Figure 6.5 The formula idea here is that where Cells = Sheep and Collagen = Wool: (a) sheep/cell producers will be embedded in the wool/collagen; (b) a sheep-wool unit would look like this in 'cross-section', much like a slice through a fibroblast or osteoblast plus its collagen. Photo (b) © Eric IsselShutterstock.com.



(a) (b) (c) (d)

Figure 6.6 (a) Flock of sheep, © US Department of Agriculture. (b) Early, low-strength cell-rich matrix, before mass collagen deposition (Cells black, collagen grey). (c) Mature, strong collagen-rich functional matrix with load carried on the collagen. (d) Post-injury, the process repeats locally, with high-density cells making bulk collagen (red dotted line = injury).

join mechanically, the whole flock would move as one. This echoes the hotel analogy, above, illustrating how the building units in a tissue are functionally integrated. If the shepherd pulled hard on two or three sheep at one edge of the flock, the whole flock would feel the tensile load and be dragged in that direction. No stragglers, and no sheepdog needed!

Consequently, we can picture a densely packed flock of sheep (Figure 6.6a). As long as we stipulate that their fleeces are not just in contact but 'superglued' together, then the flock is equivalent to cells with only a little collagen (extracellular matrix) in a cell-rich tissue (Figure 6.6b). This would be the sort of tissue we expect in an early wound repair tissue. But notice how the *cells* dominate over the matrix in this situation (Figure 6.6b).

As cells are much weaker than the collagen material, such cell-rich tissues are not mechanically strong; or at least that is true until these cells push out *much* more collagen, as extracellular matrix (ECM), between themselves. As this process of collagen deposition continues, the proportion of matrix to cells rises until the collagen/ECM begins to dominate the mechanical properties. This corresponds to stages when a new connective tissue begins to mature into a strong, stable structure (Figure 6.6c).

Here our analogy becomes really bizarre, as we are unlikely to encounter sheep fleeces that are

more than 20 cm thick. Yet adult tissues can have inter-cellular matrix several cell-diameters thick, which would translate into several sheep-diameters at our scale, implying that they can grow fleeces of some metres thick. However, this is a fantasy illustration – and cells are remarkable! In the case of collagen-producing cells, layer after layer of collagen is produced, pushing cells further and further apart and making the tissue stronger. It is as if there was no annual shearing of the fleece, or the collagen layer for cells, so that one flock eventually is comprised of lots of wool and only a few sheep. This sort of cell-to-matrix ratio is exactly what we see in mature connective tissues (without the wool!).

Surprisingly, this analogy is useful for one last lap. We can illustrate this in terms of events which follow an evening visit to the flock by a wolf. Where the flock/tissue is injured (red line in Figure 6.6c) and loses some of its bulk (viewed from the wolf's perspective, some sheep are taken out to dinner!). The resulting space will be filled by new sheep which have thin fleeces. The repaired defect will, for a while, again be sheep/cell rich and fleece/matrix poor, and therefore weak (Figure 6.6d). The process then repeats with new collagen (or wool) once more pushing apart the cells (sheep), until we return approximately to a joined-up, fleece-rich, sheep-poor flock (Figure 6.6c). And that is essentially how a wound repairs, if we leave out the sheep[10].

To conclude this section, have we, then, found a promising starting point for the composition of simple building units for layer engineering – cells in a collagen fibril network?

Collagen fibril networks (predominantly type I collagen) are at the heart of connective tissues, both in terms of tissue bulk and mechanical function. Around 25 per cent of our protein tissue dry weight is collagen. This is a truly huge synthetic investment on the part of resident tissue fibroblasts, and something which, incidentally, they are not keen to do in culture. Clearly, then, collagen is a great candidate basic building material. The more of it we can engineer into the fabric of our simple tissue templates, the less the cells will have to labour to produce.

So we have reached a possible watershed. Using 'extreme tissue engineering' concepts and working from first principles, we have a candidate tissue fabrication approach:

1. Fabricate simple tissue layers out of (a) appropriate cells in (b) a thin sheet of collagen.
2. Build up both bulk and spatial complexity with many of these layers in 3D.

Two niggling practical problems hover over this stage:

- How do *we* make native fibrillar collagen materials with predictable properties, when at present only cells seem to be able to do this?
- How do we then get the layers to stick together?

But these questions are for later.

6.5 Making a virtue of hierarchical complexity: because we have to

So far we have tried to rationalize tissue 3D complexity, hierarchies and the asymmetrical cell-matrix structures which make them up. The aim has been to get beyond some of the traditional simplifications which are so ingrained in the world of isolated cell biology and '2D' cell culture.

This is perhaps the first nettle which it has been common to avoid grasping in much of tissue engineering. The bulk of what we know about, and how we think of, cells relates to monolayer cultures attached to flat plastic. Before the appearance of tissue engineering, few of us even bothered to grow cells in 3D, as it was considered impractically complex and largely irrelevant to the questions being asked. Clearly, the habit of some cell

[10]Note, though, that we have built up an analogy where the 'tissue' is represented not by the sheep but by the imaginary integrated flock. Note also that we have a flock/tissue-building unit which is very simple in composition (sheep + fleece or cells + collagen). However, sheep being sheep, with feet on the ground and backs to the sky, it is only one layer thick. So, our analogy leads us towards a promising-looking 'tissue building unit', i.e. a *thin layer* of cells in a fibrillar collagen mesh, **ready to repeat and layer-up into complex architectures**.

types to overgrow one another in culture, forming spontaneous multi-layers, can be regarded as a very simple form of 3D culture, but hopefully the reader will now understand how minimal this is in terms of the target complexity we really need to aim for. Once we claim that our vision is fabricating functional 3D tissues, this nettle can no longer be avoided.

In one respect, though, our understanding of 'simple sheets of cells' may turn out to be a bit of a bonus for tackling the second of our high(er) targets. In a way, this is the opposite sort of problem, and it relates to the way in which tissue structure just seems *so* complex when we look at it in our familiar histological slices.

This second blind-siding factor is perhaps even more deeply rooted in biological training. Although many modern imaging techniques can reconstruct structures in 3D, a large part of our understanding of tissue structure is still based on histological techniques where the information is essentially 2D, derived from very flat, very thin slices of tissue. The reason for the thinness is similar to that for imaging cultured cells in monolayer – they produce the most beautiful images because the optical depth is optimal. How far this is from our target of complex 3D tissues is clear from a critical glance at Figure 6.7, an idealized diagram of skin. At its simplest, skin is regarded as at least three 'layers' (meaning in a plane parallel to its surface). These layers are the epidermis (keratinocyte layers), dermal (fibroblasts in a collagen-rich stroma) and subcutaneous or hypodermis of fat, plus or minus underlying muscle.

