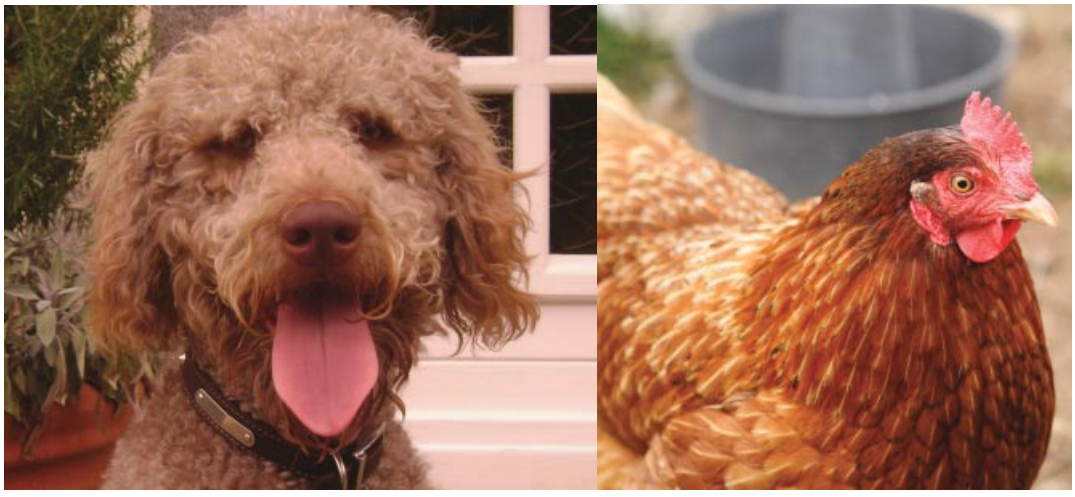

This chapter is all about spatial perception and cells. Understanding another person's perceptions is tricky even when we speak the same language. Understanding how animals sense and perceive the environment is more difficult still, even where we can recognize similarities in the structures and sensing machinery. Even then, much of it is a guess.
Understanding how spatial signals are processed and interpreted in single cells or cell clusters is a whole different ball game – not least at the cell-scale of 10–50 μm, where many of the basic assumptions do not apply, or work differently. Perhaps, then, we should give much more time to our concepts of how cells detect direction and movement.
As an example, we understand the spatial perceptions of the dog – his forward-facing eyes give stereo 3D images of the world he is pointing into. In contrast, the chicken seems to have two non-overlapping, independent and sideways views of the world, useful for all-round danger-warning but rather less so for precision 3D perception – we think! We struggle as much with chicken vision as with the dog's tongue-perception, because we cannot experience what they do.
Working out the basics of how cells measure asymmetry in their μ-environment is similar if we can try thinking at the cell scale and eliminating the implausible. For example, it is likely that cells rely much more on a 'tongue and whisker' type of sensing (i.e. chemical and mechanical) than the light/sound systems of our two multi-cellular chums above.
Chicken photograph reproduced with permission © iStockphoto.com/Peter Seager.

3 What Cells 'Hear' When We Say '3D'

or: How do you know you are moving when you close your eyes?

3.1 Sensing your environment in three dimensions: seeing the cues

In many European cities, the walkways and crossings now have a bewildering collection of accessories to help blind people. It is instructive to take a walk with a stick and learn how to read the information available from these. All the time, extra clues are being provided about what is coming up or how you might move between static objects (e.g. walls, edgings, barriers) or moving projectiles (avoiding cycle-ways (Rotterdam), finding gaps between cars (Barcelona) or buses (London)). These are designed to give instant, functional mechanical information (touch through a stick or vibration through sonic pedestrian crossings) about the static and dynamic space surrounding the footpath.

Although there are a few exceptions, there is far less useful and reliable information to be gained by sniffing or tasting the air. So it must be in the micrometre (μm) scale 3D physical space which cells inhabit. *Physical* measurements (e.g. of

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

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mechanical contacts or reflectance of sound or light) must provide much better information streams on the physical 3D space than (bio)chemical signals. Chemical signals are good at telling us we are near to doughnuts or cattle but less good at indicating position, size or rapid movement (shop, farmyard or delivery truck). For this analysis, we shall assume that most cells are functionally 'blind'.

In this chapter, it is useful if you can imagine what it is like to 'be' a cell within its 3D space, and so identify what types of basic spatial information it needs and whether this information is available (Text Box 3.1). In particular, the key question is how cells gather information about their immediate and neighbouring 3D environment – their location within that space.

The first obvious factor here is scale: that space is very small to us. However, while this space measures, at most, tens of microns in any direction, it operates under many of the same physical laws as our human space. The monitoring of physical signals and cues represents the main source of this type of data for any system. Since direct use of sound (i.e. significant pulse frequencies), optical or gravitational attraction by cells is considered specialist and rare, much physical information will come from monitoring of changes in the mechanical environment.

Clearly, if we aspire to control how, when and in what form cells build 3D tissue structures, it is essential to understand the mechanisms by which they 'find out' where they are and what is within their space. In other words, in which language must we communicate spatial instructions to cells?

As with any complex question, it is always good to start by describing the question in the simplest terms possible, focusing on the most dominant factors. In the case of spatial matters, orientation and material mechanics are good starting points (for example, which way is up, down, left and right and which is harder and softer?). For this, there is a particularly potent analogy with the human scale (Figures 3.1a to 3.1c).

The activity of sea travel illustrates how it is essential to adapt to the **two basic positions**: on and in. Each requires profoundly different forms of structure and environmental monitoring. The submarine (Figure 3.1a) is the sort of craft we are familiar with for travel *through* the bulk of

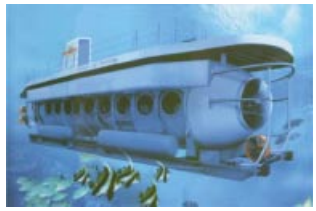
Text Box 3.1 What does '3D' really mean for different cell types and (why) does this matter for tissue engineering?

If cells could 'feel', then, how would they feel about 3D? In biology, we fully expect that cells can build and maintain their home tissues with exquisite fine structure in a way which is only plausible if they are able to detect complex orientations and forces within their 3D space. Because space and 3D structure (morphology) are so central to our aims in tissue engineering, this is not a bullet that it is possible for us to dodge.

Unfortunately, despite increasing recent interest, we are working from a low base and presently we have only a sketchy understanding of how cells collect, process and use physical information about their space. In contrast, we have a far more complex and sophisticated understanding of molecular control mechanisms. In this chapter we shall explore how this is made more difficult by nomenclature-logic clashes in traditional (*in vitro*) cell biology. This is visible in the very idea that 3D cell culture is a special state, when to engineers and physical scientists, cells in practice *always* operate in a 3D environment.

To hunt out some truths around this paradox, we must burrow into what is really meant by the 'physical cell space', with the aim of identifying what information different cell types *may* be able to collect and use. Important conclusions are that the answers depend on the types of cell in use. This analysis draws as little as possible on chemical/molecular signalling, primarily because many familiar pathways have already been elaborated, but also because monitoring of the physical space must be dominated by data about its physical properties. Even at the human scale, if you are wearing a blindfold and earplugs, how much help is it to sniff and taste the air for getting across the street or making a bed?

What is the use of this understanding? The hope is that by knowing which physical cues and information cells use to make and maintain their space, we will be able to design rational systems to control growth, both in the body and in culture. In a way, we are trying to learn the body language of cells.



(a)

(b)

(c)

Figure 3.1 (a) Submarines are designed to work within a relatively homogeneous, single phase (fluid) environment. Clues as to where you are, especially orientation in 3D within that medium, are few, and are non-trivial to collect. © Balicruises.com. (b) In surface ships, we know so much more about our 3D spatial location from the obvious fact that we are working at an air-water fluid *interface* (we assume mariners find 'sinking' obvious). This is particularly clear where the air-liquid interactions are used for propulsion and steering. © Europeanbarging.com. (c) Once this airship takes off, it is in a similar homogeneous environment to the submarine, except the fluid is now a gas-air. Like the submariners, the airship's pilot must measure how high/low the vessel is and how close to horizontal or vertical it is. © US Government, Library of Congress.

the medium (in this case, the bulk-water phase). Interestingly, we call it 'underwater travel'. The second type is the surface ship (more familiar to us only due to the accident of our own primary habitat). This operates at the air-sea interface (Figure 3.1b).

Both the craft construction and the sort of information collected by their crews as they move around 'their 3D space' are very different. The submarine is characterized by symmetrical structure, mechanically equally robust in all planes, with streamlining/elongation in the direction of principle axis of movement. The surface vessel is highly asymmetric, with profound differences in its upper and lower shape, structure and material properties (strength). Characteristically, this leads to a great deal more variety (asymmetry) in the overall shape of interface/ship than we see with undersea vessels.

Incidentally, there is a third means of ocean travel, namely over the water phase (e.g. the airship, Figure 3.1c). Because this again involves motion through the bulk of a fluid (in this case, air) it has more structural similarity with the submarine – tubular and symmetrical. The point here is that these systems are adapted not to the water or the air fluids themselves but either (i) to being surrounded (symmetrically) by the fluid, or (ii) to operation at the (asymmetric) interfaces between different fluids or fluid-solid interfaces.[6]

The earthworm (Figure 3.2a) is clearly well adapted to life embedded in a relatively uniform bulk material (moist soil), but much less so to the stark, symmetrical contrast of life at the surface – the air-soil interface (Figure 3.2b). In contrast, the limpet is another invertebrate with the alternate space adaptation, having evolved to a tough life at the rock-sea interface of tidal zones (Figure 3.2c). Here it is harshly reminded of 'up and down' twice every day.

[6]It might be assumed that heavier-than-air aeroplanes represent an exception to the rule of symmetry and within-fluid travel, but this would be a mistake. Planes are a special case as they are *forced* to use interface effects (lift-generating surfaces) to overcome their weight. On the other hand, rockets use raw blast energy to dodge this, and so they are again made as tube-shapes.

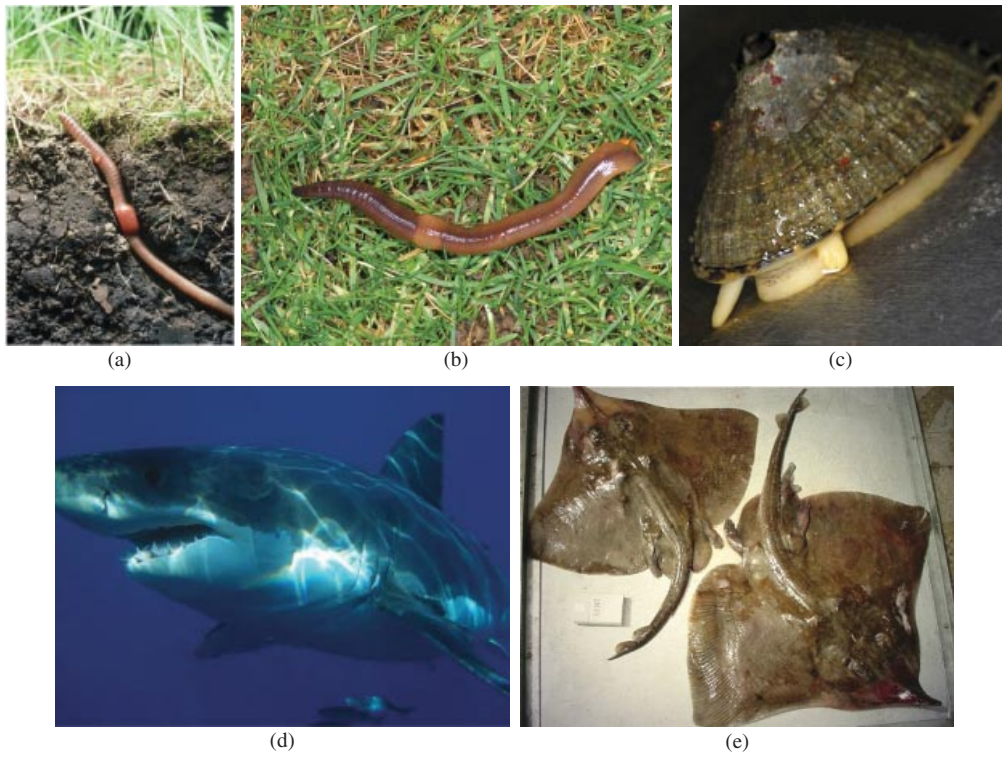


Figure 3.2 Animal adaptation to life in bulk, symmetric surroundings versus asymmetric surfaces (i.e. interfaces). Illustrated: An earthworm in its burrow (a) versus on the surface with the air (b); and (c) a limpet on a rock-sea interface surface; (d) a shark in mid-level swimming, and (e) a bottom-adapted skate. Credits: (b) © Steve Hopkin/ardea.com; (c) reproduced with permission from John Banks, Caithness Biodiversity Collection; (d) © US Government.

To bring the analogy back to vertebrates, sharks and skates are closely related but extremely adapted forms of fish. While sharks (Figure 3.2d) are well known to range freely through the bulk of ocean depths – again a largely symmetric aquatic space – their cousins the skates are adapted to an asymmetric sea-bottom life. Sharks, are characteristically round-bodied (with roughly radial symmetry but axially elongate and streamlined. Skates are flattened in structure to hug the interface. They are axially more symmetrical, with their eyes on top and mouth underneath (Figure 3.2e). Neither sharks nor skates are particularly happy in the other's space – in Darwinian language, they are poorly adapted to compete and survive there.

These examples illustrate just how important certain features of our 3D space can be – particularly its symmetry – and how the information collected from these spaces can be taken for granted.

3.2 What is this 3D cell culture thing?

There has, for some time, been a growing awareness in the biomedical and biotech worlds–accelerated by tissue engineering – that future concepts of cell physiology must take more account of the 3D cell living space. At the same time, there has been a rather superficial view that moving from '2D' to '3D' culture models will glide us gently into these new and fruitful waters. Why, then, has this apparently gentle gradient of logic given so much trouble and so little new understanding? While all we now know shouts that the basic premise of '3D' is good, could it be that

the simplicity of the idea is concealing a complexity of gargantuan and shocking proportions. Only a few hardy souls seem to have begun to grapple with what is now revealed to be a multi-armed monster at anything like the level it demands. In fact, the beast lurking in this deceptive Pandora's box may have been made more forbidding because of our own naming habits, and particularly in the 'cell biology' philosophy of what 2D and 3D really mean.

It is often good practice, where we are 'surprised', to initiate a paradox hunt, since it is common for our surprise to be rooted in contradictions or definition-anomalies. In this case, there is a clue within the seemingly fatuous question of 'what do we *really* mean by 2D', as this will elicit different answers from the cell biologist and the physicist. In cell biology, the terms '2D' and 'cell monolayer' or 'cell sheet' have become synonymous. This is potentially a dangerous definition slip because, in the present world of nanotechnology and molecular-scale surfacing, there are many tribes who do not understand how such a fundamental concept as spatial dimensions can become 'cell-dependent'. Once this problem is pointed out, we can probably all agree that two-dimensionality or 2D is either:

1. (practical usage; *effective* 2D) where the dimension of a structure in its *z* plane is functionally insignificant relative to its two other dimensions (*x* and *y*, or length and width, i.e. for practicalities of the system, the thickness is negligible); or
2. (absolute terms) a theoretical state in which a structure has substance in the x and y planes but non in its 3rd (*z*) plane. To illustrate: Hawking has pointed out that a truly 2D dog would fall into quite separate top and bottom, bisected by its gut (see further reading).