We take such images and μ-structural/anatomical analyses completely for granted now. But as a spatial analysis, they contain an assumption, they are selective in a very particular way. The plane in which those *slices are cut* is selected apparently for optimal teaching properties. They are ideal for showing off the main 'layers and interfaces', as in Figure 6.7. This means, though, that many tissues are commonly only really cut and illustrated in one of two planes, *not* three. Look in your text books at the structures of gut wall, blood vessels, oral tissues,

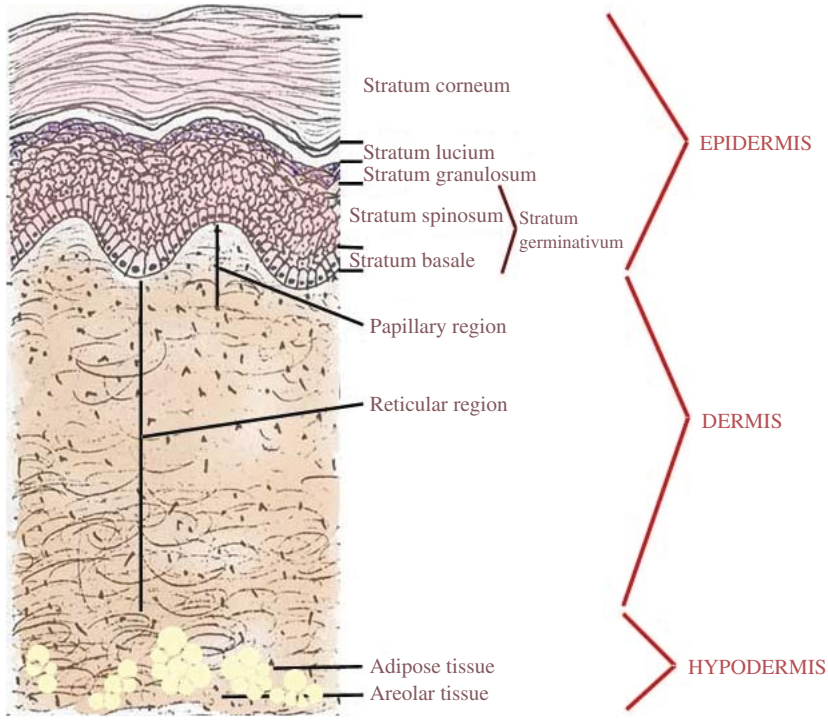


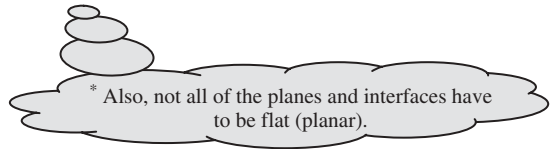
Figure 6.7 Three layers of skin, in 2D.

trachea, bladder, etc. They all resemble the skin shown here, in that the histological slices go across the maximum number of *layers*: surface to deep. So why do we so rarely see or consider the μ-structures of these tissues through or between the layers – that is, in the 3D plane parallel to the surface in skin?[11]

Simple, really: *if* you could get such sections cleanly through the layer (difficult as they are thin and not flat), they would be really boring. They would show an unappealingly constant structure over monotonous lengths of tissue. The dermal layer, for example, changes only slowly from one part of the body to another. We can, in fact, do this now with modern imaging – but we still don't often bother. However, once it is pointed out that there is *another* 'view' of these layered tissues (and almost all are 'layered' at some scale), we can suddenly see that making them need not be as complex as we thought. After all, this **third plane constancy** (monotony) means that only two of the three are different and complex (see Figure 6.2)!

To the extreme tissue engineer, this limited asymmetry is the source of great joy and promise. It means that, once we can identify a series of planes of *constant* structure, we can design simplified tissues as a series of layers (Figure 6.8(left)).

The epidermal and dermal layers are illustrated in Figure 6.8(left) in 3D. As we can see, these can be separated, arbitrarily, into at least three 'single layer' planes. The epidermis could, in theory, be split from the dermis in plane (a), while a parallel, but deeper plane (b) would peel apart the dermal and subcutaneous layers. However, there are clearly many more identifiable layers in this diagram, if we do the cake-type analysis. Indeed, there are more planes to analyze than those which are parallel to the surface*.



Tubes running through the tissue have concentric layers, and such radial layering is about as common as you can get in biology. These are indicated in Figure 6.8(right) as multi-layered, concentric cylinders running either from surface to deep or parallel to the surface, respectively ((c) and (d) in Figure 6.8(right)). Some of you will have noticed that with this step, we tend to have dropped one scale hierarchy, from mm of the dermis to tens of μm for a capillary or nerve. The example plane (c) includes structures which run down into the skin, such as hair follicles and sebaceous glands. Coaxial structures in the (d) plane might be blood vessels and nerves, or even small muscle groups and connecting tendons.

Contrary to non-specialist understanding, skilled plastic surgeons do not satisfy themselves with just concealing large facial defects with a layer of skin following trauma or tumour surgery. Where they can, their aim would be to replace/reconnect the complex facial muscle blocks and their fine nerve and vessel routes which give a balanced smile or a social blink. This is the target of *regeneration*, as opposed to *repair* with a covering skin graft. It also illustrates that analysis of layers and asymmetrical tissue hierarchies is no esoteric whim of micro-anatomy, but can hold the key to functional *regeneration* (as opposed to repair or 'filling the gap').

So, perhaps we can use these 3D hierarchies of structure – layers – as the basis for building up (fabricating) the *apparently* complex tissue structures that we take for granted as everyday users. We should be modest, though, as the idea of tissues as assemblies of precise, geometric layers in fact paints a highly stylized, grossly simplified version of natural reality. The point, though, is that even a simplified layer tissue would be a great advance on what we presently have. Furthermore, as we shall

[11] This piece of logic can take a few minutes to sink in if you are less familiar with spatial biology. It helps if you close this book and imagine you are a bookworm, munching through the pages. Now you can see that you would experience the same meal if you move in two of the three planes (between two leaves), but not when you try to eat through from page 13–133. The book is two-thirds homogeneous!

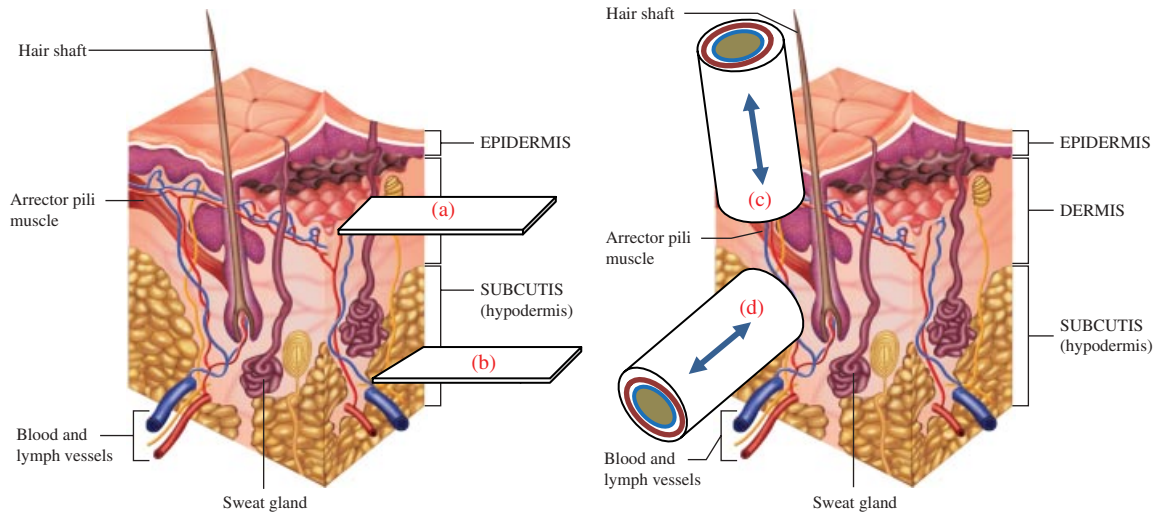


Figure 6.8 Contemplating the planar (left) and radial (right) layers of skin.

see, it also has great potential to be customized with greater and greater complex variation – just like real tissues.

In summarizing this section, we again glimpse the 'aiming low' tissue engineering paradox. The low target here comes from a naivety and limited cross-disciplinary understanding (e.g. the plastic surgeon's dream). It has led us too often to think we must engineer the *whole* tissue lump in one go, as it is too complex to sectionalize. Could it be, then, that tissue engineers who avoid the 'low target' of a single tissue lump might, in fact, fabricate much more controlled customized 3D structures by building in thin layers – deconstructed hierarchies, like cake-makers?