Panels (a) and (b) of Figure 3.3 try to express this graphically and, although they seem at first to be drawing out the completely obvious, it is important, as we approach any paradox, to be crystal clear. A layer with large x–y surface area (i) but insignificant thickness (z) still has a finite thickness, shown in the (ii) plane. Panel (b), shows the same cross section

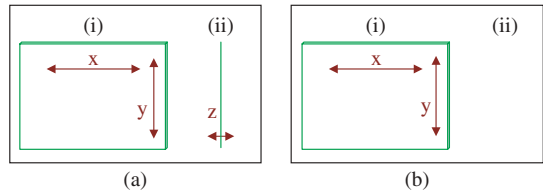


Figure 3.3 Diagram to illustrate the distinction between functional (a) and absolute 2D (b) in terms of *x*, *y* and *z* plane dimension. In absolute terms, 2D has absolutely *no* *z* but, practically, it can be just 'insignificant' – **but insignificant relative to what**?

plane (i) but with no thickness at all (ii). Although it is simple to draw (b) and to talk about it, it is quite rare to experience it (outside of theoretical physics). Indeed, it will not exist in our practical world. The point of this illustration is that it highlights the heavy burden which we must accept (in claiming '2D') to fully define and explain why z is not significant *in the system we are working with.*

Perhaps most telling – as we shall see – is the question, 'at what stage, then, does a stack of 2D layers become in reality 3D?

3.3 Is 3D, for cells, more than a stack of 2Ds?

The idea that cells have insignificant thickness (even when seriously flattened onto a plastic culture dish) would definitely not be a happy position for even the most traditional cell biologist. They quite clearly have significant aspect ratios (length to thickness) – normally many thousands of nanometres or hundreds of molecular diameters. In addition, there are entire texts and journals dedicated to the study of trans-cellular transport of drugs and proteins across (the thickness of) single cell layers which line our organs.

It is equally well established that some of the most essential (i.e. significant) cell functions take place at scales far below that of the cell itself. These are dependent on complex membrane structures, surface and membrane receptor proteins, enzyme systems and critical molecular-scale control systems, all operating in the nano-scale – orders of

magnitude smaller than the thickness of cells themselves. No, the real source of the paradox here is not that cell scientists are unaware of the *normal* meaning of 2D/3D. Rather, they have (almost unconsciously) invented *another* meaning which only has cellular significance. This now lives in a parallel cytological universe, as convenient shorthand. In fact, it more correctly relates (like the sharks and skates) to the stark difference between life in symmetric and asymmetric spaces.

'2D' is in fact being used in place of the term 'monolayer' (Figure 3.4), in a way which damages the concepts of our students. In this 2D world, cells are attached to a solid surface, though only on one surface, normally the basal or 'dorsal' surface (Figure 3.4). Cells in 3D have other 'things' all around (to which they may or may not attach).

This concept of '2D' is made more remarkable (paradoxical?), not by the fact that cells in monolayer culture *do* in reality have material on their non-attached (dorsal) surface, but that it is a clear fluid (i.e. different phase, with very different mechanical properties; more of this later). Neither is it that this form of cell growth (with all solid attachment on *only* one surface) is typical of most cells in their natural life. In fact, only a small proportion of specialist metazoan cell types are adapted to living at fluid interfaces.

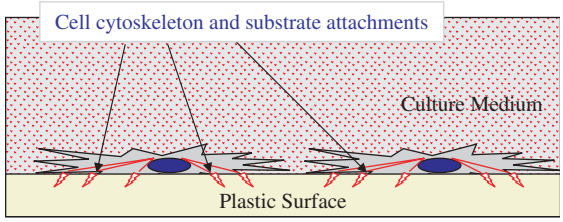


Figure 3.4 Although it is common to *call* cells grown in monolayer '2D cultures', the reality could not be more misplaced. The cell thickness (z-plane dimension) cannot be regarded as negligible in absolute terms, being around 5–10 per cent of the total cell width and more than 1,000 times larger than the thickness of essential components which hold the cells onto the plastic, such as integrin cell-substrate receptors or tethering rods of the actin cell skeleton (arrowed below). Paradoxically, though, cells grown in monolayer on planar surfaces (especially epithelial cells adapted to fluid surfaces) do have unambiguous cues available about their 3D space. These include both the presence (down) and absence (up) of attachment sites and the mechanical properties (e.g. compliance) of the two surfaces.

The real paradox is that these are the cells in nature which have the *least* problem in obtaining simple, accurate spatial and mechanical data about their 3D environment. Interface cells, in fact, live in a crystal-clear 3D world where the difference between 'up', 'down' and side-to-side could not be clearer or easier to monitor. It is reasonable to assume that the bulk of the animal cell universe is adapted in the struggle of detecting and interpreting far more complex and ambiguous 3D clues which hint at the changing nature of their native space. We can only guess, then, how such cells might be 'blinded' and their 3D responses completely modified when dropped onto a fluid-plastic interface, where information is stark and glaring, in the same way we imagine that bats must struggle with light-information overload when they are in full daylight (Figure 3.5).

Epithelial cells characteristically 'line' many tissues, forming the interface with other tissues or external fluids. These include the gut, bronchial and corneal epithelia, urothelial linings (bladder, urethra, etc.) and the many varieties of vascular endothelial cells. These cell types are adapted to a general positional existence where (in nature) they grip tightly to some form of (fairly) solid underlayer and to all of their adjacent neighbouring cells

Figure 3.5 Bats and daylight: A well-known example of sensory confusion – deprivation by overload. Eric Isselée/Shutterstock.com.

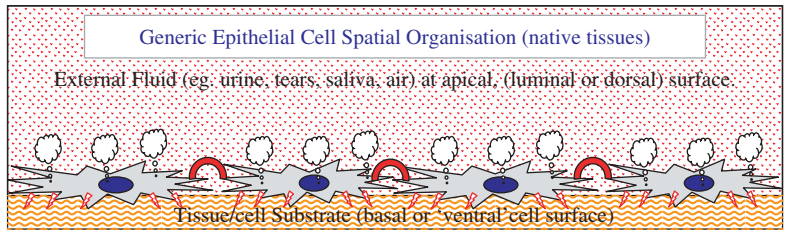


Figure 3.6 Epithelial cells (including endothelial cells lining the vascular system) are adapted to life at very special 3D locations, on surfaces and interfaces. The general pattern of 3D arrangement of these cells is remarkably consistent. They attach down to the underlying solid substrate (lower cells or extracellular matrix) by receptors (integrins) which pass through the cell membrane. At the same time such cells attach laterally to their closest neighbours within the sheet, via different receptors (frequently cadherins). In this way, they form fluid-tight sheet coverings. Again, contrasting with the integrin receptors over their basal surfaces, these cells express and segregate yet other forms of receptor on their outer or luminal surfaces (normally adapted to recognising key, soluble components in the overlying fluid). These cells define the term '*direct* environmental contact' with a fiercely bipolar adaptation, leaving little scope for confusion between up and down.

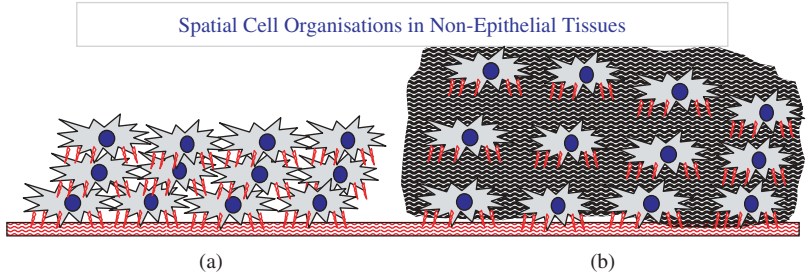


Figure 3.7 Non-epithelial cells, particularly cells of the matrix stroma, are characteristic of the bulk 3D mass of tissues. Some tissues or stages may grow to form dense cellular masses (a), where the emphasis is on cell-cell contact. Such configurations would contain little extracellular matrix material. However, where such cells are matrix-producing, connective tissue (stromal) cells such as fibroblasts, chondrocytes or bone cells, the organisation in (a) quickly gives rise to that in (b), as each cell produces more extracellular matrix material around itself, like a 3D coat. Since these matrix coats are effectively trapped in space, the neighbouring cells are progressively pushed further and further apart. This produces the typical stromal cell (matrix-rich) 3D organisation, characterized by variable cell densities within and attached to the hydrated (gel-like) extracellular matrix, whose density is inversely proportionate to that of the cells.

(Figure 3.6). At the same time, they must expose their unattached (upper or dorsal surface) to liquid or air, with all that this brings with it in terms of fluid shear forces, random passing contacts, unstable gradients and, most of all, asymmetric mechanics.

Most other cell types (e.g. those living within extracellular matrices or stroma – stromal cells) do not have this natural extreme of polarized attachment in their native tissues. They are variably connected to cells or extra-cellular matrix components of many forms, all with distinct mechanical properties (Figure 3.7). This is particularly true in the connective tissues. Not only can the nature (strength/stiffness), spatial pattern and number (density) of these attachments be unpredictable, but they inevitably change with time as the cells and matrix move or are remodelled/reshaped. These, then, are the starkly contrasting lifestyles of epithelial and non-epithelial cells in nature.

The answer to our question (is 3D for cells more than a stack of 2Ds ?) now becomes a little easier to predict, accurately, through the use of biological and time caveats. Obviously, in absolute terms, if we make a stack of a sufficient number of sheets which are 'effectively' 2D sheets (i.e. thickness is functionally minimal), that stack will eventually become functionally 3D. But here we come to glimpse the flaw in the question that is so informative. In fact, it

never *was* the simple 'thickness' which distinguished the cell biology term '2D'. It was the asymmetry of being at an interface. Consequently, the answer to our question is 'No'. As far as cells are concerned, a stack of layers still provides a cell-space which remains a *stack of interfaces*, no matter how many there are. Functional thickness was never the issue anyway, as illustrated by 'epithelial stratification'.

However, there is still that 'time-caveat'. While a stack of layers does represent a series of parallel, '2D' interfaces at set-up time (time zero), the resident cells will remodel that structure during culture or after implantation. One outcome of remodelling can be new physical attachments formed between the adjacent layers, so producing a more symmetrical 3D bulk structure.

When, where and how fast this transition occurs will depend on the resident cells in question. Epithelial cells would initially see this as a familiar series of adjacent interfaces, but non-epithelial cells will routinely attach to both available surfaces between the layers, so degrading the asymmetry. The 'time caveat', then, as so often in tissue engineering, becomes part of the process. Indeed, 'time' is where biological (cell-based) activities of our tissue engineering will have their strongest role (see Chapter 9). Cellular activities can generate huge functional diversity (i.e. the tissue detail), with all that this implies for the versatility of our clinical applications. But it needs time.

3.4 On, in and between tissues: what is it like to be a cell?

It can be intellectually risky to 'humanize' your cells (i.e. imagine that they have complex sentient attributes). It is classic to hear, even at major conferences, how cells in this culture or that system are 'happy' or 'looking' for receptors. However, it can often be very helpful, for example in understanding the basics of environmental sampling and data handling at the cell level, to think of ourselves as cells. The trick here is to imagine what basic information they would need in any particular situation.

The first cell type example, epithelial/endothelial cells, live in a thin layer (Figure 3.6). Information on the properties of their narrow 3D space (at most a few cells thick) comes to them directly in a glaringly unambiguous manner. This might be like sampling the kaleidoscope of changing sights and sounds from the sun-deck of a cruise liner. You can tell pretty well immediately which way is up, down, left and right, which way the wind-shear and sun-heat are coming from. In fact, spatial information can be monitored *directly* on a minute-by-minute time base.

At the other extreme, stromal cells have an embedded existence, deep in their surrounding extracellular matrix (Figure 3.7). They might be imagined as living deep in the midst of a 'muted cacophony' of complex mechanical hints about their spatial location. The effects of external loads (e.g. strain magnitudes and vectors) are altered, deflected and reduced as they pass through surrounding materials (the ship's hull). These surrounding, modifying materials comprise neighbouring cells and the visco-elastic extracellular matrix in which they are embedded – their extracellular matrix.

If we use the human analogy of monitoring your 3D environment from the sounds you hear, it is almost as if the stromal cells have adapted to detect the groans or creaks that would come to you deep within the hull of an old steamship. They respond to such noises and vibrations, based on what they imply is happening to the ship and their micro-environment within the ship (e.g. a storm, docking, collisions, etc.). Unfortunately, such signals would inevitably come with complex, confusing echoes and harmonics – a far cry from the clear bangs, hoots and whistles available to the surface epithelial cells living on the metaphorical top-deck.

It is possible to get an impression of this from the diagrams within Figure 3.7. Figure 3.7a illustrates a cell-rich mass (typical of growing, repairing tissues, contractile, glandular and neural tissues), with little matrix and mainly cell-cell connections. Environmental physical cues, in this instance, are extremely variable and presumably among the most difficult to interpret usefully. The mechanical properties of surrounding cells are inherently soft (or compliant).

However, there will be additional and conflicting information streams from the high and low stiffness layers, which are in other deeper planes (e.g. tough extracellular matrix sheets, bone or natural inter-tissue gliding surfaces). Detection of these planes seems to depend on movements of the cell against its surroundings using the stiff rods and contractile filaments of its cytoskeleton. Furthermore, since the cytoskeleton can and *does* change rapidly in the surrounding cells, these stiffness signals will also vary rather unpredictably in direction and time.

Again, some cell-types and cell-stages are adapted to relatively active, constant motion – for example, many stromal cells and cells within a repair site. Others, such as mature epithelial cells and stromal cells deep within healthy, adult connective tissues, are relatively sloth-like, tending to stay in one place – and for all we know, they just contemplate their happiness-factor (see above).

In many cell-types, this very motion generates its own localized mechanical feedback signals by applying tensile forces which pull and distort both the neighbouring cells and surrounding matrix, independent of external loads. These contractions are generated by the inherent cell cytoskeletal motor activity, based on actin-myosin fibres within the cell cytoplasm. To continue our humanized analogy, we might imagine the problem of gathering spatial information again from deep in the steamer's hull, but now surrounded by a cargo of assorted farm livestock. This situation can be understood a little better by studying the scale and structure of life as a cow in the old type cattle-transport ship (Figure 3.8a). In this case, cattle squeezed together below decks would pick up clues about sudden jolts as the ship was docking, or about the appearance of storms, or even the direction of large waves. However, they would be vague and confusing clues, affected by the size/position of the adjacent cows on each side and whether one or more of these neighbours started moving themselves.

However, in the case of stromal cells, this position of being packed in between lots of other squishy, moving cells (or cows) changes as they synthesize and deposit extracellular matrix proteins. This matrix provides the resident cells with a relatively

(a)

(b)

Figure 3.8 (a) Historic picture of cattle being transported by ship; loading them deep into the hold. (b) A coastal freighter loading with timber. Small animals living between the wood during the voyage will have a simpler time interpreting what is happening when the ship rolls or turns than they would if they were all packed together like the cattle in Figure 3.8a. Photo in (b) © Atlas Marine Services.

stiff, predictable support material to which they can attach, and so gather more reliable spatial information (Figure 3.8b).

This might occur, for example, during scar formation after a skin/dermal wound. At this point, the monitoring situation of resident cells will improve dramatically. This embedding material is mechanically *far* more stable than animated, moving cell bodies, both in terms of its lack of change over time and in their overall material stiffness. For any given, fixed volume of tissue, there is an inescapable inverse correlation between this increasing accumulation of extracellular matrix material and the overall cell density (expressed as cells/mm^3 of tissue). In other words, more matrix = less cells. As a result, the progression from cell-rich to matrix-rich composition

brings with it both greater biological (improved space monitoring) and mechanical stability, in proportion to the reduction in cell density. Importantly, that cell-rich : matrix-rich progression is almost inevitable in connective tissue growth and repair, and also during connective tissue engineering. In our shipping analogy it is as if the steamer trades some of its cattle for timber at each port of call, progressively improving and simplifying the collection and interpretation of spatial information as it goes.

A key factor here, then, is that our considerable understanding of the different general cell types (e.g. **epithelial** cell sheets and extracellular matrix-rich **stroma**) suggests that they are adapted to living in (and gathering information from) their very different natural locations. Therefore, just as we are comfortable with the idea that the sensitive hearing of whales may be disorientated by unnatural and random shipping sonar (Figure 3.9a), and night-flying moths are confused by electric lights, it should not be surprising that the monitoring systems of cells adapted to complex, low intensity information can be overwhelmed by excess spatial information.