The process then becomes one of identifying and designing the necessary series of *layers* which could form our tissue template. To return to our Blue Mosque analogy, we should avoid the mistake of being overawed by complex beauty of the finished item and concentrate on finding the repetitive simplicity which is inevitably present. Completing the whole building is not yet the task of tissue engineers. We must first construct good, but simple, tissue templates. In the case of the Mosque, the ornate plaster and gold leaf is added later; and in the case of facial reconstructions, that is the task for reconstructive surgeons.

6.6 Cell-layering and matrix-layering

What, then, do we need to do to fabricate such 3D structures through layer generation, even the simple ones? Is it really practical to use these natural planes of tissue layering and zoning as the basis for making complex, anisotropic bio-artificial tissues? In fact 'tissue layer engineering', or just **layer engineering**, is already in use. Perhaps even more surprising, there are two basic types of layer engineering and both are already available, though based on quite distinct technologies. These complementary approaches, cell-layer and matrix-layer engineering, neatly reflect the two basic forms of tissue we have identified already as our basic targets: cell-rich and matrix-rich tissues.

The first of these, cell-rich tissues, can be tackled by 'cell-layer engineering'. This uses many similar cell layers, grown as coherent sheets and then stacked to form multi-layers. The cells used for cell-layer engineering are commonly natural sheet-forming epithelia or cooperative cell types

which work in the body at very high densities, such as muscle or liver cells.

The key enabling technology behind cell-layer engineering was the development of cell-culture substrates whose surface chemistry changes from hydrophilic to hydrophobic with temperature, commonly between 20–40° C. This transition in surface chemistry is key, as the hydrophobic or hydrophilic nature of a cell culture surface governs how, and if, cell attachment proteins stick to that surface (as we have seen before, cell adhesion proteins commonly include fibronectin and vitronectin from serum in the culture medium).

In this case, the poly N-isopropylacrylamide (p-NIPAAm) thermo-responsive coating which is applied to the tissue culture plastic supports protein-binds (and so cell adhesion) at temperatures around 37° C, at which cells are routinely cultured. However, if the culture temperature is reduced below say 30° C, its protein binding capacity flips. The adhesion proteins holding the cells down detach from the plastic, and take with them any cells which were using them.

The big plus about this system for cell detachment is how gentle it is. If cells are grown on (adherent to, at 37° C) such surfaces for some time – often in the order of (7–14 days) they form a confluent/continuous and stable sheet, incorporating small amounts of extracellular matrix, commonly a basement membrane. Such cell-rich sheets are then detached from the plastic culture surface by reducing the temperature for a short period, and then they can be used individually or stacked into multi-layers. The key point is that the cell-sheet remains intact as a floating, coherent layer of, for example, epithelium. This is unlike classical cell recovery by digestion of the cell monolayer with enzymes, which destroy the cell attachment and matrix proteins, so breaking up the sheet.

This technology has been used to produce a number of multi-layered tissues, including mucosa, cornea and muscle. Multi-layer engineering of muscle means that beating muscle sheets can be fabricated with reduced (and controllable) tendency to suffer serious hypoxic damage. Figure 6.9 summarizes the stages and mechanisms by which cell layers are fabricated on thermo-responsive surfaces.

The second type of layer engineering – this time producing *matrix-rich* tissues – aims to fabricate sheets containing cells within a mechanically strong extracellular matrix. The enabling technology supporting this matrix-layer engineering comes from technologies to construct native protein-based (often collagen) neo-tissues containing cells. A completely different approach, involving rapid shrinkage by fluid expulsion (collagen plastic

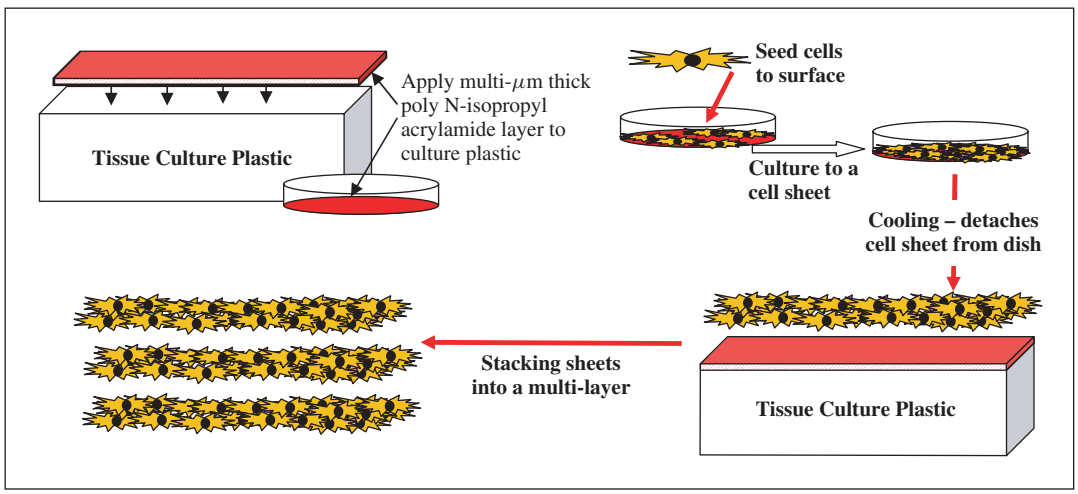


Figure 6.9 Cell-rich layer engineering: using poly N-isopropylacrylamide thermo-responsive surface, on conventional tissue culture plastic.

compression or CPC), has proved successful and avoids mechanical cell damage. The products of this CPC technology are thin, cellular sheets made predominantly of a dense, tough network of collagen nano-fibrils enclosing a closely enmeshed population of living cells. In other words, these are sheets of simple connective tissue.

It is worth explaining briefly just how CPC works to produce connective tissue building sheets. As we have seen, the two 'big problems' for engineered connective tissues are:

(i) production of collagen-fibril densities which even approach those of tissues; and
(ii) construction of even modest 3D tissue-like architectures, at the μm (i.e. cell-) scale, around the resident cells.

The trick with collagen plastic compression is that the basic tissue templates or replicas are formed initially out of hyper-hydrated, but native, collagen gels. The starting collagen for making these gels is already routinely extracted for clinical uses as acid-soluble tropocollagen, from animal tissues such as tendon and skin (see Chapter 4). Under physiological conditions (temperature and pH), the soluble collagen monomers aggregate to form nm-diameter fibrils. These nm-scale fibrils form around any cells which are suspended in the mix, enmeshing them, as they do in natural tissues. This 'fibrillogenesis' forms very soft cell-collagen gels, over approximately 10–15 minutes, comprising around 0.5 per cent collagen and about 99.5 per cent water (Figure 6.10).

Initially the excess of water, with resulting terrible mechanical properties, looks like big problems.