To extend the previous shark analogy, bottom dwelling flatfish such as rays or plaice (Figure 3.9b) are poor at comprehending or processing visual cues, particularly those related to perspective and distance, such as complex 3D motion. This is not a little affected by the location and structure of their eyes. After all, their needs are adapted to utilize a spatially distinct sea-earth interface. They make short movements – quick bursts of shimmying,

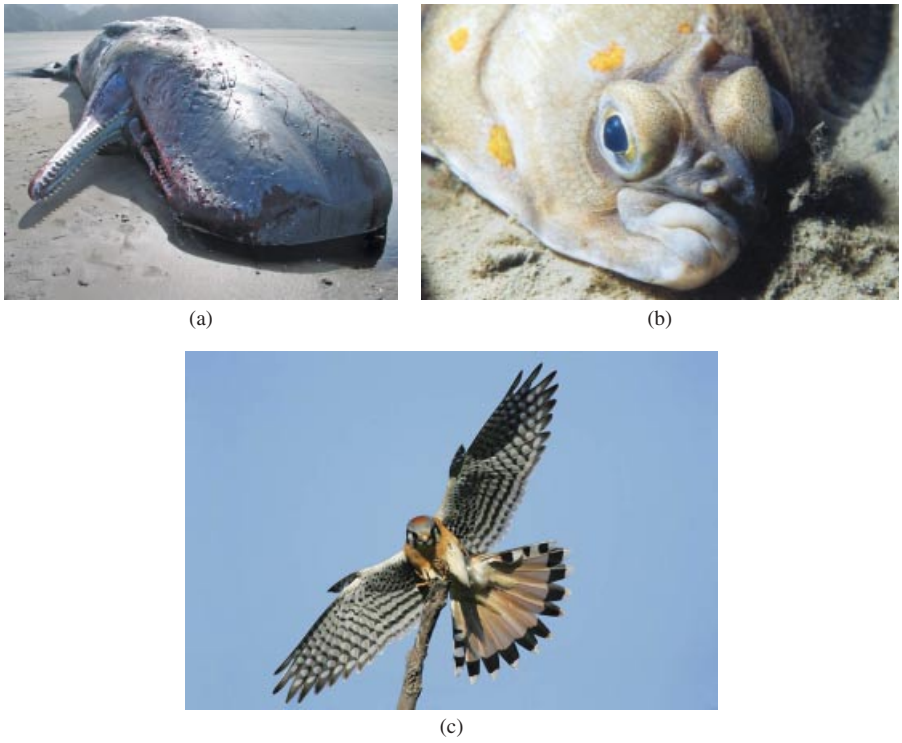


Figure 3.9 (a) Whales may sometimes become spatially confused and beached due to the effects of additional man-made sonar information. (b) Flatfish have evolved to live *on their sides* on the sea-bottom. One of their eyes has migrated round the head so as to be on the 'top-side' and the jaw has swivelled – to look ugly. Now that is spatial adaptation to life at an interface! (c) A bird of prey coping with life in the bulk 3D. In this case, it is rapidly computing subtle movements of its landing branch, while falling forward and down against a gusting swirling air-flow; now that is complex spatial adaptation. Credits: (a) © iStockphoto.com/Alan Drummond; (b) Photo by Tim Nicholson (timnicholson@manx.net); (c) Reproduced with permission © Russ Kerr.

inconspicuous movement as close as possible to the water-sand interface.

By contrast, hawks are clearly adapted to move through and sense a spatial environment where almost everything in its surroundings moves constantly and independently (Figure 3.9c). Such bulk media-adapted animals gather and use the information they need as it becomes available in their 3D space. By analogy, cells which live *within* a relatively homogeneous bulk material must have similarly adapted systems for gathering the spatial information they need, e.g. to distinguish between stiff, impenetrable areas and crevices where movement is possible. This adaptation is likely to have a profound influence on how they interpret the 3D environment in which they operate.

Unlike the cases of hawks and flatfish, though, it is now common for many cell types to find themselves deep within spaces made by tissue engineers, where their monitoring systems are rather poorly adapted. As with any biological adaptation, any advantages the cells would normally have can become a very mixed blessing if the environment changes. However, despite obviously being unnatural, our tissue engineering culture systems can be sufficiently supportive to eliminate the most damaging aspects of the mismatches. In other words, we can say that our culture conditions 'support' cell survival/division. It is far from clear, though, that they are based on any substantive analysis of how the cells are adapted to monitor and use the 3D space.

This does not mean that our tissue engineering systems should try to perfectly mimic these adaptations – far from it. It does mean as with any good engineering design, that it is critical for us to understand the demands of that space-sensing adaptation and *match* this to the functions we hope to produce from this or that combination of cells.

The conclusion, here, is that epithelial cell types grown in monolayer culture may be as near to biomimetic heaven as it can get; comfortably monitoring and remodelling their 3D space, *next to the surface*. By epithelial standards, this monolayer is actually a good 3D culture! Similarly, if and where we can grow stromal cells, such as skin fibroblasts, deep within an appropriate, dense material, they will also find this to be a biomimetic 3D system.

However, each cell type will find the alternative environment variably 'confusing' (here we go again with that anthropomorphism) in terms of spatial and mechanical signalling. Under these circumstances, it would not be unreasonable for such cells to show extremes of behaviour, such as escape (migration), proliferation or even death (apoptosis being a form of programmed cell-suicide). These tend to be exactly the sort of undesirable, disappointing responses so often reported for tissue engineering systems. The epithelial/stromal cell example used here is simply the most clearly understood, because of the stark differences in their 3D space, but it seems certain that this will be true to some extent of any 3D engineered cell system.

It is, then, increasingly the task of serious tissue engineers to understand the nature of the cells we intend to use and the space in which they are being expected to work for us. In fact, this has been the hallmark of excellence in traditional engineering by humans for at least two centuries. So, when next you fly, do ponder how well Airbus or Boeing have analysed and engineered their systems. After all, *our* earth-adaptations do not react well to the temperatures, wind pressures and oxygen levels found when travelling near to the speed of sound on the edge of space. Yet we, as passengers, happily pull out our laptops to concentrate on that last minute report or exam revision without a thought for the proximity of the jet-stream. The air industry has engineered our environment.

To return to our original analysis, then, it is simply no longer reasonable to consider monolayer culture or culture in solid (3D) scaffolds as '2D or 3D'. It is even less relevant to label them in any given system as 'less or more physiological'. They really are neither of these. They are either appropriate, or not, to the cells under culture and the task and the bio-mimesis that we have set as our target. After all, why else would it be called tissue *engineering*?

As we shall see in the next section, this simple concept is both seriously liberating and hugely illuminating.

3.5 Different forms of cell-space: 2D, 3D, pseudo-3D and 4D cell culture

3.5.1 What has '3D' ever done for me?

Although the analysis so far might sound like harmless eccentricity, it begins to reveal why the language we use around '2D-3D' (monolayer-multi-layer or surface and embedded cultures) is at best unhelpful shorthand, and at worst the source of confusion between cell biologists and their students and collaborators in other disciplines. This, in general, is an ever-present problem in tissue engineering and regenerative medicine. Where ideas cut across disciplines, concepts can have different meanings (notably between engineers and biologists).

Cells in monolayer ('2D') culture are conveniently anchored down to a flat surface or interface through their basal surfaces. Anchorage as a single sheet also has major implications for access to the culture fluid, for supply of nutrients, oxygen and control-proteins and egress of wastes and synthetic products:

(i) maximal (subject to surface fluid mixing); and
(ii) equally accessible (i.e. same concentration) to all cells in the culture (more of this later).

Over the decades, the monolayer configuration has been used mostly to understand and test the many, many effects of soluble or exchangeable molecular agents as they pass into or out from the free, unattached upper surface of a cell sheet. Cell anchorage is convenient for cell handling and is frequently essential for division of the cells of interest. The directional attachment involves the bonding of cell membrane receptors (in this case mainly integrins) which come into contact with the culture surface. This integrin-binding more correctly links the cell membrane to other proteins which are attached, mainly by charge attraction, to the plastic culture surface. In other words, integrin receptors bind indirectly, through structural proteins – *not* directly to the plastic.