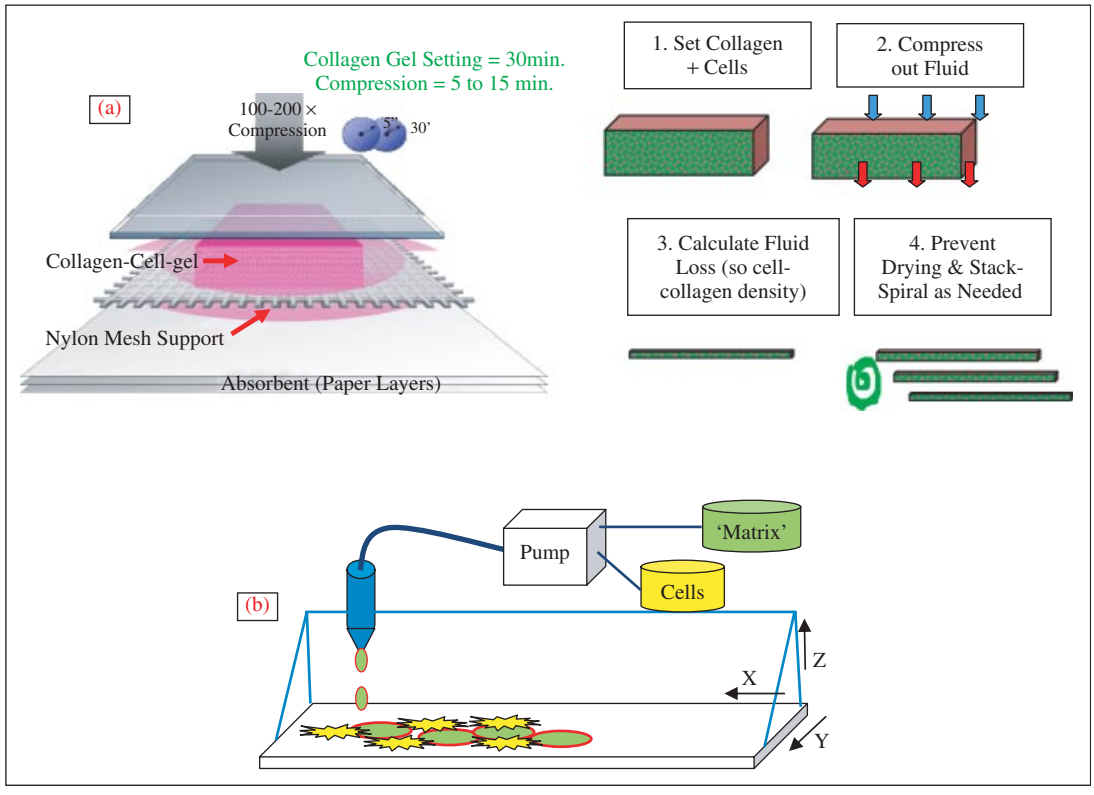


Figure 6.10 Enabling matrix layer engineering. [a] Collagen Plastic Compression: set-up and process shown on left (with thanks to Michael Anata for the diagram). Right hand side (1) to (4) show the main basic stages, including multi-layering. [b] Schematic of basic bio-printing. A delivery unit (e.g. a syringe), pump-fed with cells and a 'matrix' or cell support/gluing material, moves in X, Y and Z planes to deliver the desired patterns of cells and gluing matrix in 3D. Supporting 'matrix' must hold the cells in place, without damage, and mimic an extracellular matrix.

However, all this bulk means that we are dealing with an 'inflated' system, where the fine, μ-scale peri-cellular architecture can be fabricated at the mm-scale. This makes complex construction much simpler. Once the basic tissue template is gelled to a 'solid', most of this excess fluid can rapidly expelled (a) in a controlled manner and (b) in a single direction; with the following results:

(i) The original template structure remains largely intact, but *miniaturized* in one plane.
(ii) Miniaturization/compression is in the order of ≈100 fold, but only in the axis of compression, giving a thin, dense sheet of collagen matrix seeded with living cells.
(iii) Miraculously, any cells present, and now trapped in the fibril network, are undamaged by this rapid fluid outflow (apparently due to support of the nano-fibrils themselves).
(iv) Because fluid outflow is along a single axis, the collagen fibrils pack most densely at the fluid-leaving-surface (FLS) and in a series of parallel μm-scale thick lamellae above that. Cells find themselves enmeshed in a dense, laminated mesh without having 'done' anything.
(v) Typically, a 5 mm thick initial collagen gel is compressed to 50–100 μm. Since initial gel height, collagen concentration, cell density and total fluid removal are controlled, the final neo-tissue can be completely predicted. Typically, ≈25 million cells/ml lie in a tissue-like, anisotropic lamellar, native matrix of 10–15 per cent collagen.

In other words, we have solved our dual big problems by directly engineering with the materials we need in the end (native collagen plus cells). Furthermore, by building big and shrinking to the dimensions we want, we have made it easy to position and vary whatever cell and matrix additions we then insert. The apparent new problem we have, of having made a 50–100 μm thick tissue (a bit like moist cling-film), is not really a problem at all. In fact, this is perfect for layer engineering and heterogeneity building – rather like constructing micro-Swiss rolls.

Other approaches are under examination with the aim of producing similar **matrix-rich tissue layers**. There are, from other fields of rapid fabrication, a number of techniques which may be adapted to lay down together predetermined patterns of matrix protein and living cells. These include electro-spinning and inkjet spray technologies, which becomes 'bio-printing' when used with cells.

Bio-printing is an area of increasing interest for rapid tissue fabrication, and it is a close relative of layer engineering. The idea has evolved from the mature field of print technology and rapid prototype engineering, especially in the automotive industries. The familiarity of inkjet printer technologies has made this an attractive idea in biological specialities. The aim is to print predetermined (μ-scale) patterns of support materials/scaffolds (with or without cells) in the same way that we would print a page of ink, but then to print another and another layer on top of this until a significant third dimension is built up. Again, progressive layers do not have to be identical, thus allowing for patterning in the 3D plane also. Clearly, this is a form of layer-by-layer assembly, at the multi-micro scale, but without the production of discrete tissue layers, as we had before in CPC and cell layering.

The automotive industry teaches us that this can work for selected materials, and indeed can make wonderfully complex and fine 3D structures, including apparently 'impossible' structures such as a sphere within a sphere. These first caught the imagination of bone tissue engineers for making hard-tissue implants with biomimetic micro-structure, customized to the particular patient defect or injury site. However, the challenges of this approach became more apparent as aspirations moved beyond making acellular replicas of hard tissue, in inorganic, calcium-phosphate-based materials.

First, the nature of the scaffold material is key. For rapid prototyping of engine parts, it was possible to use synthetic polymers and polymerize these as the process went on, then to cross-link or bond the various parts and μ-layers together with harsh, non-biological agents and condition. But these materials are unsuitable for tissue engineering, often not even undergoing simple dissolution (i.e.

'*biodegradability*' of conventional tissue engineering synthetic polymers).

There is also the logical driver, discussed previously (see Chapter 5), to place the required, living cells into the 3D micro-structure of the matrix *during* the bio-printing process[12]. However, the polymerization and cross-linking processes which are integral to bio-printing are normally incompatible with cell survival, at least for conventional synthetic polymers. Furthermore, the rapid transit of cells through tubes (or sprayed across air-gaps in the case of electro-spinning) poses considerable problems for cell survival. Reduction of fluid shear favours processes which are slower, but '*slower*' in soft, tissue-like structures makes it increasingly likely that each newly deposited layer will sink into, mix with or migrate through previous layers. In turn, this pushes the process designers more and more towards cross-linking the matrix or support-scaffold elements earlier and more tightly.

For all of these reasons, many research lines in this area are starting to use natural protein support materials, with natural cross-linking (e.g. fibrin or collagen: see Chapter 4), highlighting the family links with discrete layer engineering processes as described above (Figure 6.10b). These are high technical hurdles on their own, and are trickier still when combined with keeping the cells alive.

The problem is that the huge potential for fine control of 3D μ-structure offered by bio-printing and related fabrication methods does not help at all with the pressing problem of how we rapidly fabricate dense, strong natural tissue matrix around the cells. Indeed, the very advantages they bring for generating high resolution shape presently makes it technically harder to achieve this without killing the cells.

[12]As we saw before (Chapter 5), dense, tissue-like 3D micro-structures are inherently rather too tightly packed together to allow cells to penetrate easily. As a result, where we make these *without* cells, our beautiful μ-structure will be compromised and potentially wrecked during the inescapable slow and protracted cell invasion-remodelling stages. Therefore, assembly of cells and matrix all in one step is highly desirable.

It is becoming clear that these approaches are no more 'technical-magical-bullets' for quick tissue engineering, than any other solutions we have looked at so far. Users of such print-related layering systems must tackle much the same big problems as everyone else: and these are not 'big problems' without reason. Focusing on the big problems which hold us all back is helpful in emphasizing the family links which exist between the various approaches to 'layer engineering'.

Building up of living tissues in layers, then, shares a great deal in concept terms across the field. The differences are more related to 'what is in', and 'how we make' the building blocks themselves. Some approaches fabricate discrete multi-micron-thick living tissue layers as a first stage, and then assemble-layer them together in a second stage. Bio-printing has a much higher hurdle, in that the whole process must be carried out in only one stage (though the rules stay much the same). In these circumstances, the history of biotech research teaches us that progress in both will be parallel. However, practical rapid tissue fabrication with simpler two-stage approaches is likely to come sooner than for single-stage techniques. In effect, the problems are the same, but we can allow ourselves more process time to tackle them.

6.7 No such thing as too many layers: theory and practice of tissue layer engineering

Here is the paradox of the Chinese restaurant. How is it that the most modest Chinese establishment boasts 6–8 pages of menu, containing perhaps hundreds of delicious-sounding dishes, yet these are inexpensive, quick to arrive, hot and (apparently) made by only a small staff? Are they super cooks? The answer, of course, is simple if you ever get a glimpse into the kitchen: rack after rack of tubs with different ingredients, thin and ready chopped, for rapid processing. Relatively modest numbers of these culinary building blocks (three racks full of vegetable tubs, two of chopped meats and one of seafood) can be combined quickly and with simple

cooking sequences to produce literally hundreds of very different dishes. The result is a multiplicity of rapid, inexpensive dishes, needing only a fraction of the individual attention required by the skilled kitchen staff in the French or English restaurant next door, who train for years to individually assemble items on a menu of just eight savoury dishes and ten desserts, at twice the cost.

Applying this reductionist analysis, based on our previous identification of the main tissue planes and layers, skin can be broken down into a series of stylized layers. Using the three principle axes of the tissue, either planar or circumferential/coaxial, it becomes possible to generate complex 3D structures which resemble those of the natural tissue. This is illustrated in Figure 6.11 in the form of a model tissue. There are three separate levels of complex 3D structure built into this model:

(i) multiple, heterogeneous layers (they do not have to be flat, planar layers shown here);
(ii) 3D zones, running through a number of these layers,
(iii) concentric multi-layered tubes and channels.

It is important, though, for the reader to understand how high this aspiration actually is, compared with where we currently really are in cell and tissue engineering.

Current therapeutic strategies for spatial complexity include:

(i) the simplest possible (injection of a cell suspension into the vicinity of a lesion: e.g. stem/progenitor cells around a myocardial infarct);
(ii) random cell seeding of synthetic polymer sponges; or
(iii) pre-seeding of whole-tissue sheets;
(iv) bi-layers in which differentiating keratinocyte sheets are grown over a single collagen gel layer, seeded homogeneously with fibroblasts (e.g. Apligraf™, a clinically used skin substitute and cell therapy).

Some of these are illustrated in Figure 6.12, as a staged increase in complexity, from the familiar (2D) epithelial monolayer, through cell overgrowth and cell multi-layer formation, to true incorporation of different cell types into a fully 3D material. The greatest level of structural complexity (normally at the scale of 1–2 mm thick) is currently tackled using duplication of these cell-based structures, as in the case of Apligraf and other skin substitutes. It is important to note:

(i) the relative simplicity and gross scale of current non-homogeneous engineered tissues; and
(ii) their heavy dependence on cell activity (e.g. division and protein synthesis) to produce structure.

What new and ambitious technology can we adopt, then, for such a leap in target ambition? The answer comes from noting how easily we could generate complexity with the Swiss roll – in other words, build compositionally simple cell/collagen sheets (lots of slightly different components, as in the Chinese menu) and *then* introduce (pseudo-) complexity of 3D structure through the assembly process. This eliminates previous dependence on

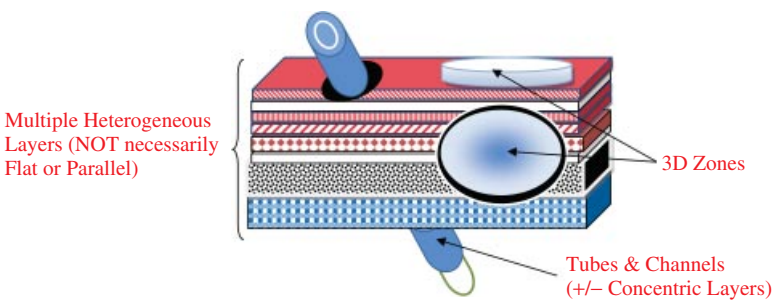


Figure 6.11 Aspiration level: complex, non-homogeneous, 3D model 'tissue' – but built from many simple layers.

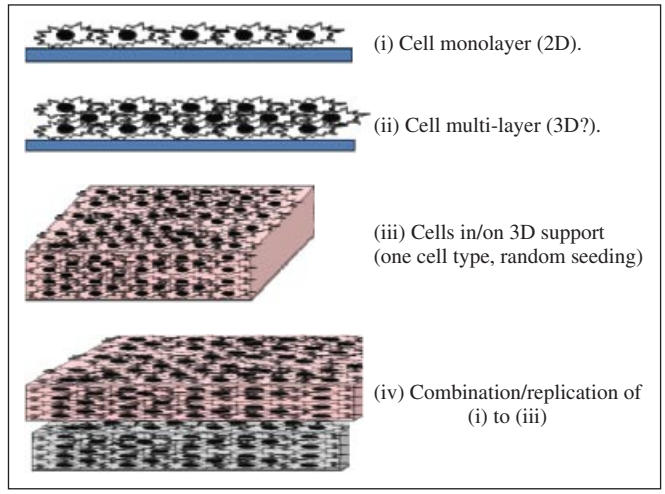


Figure 6.12 Examples of current targets and strategies for engineering and assembly of 3D structure.

protein synthesis/assembly by cells. An attractive part of the strategy is the rapid assembly into multi-layer, thick tissues (Figure 6.11). The technical trick which makes layering such an attractive route to ever greater complexity turns out to be the ability to rapidly fabricate collagen networks with any number of different added particles, including cells.

6.7.1 Examples of layer engineering

We shall now look under the surface, into the detail of how we can 'make' layers and zones through use of examples. We can illustrate the principals through one main example, with a summary of the alternative possible technologies which follow the same track.

Let us develop this idea by looking in more detail at the building block layers which went into the structure in Figure 6.11. Figure 6.13 shows the nature of one such a matrix-rich building-layer for connective tissue engineering, with a scanning electron micrograph image of the dense collagen fibril matrix of a single 100 μm PC layer.

If Figure 6.13a shows a cell-free collagen layer, the next layer(s) in the serial stack might be seeded with fibroblasts (Figure 6.13b). This fibroblast-seeded layer is mid-range cell density (perhaps more comparable to mature rather than repairing or growing tissues). In effect, cell density can be defined, layer by layer, as millions of cells/unit volume of tissue (e.g. per mm^3 or per ml). This might, for example, be used to give a gradually reducing cell density deeper in the construct (i.e. further down the layer stack) by using a lower density in each successive layer. Such a structure would mimic dermis.

Clearly, any number of such separate sheets can be layered onto each other in the stack. Furthermore, they might be seeded with different cell types. The difference can be either as:

(i) one cell type in one layer and another in the next; or
(ii) two cell types in each layer, but in a ratio which changes gradually down the stack, giving a gradual transition.