Direct cell-plastic attachment seems to be minimal in conventional cell culture. These structural matrix attachment proteins commonly derive from the serum in culture medium and, after a time, they are produced by the cells themselves. More recently, the culture surfaces have been pre-coated with such proteins (e.g. fibronectin, laminin, collagens) to avoid the lottery of how and when attachment progresses. This basal-surface-only attachment, then, is the source of perhaps the cell's most unambiguous spatial signal for 'up and down'.

Where cells attach to each other (either side to side or in forming multi-layers), this often uses another type of membrane attachment receptor, most often the cadherin receptor family. Occupation of both integrin and cadherin receptors (by matrix protein sites or cells, respectively) generates complex intracellular signalling cascades which may provide data to the cell – not only that it *is* attached, but also on the density of attachment sites, their cell surface location and substrate mechanical strength. Clearly, and by definition, cells in a monolayer will use their basal-surface, integrin-mediated attachments for simple anchorage.

At low densities, cells will have less ability or opportunity to form cell-cell (side to side) cadherin-based attachments, so basal (integrin) receptors will be the main source of signalling. As cells divide or migrate to form clusters on the flat culture surface, cell-cell contacts will become more and more common as a statistical inevitability, so changing the incoming receptor signalling data to participating cells. This shifts from being predominantly single (basal) surface, relatively stiff (protein-plastic) and integrin-mediated, to an increasing proportion of other surface (lateral), less stiff (another cell surface) and cadherin-mediated binding sites.

The fact that this shift represents an increasing ratio, or shift in *proportion*, of receptor signals makes this an inherently powerful, graded form of monitoring mechanism (Figure 3.10). As long as the cell is able to 'count' in some way, this represents a data stream indicating, with some precision, where and (in general) to what it is attached. In fact, the most plausible cell equivalent to 'counting' is likely to be their ability to react differently to different or

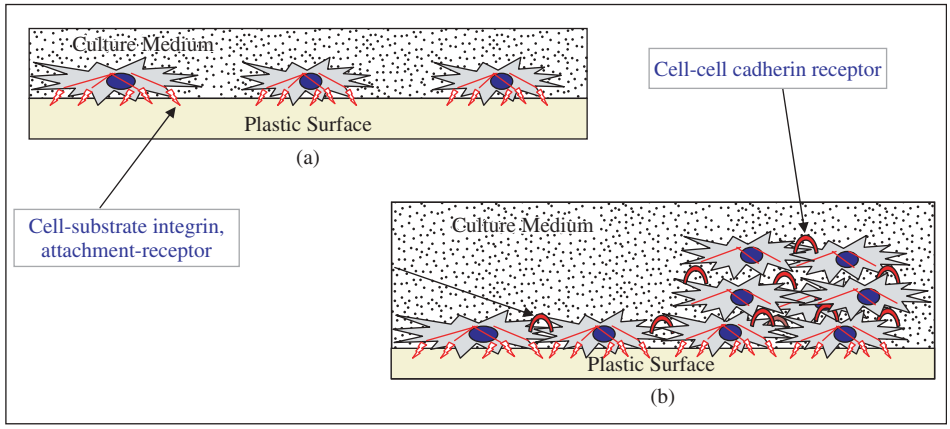


Figure 3.10 Cells have membrane attachment receptors (mainly integrins and cadherins) which pass through the cell membrane to attach, on the exterior, to either the matrix/substrate (for integrins) or adjacent cells (for cadherins). (a) At sparse cell densities cell-matrix, integrin-substrate binding predominates. (b) As cells divide or clump to higher densities cell-cell, cadherin interactions will increase in proportion and distribution. Receptor binding produces both physical links from *external* surfaces to the *internal* cell skeleton (cytoskeleton) and triggers internal cell-signalling pathways. The first of these is 'in-to-out' mechanical signalling. The second is biochemical signalling. Where clustering forms cell multi-layers ((b), right hand side), integrin attachment may be lost completely, leaving only cell-cell cadherin binding on all surfaces for some cells in the cluster (i.e. loss of direction signalling).

changing levels of chemical metabolites, generated either by receptor occupancy or clustering – i.e. as biochemical concentrations. Cells are, after all, first and foremost, sophisticated biochemical-mechano processing units.

As a result, we can glimpse possible mechanisms by which cells detect (and so react to):

(i) basal surface attachment to a planar surface, i.e. anchorage-dependent or substrate-mediated responses;
(ii) lateral edge, cell-to-cell attachments, i.e. confluence or density-dependent responses.

These responses have clear equivalents in native tissues and for certain cell-types.

The involvement of different *proportions* of receptor types (cadherin or integrin-attachment), along with distinct mechanical properties of these attachments, can be the source of information likely to push cells down one of a number of programmed responses (Figure 3.10). These responses are 'programmed' by the particular lineage and stage of differentiation (or adaptation) in which the cell finds itself when it receives the signal pattern. This cell state is, in turn, a function of its pattern of gene expression at that point in time.

We have many examples where even subtle changes to the mechanical properties of these attachments lead to dramatic shifts in both gene and protein expression by cells. This brings about visible changes in cell behaviour and so leads to the conclusion that such signalling will, itself, cause changes in cell differentiation or adaptation state. In this sequence, then, we can glimpse from the cell perspective how incoming spatial information can shift or deflect how that cell behaves. However, for any set of spatial properties, the cell reaction will be heavily dependent on cell type or stage, in just the same way that people from different walks of life will hear different messages in the same speech.

3.5.2 Introducing extracellular matrix

Let us, then, be more critical about why it is useful anyway to culture cells deep within a porous support material (i.e. '3D'), as opposed to asymmetrically on a flat surface ('2D'). The aim here is to avoid the rather weak justification (see the case above) that '3D' is 'more physiological' than '2D'.

How, then, do the cells 'see' this situation? Two key factors inevitably assume much greater, even front stage, importance as cells find themselves living deeper and deeper within a material. Paradoxically, both tend to take the form of lost or diminished signals. As we have seen, the first is a profound change of spatial mechanical signals to the cell. This is chiefly a loss of asymmetric attachment, or at least loss of major directional differences in attachment stiffness at different parts of the cell.

Cells shown diagrammatically in Figure 3.11a in a matrix-rich culture scaffold are surrounded by non-living material. This extracellular material (be it natural connective tissue matrix or synthetic polymer scaffold) will have stiff or compliant mechanical properties, which will dominate the cell space similarly in all planes. This means that, as cells develop their **internal forces** and pull on their support material, it will resist those forces from all directions, in much the same way that water resists and moves similarly on all sides of the tail of a shark swimming through its symmetrical bulk environment. Exceptions to this relative uniformity will develop at the edges of the matrix material, where cells once again meet other 'surface' materials.

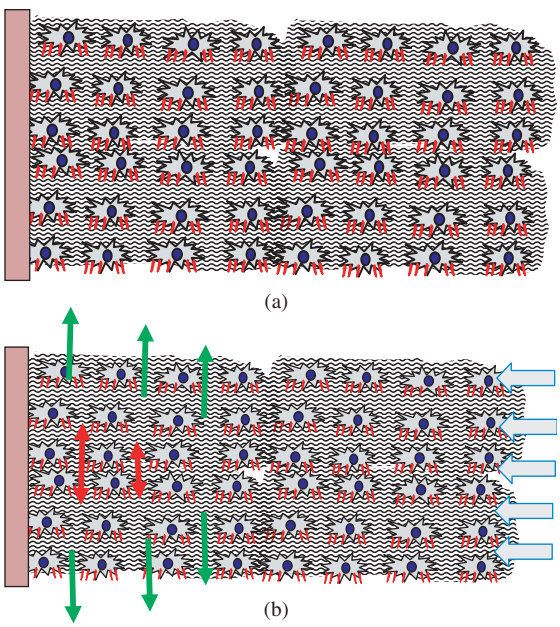


Figure 3.11 (a) Cells seeded and living throughout a material. Note: their environment is already made asymmetric by the stiff support on the left. (b) Asymmetric external loads on cell-seeded materials generate complex strains. Note: even apparently *symmetric* loads (e.g. the tension shown in the green arrows) act asymmetrical because of the asymmetric material with its stiff left-hand edge.