Real tissue examples where these structures would be useful are easy to find. Larger blood vessels typically have an outer layer of fibroblasts which gradually grade into smooth muscle cell-rich layers which suddenly (innermost surface facing the blood) becomes a single-cell-thick layer of endothelial cells. Similarly (but this time a planar stack), the abdominal wall inside the skin changes suddenly from skeletal muscle sheets in different orientations, to a fibroblast in woven collagen layer (transversalis fascia), lined on the inner abdomen wall surface by a peritoneal cell layer.

Text Box 6.4 Well? *Can* we have too many layers?

Actually, there is a progressive advantage to tissue precision, and the ability to customize, in having as many layers as possible per millimetre of total tissue construct. This we can call '**resolution**'. It is a bit like the pixels in a digital photograph – the more there are, the better the image looks. This carries the predictable cost of more layers, more cost, and perhaps being slower to build up, but this another matter, as it is in digital photography.

There is a more pressing limitation which is easier to see if we look at a real example, say, skin. If we have only three layers – epidermis, dermis and fat, as mentioned above – each would be in the millimetre scale and the resulting tissue would be *very* simple. We could make the dermis ever more complex, with 10–20 layers of, say, 100 µm or (just) of 100 layers of 20 µm thick but, at much smaller than 20 µm, there is no space for the cells. This means that the theoretical limit for a cellular layer is a little over one cell diameter. There is, however, the possibility of adding in non-cellular layers, such as tough matrix, anti-adhesion or drug/protein carrying sheets, interspersed between cell layers or even cell-rich layers, such as epithelium.

So, there can be limits to the number and density of layers, but these can be predicted quite logically.

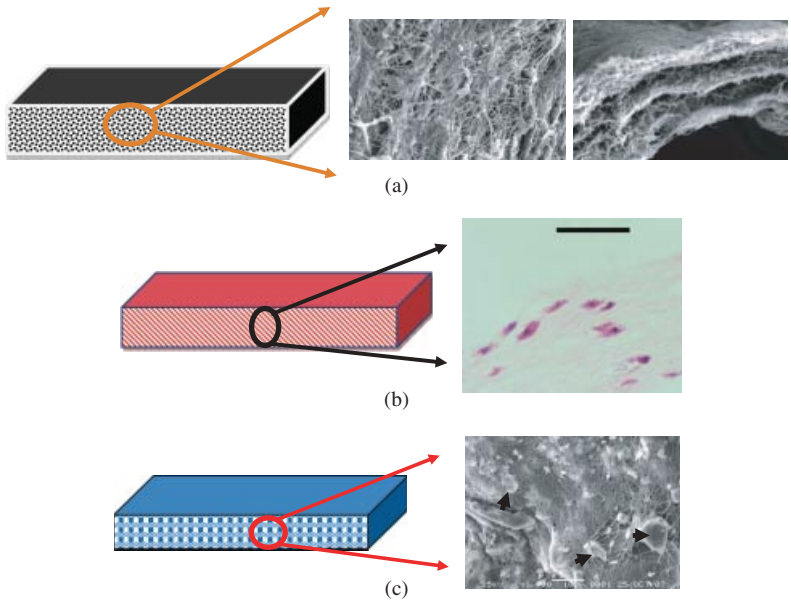
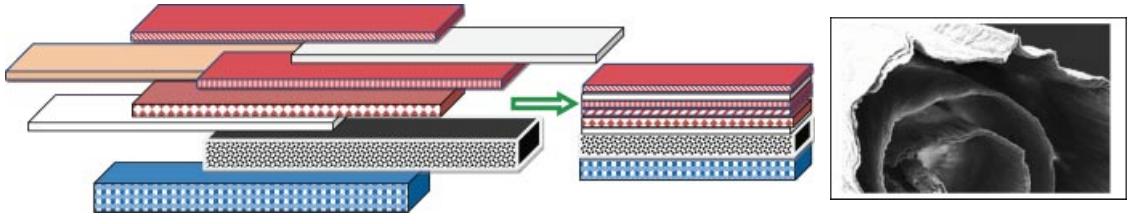
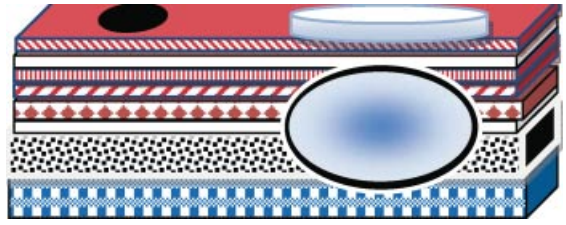


Figure 6.13 (a) Basic building-sheet for matrix layer engineering showing the dense fibrillar appearance of a 100 µm thick PC collagen layer (Figure 6.10). Left hand panel: bulk collagen. Right hand panel: cross-section of layer. (b) Fibroblast-rich collagen layer. H&E stained micrograph (inset) shows the distribution of (purple) cells within the orientated fibrous matrix of the single layer. (c) A hard tissue layer can be fabricated by the mixing of mineral particles with the collagen prior to gelling and compression. This leaves particles (in this case phosphate glass (arrows)) trapped interstitially within the fibril network, as in the inset scanning electron micrograph. With thanks to Dr. Tijna Alekseeva. (d) Assembly of layer series: Layers of differing, or similar composition can be stacked to give tissue-like structure at the multi-micron scale. The scanning electron micrograph (right) shows a stack of identical layers, formed by spiralling a single, large compressed collagen sheet (layers shown separate here for clarity, but would normally be in contact). Reproduced by kind permisson of Nelomi Anadagouda. (e) Local zones or depots can be inserted into position at either the layer-stacking or the gelling stages. (f) Micro-channels are formed to order through dense collagen tissues by co-compressing soluble (phosphate) 40 µm diameter glass fibres into the initial collagen gel. Far right inset shows fibres between rolled up collagen layers (arrows): the left hand SEM image shows a channel produced right through the construct when the glass dissolved. (e) and (f) reproduced with permission from Real-time monitoring of the setting reaction of brushite-forming cement using isothermal differential scanning calorimetry. M.P. Hofmann, S.N. Nazhat, U. Gbureck, J.E. Barralet. Journal of Biomedical Materials Research (Applied Biomaterials) 79B, (2006) 360–364. © Wiley. *Parts (d), (e) and (f) on the next page.*



(d)



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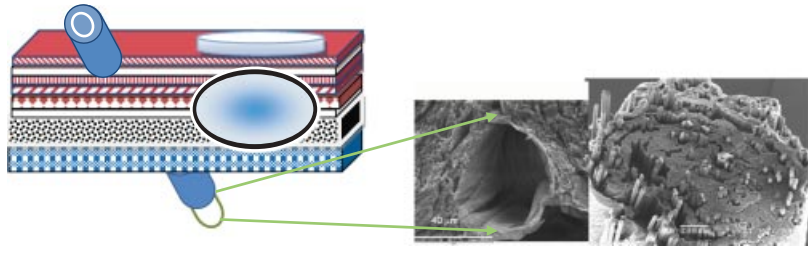

(f)

Figure 6.13 (*Continued*)

In fact, we can go yet further with the types of layering available; for example, the 'particles' trapped within the collagen network need not only be cells. Hard tissue layers can be formed by mixing mineral particles (again at any selected density and particle diameter) in with the initial collagen gel, with or without cells. Mineral particles can be, for example, hydroxyapatite, tricalcium phosphate or phosphate soluble glass across a size range of ≈100 nm to ≈20 μm (Figure 6.13c). This size range allows them to be retained by the fibrillar net at the lower end, and yet is a comfortable fit into the 50–100 μm thick collagen layer at the larger end.