External forces, applied to the support matrix, commonly act as indicated in Figure 3.11b. The blue arrows are acting on the left hand (softer) surface of the material-and-cells construct to compress against the rigid support to its right. In this example, cells are compressed by the applied load (from the left), but there is an equal and opposite reaction from the fixed plate (right). Since the remaining faces – top and bottom – are unrestrained, this uniaxial compression will generate internal *tensile* forces acting at right angles (up and down, indicated by green arrows) as the soft construct bulges up and down.

Furthermore, where the cell-support material bulges and deforms (i.e. is strained (green arrows)), this generates shear forces around core cells (red arrows), tending to split between 'shear planes' (Figure 3.11b). It is clear, then that apparently simple external loads will affect many levels of cells in an apparently complex manner. The details of force/strain magnitude and direction (vectors) will vary dramatically, depending on exactly where in the support material each individual cell lies. This is totally different to that for cell monolayers.

We shall return to the main caveats to this model (Figure 3.11b) later – namely, its implication, from the cell perspective, that the support matrix is equally stiff/compliant in all planes and all regions. Happily, engineers and materials scientists can predict/quantify at least some of these changes, in detail, providing we can tackle the caveats.

However, this does illustrate (if we needed it) just how much more complex and *dynamic* (therefore harder to interpret) mechanical signals can be to cells-in-a-support-material than they seem to us when we 'just apply a compression'. The take-home message from this illustration should be, 'the support-material-matrix rules, OK!' If the reader has is any lingering uncertainty as to the *huge* increase in complexity, perhaps I should point out that you

have deliberately been given *only* a 2D diagram. Imagine what happens when the system above is extended down into the page.

3.5.3 Diffusion and mass transport

The second critical difference felt by cells growing deep within a material is a profound change in the manner (rates) by which they can collect or get rid of nutrients, waste substances/metabolites, molecular messages and export products. Such molecular movement is also known as mass transport to and from the cells, and it is critical to cell survival and function. Clearly, even where mass transport to deep cells is rapid, there is again a loss of polarity or 'direction', relative to monolayer culture where, in one plane, these distances are negligible.

It is equally clear that the rates of mass transport will be significantly reduced by the presence of natural or artificial matrices or the presence of other cells. From the cell's standpoint, then, we can predict a loss of the rapid and highly directional molecular exchange which is inherent in surface monolayer cultures. Embedded in support materials, this will give way to an increased number and complexity (i.e. direction) of nutrient/waste *gradients*. The extent of depletion/excess of any particular molecule and the gradient direction will depend on cell position and density relative to the support material and neighbouring cells.

Before we concern ourselves with the damage benefits of these gradients on cells (a bio-tendency exaggerated by the habit of humanizing cells), we should first remind ourselves that they will primarily result in new, more complex signals. Interestingly, but far less well studied, there will be a parallel but inverse effect on the export of macromolecules, secreted by deep cells, which will be slowed or blocked by their surrounding matrix. This effect will be governed by the size of the cell products and diffusion properties (e.g. average pore diameter) of the surrounding matrix.

The factors affecting mass transport in model materials are well understood from basic physics and engineering. Key factors (Figure 3.12) are the diffusion path-length, diffusion properties of the matrix

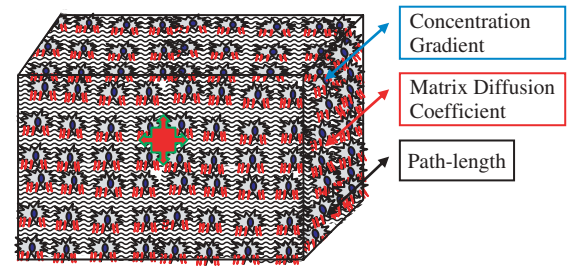


Figure 3.12 Illustration of idealized 3D tissue culture construct at t_0 indicating three of the key physical factors controlling mass transport, which will change substantially with time.

along that path and the concentration gradients of the molecules in question from one end of the path to the other (the diffusion gradient). Unfortunately, the caveat here – '*in model materials*'–dominates. We immediately introduce complexity to the basic model by seeding the materials with significant numbers of cells, distorting both diffusion gradients and properties of the material.

While the effects of cell seeding can be incorporated into defined culture systems *at time-zero* (t_0), each cell is a dynamic bio-factory. This means that the starting conditions will break down – and sooner rather than later for active systems. This key factor is often not incorporated into TE strategies. The very fact that our culture systems are designed to generate new tissue material structures makes it *inevitable* that t_0 'design' conditions for mass transport will alter. The only point in question is how fast we reach a point where we no longer understand, or even superficially control, the 3D culture. In fact, the very properties of bio-mimetic tissue structure that we aim to generate though cell action will change initial mass transport and cell activity. Consequently, many 3D culture systems tend to go out of operator control relatively rapidly and move towards cell 'autopilot'. Increasing the culture period, probably over just a few days for many cell systems, is likely to take this far from its design objectives.

This effect, then, helps to explain the strong tendency in this field for serendipitous advance as opposed to design prediction, i.e. conventional engineering. More importantly, it also makes the control

of batch variation and industrial or production scale-up into major headaches (see Chapter 9). In such circumstances, poor reproducibility can only be tackled by the strictest possible technical rigour. In short, without complex, inbuilt adaptations over time, '3D' culture TE systems have an inherent tendency to go out of control – paradoxically, as a consequence of their own success.

The challenges which spring from this analysis are illustrated in Figure 3.12, representing a homogeneous material construct, seeded evenly with a homogenous cell population (a t_0 ideal in itself). Mass transport of nutrients and export proteins will rapidly produce diffusion gradients across the thickness of such constructs, proportional to (i) average cell activity/density and (ii) matrix density/diffusion coefficient. Both of these factors will, by definition, change over time, so altering the 3D cell space and feeding into the spiral of change.

Over culture periods of days (and especially of weeks), the very aim of the culture process requires that cells will:

(i) divide or die (altering cell density/distribution);
(ii) deposit or remove (i.e. remodel) the extracellular matrix differently in different directions to give anisotropic material properties (different in different planes) as they are in nature;
(iii) adapt (or differentiate?) to conditions, e.g. altering metabolic activity or behaviour patterns;
(iv) migrate within the matrix or artificial material.

All of these cell responses are *exactly* the events which are commonly assumed will occur in a culture (e.g. a 3D tissue bioreactor) as the target tissues such as skin, tendon or blood vessel begin to grow. But equally, all four will profoundly affect path-length, diffusion characteristics and key concentration gradients, not to mention matrix mechanical signals.

Perhaps the most basic law of system-design states that it is essential that your process can cope with the consequences of its own intended success. Consequently, great care is needed in setting the criteria for 3D culture success.

To sum up, then, from this analysis we can reliably predict that there will be a time-dependent increase in complexity in the resident cell 3D environment. The current challenge is that the nature of this complexity and the speed at which it forms leaves the tissue engineer unable to control the system. Next-generation tissue bioreactor science will need to wrest a greater degree of control back from the cells themselves, which is only likely with our current level of understanding, by detailed real-time monitoring of the properties of the cell space over the culture period. This will allow dynamic feedback-control to be fed into the 3D culture conditions.

This analysis indicates that long term culture systems will prove disproportionately problematic (even aside from economic constraints). As a result, it is clear that minimal culture duration needs to be a first-order design target, far higher than it is at present. Also, to minimize the rate of change of complexity and so simplify the monitoring, it is important to avoid cells at a 'volatile' stage of their development or adaptation (i.e. prone to undergo rapid shifts in behaviour). Such cell types will generate rapid changes in 3D space, amplifying system uncertainty. This suggests, for example, that stem or progenitor cells are best used in 3D cultures only after pre-processing to a more stable, committed state. Good practice would aim to expand and differentiate cells *prior* to seeding them into a bio-mimetic 3D space. In effect, this is the logic of segregating critical process stages to avoid compromise conditions.