Cells seeded along with the mineral particles largely survive co-compaction at modest mineral densities. This introduces the potential for fabricating hard tissues or hard-soft tissue interfaces. Such hard or hard-soft interfaces have a wide range of clinical and model tissue testing applications in the bone, calcified cartilage and dental fields. In particular, there is considerable current interest in engineering of the osteochondral[13] interface or junction. This is a major objective for surgical repair of joint damage. Tissue models of the osteochondral junction could help in understanding the origins of osteoarthritis, as it is suspected that changes in this layer could lead to some forms of osteoarthritis.

[13] e.g. the layer where the joint-end of a bone surface is bonded into the overlying articular cartilage.

By the same logic, this type of particle entrapment technology can be used to locate defined types and densities of cargo-carrying nano-micro vesicles or even carbon nanotubes. These can be pre-loaded with drugs, growth factors, gene-sequences, etc. and localized precisely within the final tissue to give new levels of controlled release and local biological control. A promising example of this has been described in the formation of ultra-high-density cell depots calculated to produce known levels of deep tissue hypoxia. This hypoxia leads to time-dependent generation of angiogenic growth factors, representing an entirely controllable pseudo-physiological therapy to promote local blood vessel in-growth. Such an ability to induce local re-vascularization at will is a long-standing clinical dream.

This train of research aims to develop a selection of both cell-rich and matrix-rich prefabricated 'layers' as building blocks. It only remains, then, to assemble these into stacks or layers *in the required sequence* for the simplified tissue of choice (Figure 6.13d). Integration of the layers of the stack (i.e. physical linkage) to form a single unit can be achieved either by co-compression of layers or subsequent cell action. Prevention of inter-layer adhesion, perhaps with insertion of a further *non-adherent* layer can be used to form gliding layers, similar to the synovial sheath in native tendon. Figure 6.13d illustrates the planar stacking of selected layers into intimate contact, as a complex tissue construct. The inset scanning electron micrograph shows an actual series of collagen layers.

As mentioned earlier, layering does not necessarily have to be parallel or flat to be biomimetic – far from it. Additional shapes and structures can be inserted into and across the layers to form localized 3D zones or depots in addition to the basic layer structure (Figure 6.13e). Depots of particles, fibres or cells can be inserted between the layers during layer assembly, or micro-injected after layering.

Alternatively, channel structures, either cutting across or running between existing planes, can be introduced as a single layer or as a co-axial tube and sheath. Figure 6.13f illustrates what is meant by layer-crossing micro-channels. These can be fabricated either as large diameter channels (>1 mm), formed relatively simply by rolling the collagen layers around a mandrel or by puncturing/drilling the constructs. However, the all-important micron scale channels, to mimic micro-vascular perfusion, or to guide capillary in-growth, are another matter. Once again, though, these can be achieved relatively simply by engineering of the soft collagen layer structure. Tissue examples of axial and co-axial layering can be found in blood or lymph vessels and nerves, as they run through other tissues, hair follicles in the skin, tear ducts and parts of hearing, uro-genital and airway systems.

One example of micro-scale channelling uses the plastic nature of the collagen PC process (Figure 6.13f). An example is shown (right hand inset) as two scanning electron micrographs. The right-hand (lower power) image shows a spiralled dense collagen construct, into which many parallel 40 μm diameter glass fibres have been compressed prior to spiralling. Such fibres can be made of any required size or composition. In this case, they were made of a soluble (phosphate) glass, where the familiar silica component of the glass is replaced by phosphate, making it fully soluble in a few hours. The glass dissolves and is flushed away soon after fabrication, to leave full-length patent channels right through the constructs.

This, then, is a 'lost-fibre' μ-channelling technique. Since dissolution products of the glass are generally not toxic, cells seeded into the collagen survive well. Indeed, recent work has shown that cells (in this case vascular endothelial cells) pre-seeded onto the glass fibres prior to plastic compaction come off the fibres and adhere to the walls of the 50 μm diameter channel, potentially forming an endothelial lined μ-channel. In nature, we would call this a simple capillary.

In a comparable approach, another group has seeded endothelial cells into 0.5 mm strands of agarose and cast this into a soft collagen gel to again produce an endothelial cell-lined channel through a gel matrix, in this case designed to

Text Box 6.5 More than one way to make a 'layer'

Perhaps it is time to take a small digression into the practical side of what interests us. We are starting to see a dazzling new collection of routes to 'assemble tissues and scaffolds' with such speed and detail that we might be forgiven for pinching ourselves. Readers and tissue engineering brethren who are less prone to swallowing whole fabulous tales of what could be coming, might wonder where the warts and the quicksand pits are hidden. The first thing, though, is to classify the different approaches and identify their family trees. After all, it is important to understand the origins of our ugly sisters before making any life-changing choices. Three seemingly loosely related examples of 'layer assembly' are:

(i) layer-by-layer nano-fabrication of surfaces on scaffolds and biomaterials
(ii) ink-jet, or 'bio-printing' of scaffolds and tissues,
(iii) tissue layer engineering (cell-layer and matrix-layer types).

All three aim to build up 3D structure by progressively adding layer onto layer in predefined sequence – clearly an attractive approach.

(i) Layer-by-layer fabrication of scaffolds involves coating surfaces with molecular (nm-scale) layers, bound by opposite charges (Figure 6.14a). This provides nm scale control of composition-structure in the *z* plane (red arrow), but mm or cm in the *x* and *y* planes.

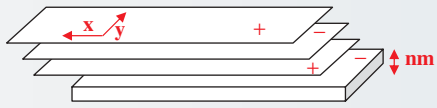

Figure 6.14a

(ii) Ink-jet, or bio-printing, deposits μm-scale drops, layer after layer, as liquid plus cells plus scaffold, to build up 3D structure in the *z* plane (Figure 6.14b). In this case, spatial control is in the μm-scale, *but this time in all three planes* (*x*, *y* and *z*).

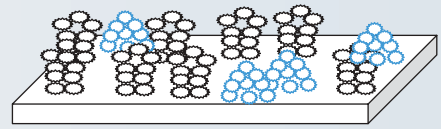

Figure 6.14b

(iii) Cell-layer and matrix-layer engineering (Figure 6.14c and described above) controls the *z* plane assembly at the tens of μm scale (arrow ≈50 μm). However, layers can be mm or cm in the *x* and *y* planes with little or no variation in structure.

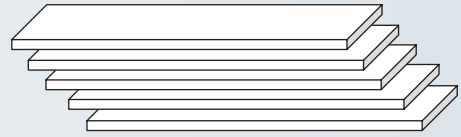

Figure 6.14c

As a result, layer-by-layer fabrication (i) gives the highest resolution in *one* plane, bio-printing (ii) provides the greatest resolution in *all three* planes, and cell-matrix layer engineering (iii) offers the lowest resolution and provides structure mainly in the *z* plane. However, because its scale reflects that of resident cells and its operation is simple and cell-friendly, (iii) is on track to produce living, functional 3D 'tissues' first.

mimic a larger vessel. In fact, there are now a number of versions of the lost fibre technique for rapid fabrication of microvascular-sized channels through solid implants. This has included the use of caramelized sucrose μ-strands (otherwise known as candy floss or cotton candy) to form channels. Colourful as this is (in fact, pink!), the very rapid dissolution of method in this case is limited to making channels in synthetic polymers where it is not a problem that such sugar fibres are ultra-soluble in water, dissolving in a second or two in the gel.

6.8 Other forms of tissue fabrication in layers and zones

We have worked through one example here of *direct* layer engineering of tissues, in this case based on controlled fluid flow and shrinkage of native cell collagen gels. This use of fabrica ted living connective tissue sheets as building blocks for layer assembly was selected as it illustrates most of the desirable targets and approaches in a single system. Clearly, though, there are other examples of approaches following partially or wholly the same general track. Not least, as we saw earlier, there are mirror image systems designed to make cell layers for assembling layered constructs. These are designed for use where little extracellular matrix is needed, aside from small amounts around the cell (pericellular matrix) such as the basement membranes of epithelia or in muscle tissues. These cell layers are, of course, entirely 'grown' in culture over time (see Chapter 8), and are then assembled to give cell-rich multi-layer structures.

Other techniques, mostly for direct matrix assembly, have been adopted from:

- biomaterials production (electrospinning);
- rapid prototyping, such as in the automobile industries (layer-by-layer deposition and photo-cross linking);
- inkjet printing adapted to 3D deposition of layers containing proteins, synthetic polymers and, in some cases, cells.

Being adaptations from *non-bio* production technologies which we might regard as rather '**cell-brutal**', many of these struggle to operate with, or even around, living cells. Electrospinning as well as inkjet-type systems tend to be inherently cell-lethal due to both physical (shear and desiccation) and chemical solvent stages. Not surprisingly, then, most past and present uses aim to produce 'scaffolds' with complex and pre-determined 3D structures, but which are seeded with cells in a separate step. Electrospinning typically has been developed to produce nano-fibrous materials (protein or synthetic polymer), often with defined alignments which can then be layered (and cell-seeded). Inkjet technologies have been used to form complex structures rich in mineral to mimic hard tissues.

Controversially, some examples are now being developed where cell deposition is possible into the polymer 'scaffold' – the emerging cell-bioprinting technologies. These are beginning to tackle problems of μ-nozzle blocking and high shear but are moving towards production times of hours for significant tissue size and 3D (layered) complexity. However, it should be clear to the reader that successful examples of these methods will inevitably follow a similar concept and strategy route map to our main example. This is because they all aim to build up thick, complex tissue structures from many layers and zones made initially from simple building-blocks. In effect, they will all build up relatively simple compositions into disproportionally complex μ-structures on the model of Swiss rolls or Black Forest gateaux.

6.8.1 Section summary

The idea of this section has been to build up to a view of how we can (and do) develop a new approach to engineering of tissues. This extreme tissue engineering message, then, involves recalibrating our image of the target tissues. This sees the native target tissue as made up of a sequence of many similar layers and zones, in differing planes. It then becomes relatively straightforward to fabricate simple but mimetic collagen-based μ-layers which can be assembled, in sequence, to build up a cellular tissue mimic or template. If we are to make full use of this extreme new view, it is important to practise the deconstruction-reconstruction process using layers in all three planes. This can include radial/concentric, planar stacks and non-parallel layering. Such an approach to fabrication process design leads naturally on to the use of tedious repetition (and very thin layers) as a means of building up hierarchical structures across the major size scales.

Figure 6.15 Bridge construction analogy. Building up complex, long-range structure in 3D space is often best achieved using repetitive segments assembled in sequence. In effect this is the layer engineering principle, seen here during assembly of the Golden Gate Bridge, where decking segments (some identical, some different) are slung into position to be joined in a pre-determined sequence.

Finally, we should now be able to see, in best extreme TE traditions, that by aiming at the apparently impossibly high target of fabricating the tissue from hundreds of basic building block layers (instead of all in one go), we can achieve previously unthinkable levels of biomimesis in a fraction of the time. To appreciate how far this has taken us, recall that one of the most successful and complex engineered clinical skin equivalents at present is Apligraf™. This comprises only two layers, takes many days to grow (so is expensive) and delivers cells in a hyper-hydrated gel, acting more as a cell delivery than as a strong graft tissue. Now compare the complexity and bio-mimicry being contemplated in Figure 6.13. Paradoxically, then, by raising this particular bar, we are opening extreme new possibilities, often resembling those common in good process engineering and mass production.

An interesting aspect of this form of layer engineering and assembly is that of growing progressively in complexity from originally simple approaches to fabricating the layers. This is beginning to spawn speedier or more effective device-based variants, but this evolution has a distinct direction, towards mechanization. It is not driven by a pre-existing device or technology which then has to be adapted to the biological demands of the cells and tissue, as is the case, for example, in bio-printing approaches.

6.9 Familiar asymmetrical construction components: everyday 'layer engineering'

'Segmental building' is a short-cut term for non-homogeneous, directional (or sequential) assembly of prefabricated segments into complex, anisotropic 3D structures. This, of course, is exactly what we are looking for in a rapid tissue assembly process. The bridge-building analogy (specifically *suspension* bridges) is useful here, as it emphasizes the potential for the rapid engineering of large, complex structures using layers and zones (in this case, decking segments). The way in which civil engineers go about erecting such structures can act as useful illustrations of the extreme tissue engineering solution which is beginning to emerge under the name of layer engineering.

It is important, of course, to keep the analogy in perspective, as there is much about bridge assembly which is anything but mimetic of cell-related assembly. However, the idea of pre-fabricating the large numbers of similar basic structural components and assembling them into position *in a strictly planned sequence* definitely has echoes of layer tissue engineering.

The key lesson here is just how valuable it is to plan the assembly sequence. Figure 6.15 shows San Francisco's Golden Gate Bridge under construction. The system of assembling prefabricated sections of deck in a directional, self-supporting sequence is clear. The direction of progression is forced by the necessity to work away from points of maximum support (i.e. the towers). Such basic mechanical logics can also dominate how we assemble layers and how resident cells work on natural tissues or template tissues.

Hanging and fixing together a long series of deck sections is a mechanically tricky process, and the bi-directional progression away from the towers is not only economical of effort and beautiful to see, but ensures a degree of balance and speed – in this case, between earthquakes!

6.10 Summary

What have we learned, then, about how to make the most of 3D hierarchical complexity? Or, to put it another way, have we answered our initial question, 'Is it worth the hassle'? Well, firstly, it is axiomatic that we have little choice but to use it and optimize it if we have any aspirations to be mimic natural tissues. It is clear, though, that once we understand how the natural hierarchical levels are built up in native tissues then, by repetitive positioning of similar parts in sequence, we can adapt well-tried strategies from other fields of construction engineering.

The take-home message seems to be to learn how to fabricate increasingly complex building 'segments' (layers when necessary). In this way, our designs for tissue assembly can be as **flexible** and **finely tuned**, as we undoubtedly need. This track is unavoidable if we aspire to mimic that most basic characteristic of natural tissues: *adaptability*.

After all, what sort of a visionary target have we produced if it is to fabricate a generic 'skin', for example? Imagine the reconstructive surgeon who needs skin for four successive patients to reconstruct (i) eyelids, (ii) a facial scar, (iii) post-tumour breast tissue and (iv) a post-burn thigh injury. Neither the surgeon nor the respective patients will be over-impressed by the ambition of our vision if they are offered a generic, lowest-common-denominator sheet of 'average skin' which looks like and behaves like the skin found at *none* of these sites!

Perhaps worse, patients needing eyelid reconstruction will be particularly unhappy with a skin equivalent which is functional and well adapted to work on the thigh. Less obvious, but no less important, is that in practice, the surgeon will also need different skin characteristics if the patient is 5 or 65 years old, or on chemotherapy, immunosuppression or anti-thrombotic drugs.

Surely, then, the 'generic tissue' targets we have adopted in the past are already too low to be called ambitious. In addressing this reality of biology, it is now becoming clear that we must either rely on the innate ability of our cells to *grow* this 3D complexity (as they do in embryos), or we must find ways to *fabricate* it for ourselves, as we have done for centuries, in other materials. This 'grow-fabricate' tension will occupy us for the remaining chapters.

But the honest answer to our 'is it worth the hassle?' question must be, YES. This is not only because, if we are frank, we don't really have a choice but also, surprisingly, that like so many other 'tall orders', *tackling the toughies head-on* can be the easiest long-term route anyway.

Further reading

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