3.5.4 Oxygen mass transport and gradients in 3D engineered tissues: scaling Mount Doom

Ask any cell biologist (non-3D specialist) what they think is the big deal (and big threat) about having cells in 3D and the answer is likely to come in two parts. Firstly, with positive enthusiasm, "... it's so much more '*physiological*'!" – but then, with an increasingly clouded face, they check themselves with the assertion, "... but all the cells in the middle will die". Put the same question to an engineer and (s)he will want to know the path length, diffusion coefficients and cell density/consumption rates.

In cell biology, there is a deeply engrained dogma (which seems not to need qualification or calculation) that cells in 3D, deeper than a few layers (or variously 200–500 µm) deep to an O_2 source, are *doomed*! We can trace its origins back to the early days of angiogenesis research, and tumour cell proximity to the nearest blood vessels.

This figure and concept of 3D cell life may well be realistic for tumours, liver, muscle and kidney, where cells in 3D are packed together into a cell-rich mass (see next section). However, it is certainly worth revisiting how valid this view is where cells are living in a 3D fibre-and-water-rich mesh – that is, your average connective tissue. In such matrix-rich tissues, the dense 3D mass of cells is dispersed throughout large volumes of watery material which is highly permeable to oxygen and other small nutrient molecules. Incidentally, this difference is exaggerated, since these cells (often fibroblast types) tend to be slow consumers and often have low cell activities relative to those of cell-rich tissues. All of this adds up to a completely different calculation and makes 3D living, in matrix-rich structures, a completely different proposition – and definitely *not* fingernail-dangling over the edge of Mount Doom!

Figure 3.13 shows what happens if we actually measure the real levels of oxygen at the core of a dense, collagen-engineered tissue filled with cells. In other words, what do we see where we monitor real oxygen transport across a living (if simple and defined) 'tissue'?

First, we find that even for relatively dense, tissue-like collagens (a gel of ≈12 per cent w/v in this example), *full* passive re-equilibration of

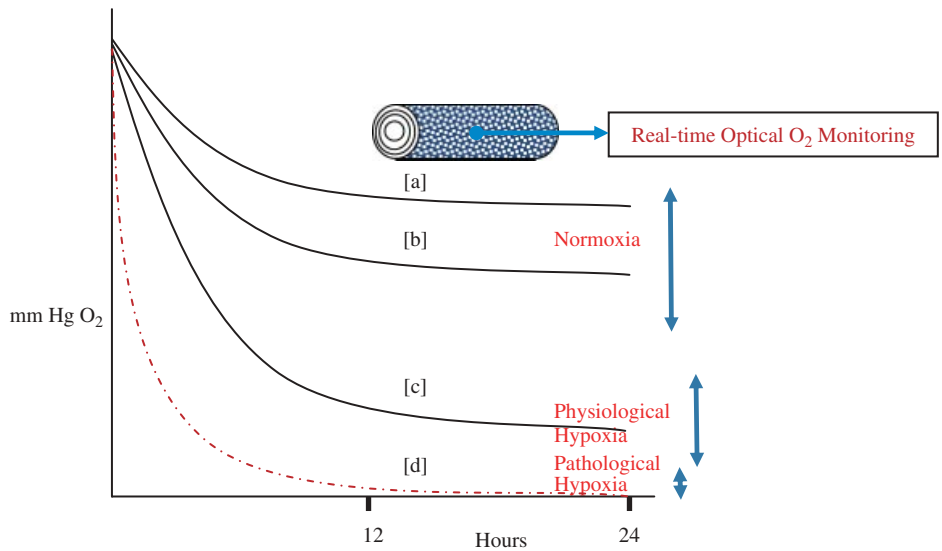


Figure 3.13 Real time plot of O_2 tension in the core of a (22 × 2.2 mm) rolled solid rod of dense, cell-seeded collagen (inset diagram, showing the indwelling fibre optic oxygen probe.) Lines (top to bottom) [a], [b] and [c] represent the fall-off of O_2 over time in culture with low, medium and high densities of dermal fibroblasts (respectively). Note: at the high cell density (≈20 million cells/ml of tissue), cells are densely packed and difficult to see through by microscopy. Line [d] represents the response for pulmonary smooth muscle cells, at the same density as in [c]. Even after 24 hours, the highest fibroblast density had only fallen to levels of physiological hypoxia (this is the same as we find in normal tissues). Only the smooth muscle cells reached pathological hypoxia. Adapted from: Cheema, U., Brown, R.A., Alp, B. & MacRobert, A.J. (2008). Spatially defined oxygen gradients and vascular endothelial growth factor expression in an engineered 3D cell model. *Cellular and Molecular Life Sciences* **65**, 177–186; and Cheema, U., Hadjipanayi, E., Tammi, N., Alp, B., Mudera, V. & Brown, R.A. (2009). Identification of key factors in deep O_2 cell perfusion for vascular tissue engineering. *International Journal of Artificial Organs* **32**, 318–328.

oxygen occurred across a 1.1 mm diffusion distance in only 20–30 minutes (in other words, if the core is depleted to zero O_2 and the surface (path-length = 1.1 mm) placed at 120 mm Hg O_2, (cell incubator levels), the core becomes fully oxygenated in 20–30 minutes). This means that the apparently 'tissue density' collagen material is not the diffusion barrier to O_2 that it is often made out to be.

The second surprise from Figure 3.13 is that the core cells do not die, at least in the short- to mid-term. There is no detectable excess core-fibroblast death (i.e. over and above that at the surface) after 24 hours culture, even at extremely high cell densities (plot [c]). Indeed, core cell death was only just around 20 per cent between days 3–7, at which stage cell division and other factors complicate the interpretation of cumulative cell viability. However, in taking a closer look at the data, we should perhaps be less surprised by this, as the minimum level reached for core oxygen (plot [c]) is well within normal tissue levels (paradoxically known as '*physiological hypoxia*')*.

* Oddly enough, we refer to the oxygen level in our cell culture incubators as 'normoxia' (selected because it encourages cells to divide rapidly). Tissues live very happily well below air levels of O_2 called 'physiological hypoxia', and only when it falls below 2% O_2 do we reach (Doom) 'pathological hypoxia' (not to be confused with hyperbaric therapies, using high **pressures**).

Some of this effect is down to the cell type, as fibroblasts seem to be good at holding their collective breaths. For example, smooth muscle cells are much more energetic, and they behave as shown in Figure 3.13 by plot [d]. At the same cell density, core O_2 drops (like a stone) straight down to pathological hypoxic levels. Also, more of these cells do die, though still only gradually over the following days in culture.

Incidentally, bone marrow stromal stem cells behave in much the same way as the smooth muscle cells, though again only when cell densities are high. All three cell types have plenty of time to steadily up-regulate their expression of angiogenic factors, as they would *in vivo*, to attract new vessels in growth (we discuss this – and stem cells – as a means to produce angiogenic depots in later chapters).

There is a very big 'however', here. It should be clear that we are no longer discussing the consequences of transport in a dense collagen material, acting as a barrier to oxygen diffusion (mass transport). Rather, we are balancing the consequences of O_2 *consumption* by other cells in the construct (i.e. each cell 'layer' between the surface and the core extracts what it can from what passes by). Even the great Colorado River was reduced to a steam at its outflow as a result of water withdrawal by thousands of farms and industries (e.g. Las Vegas) along its 2400 km track.

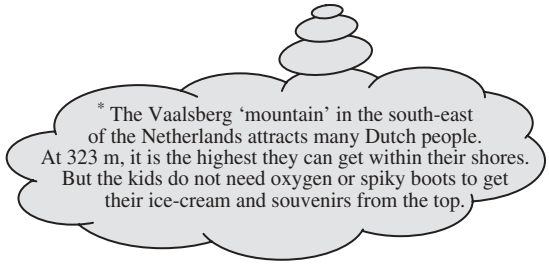
Now we can see why our great biological obsession with 'oxygen diffusion and 3D' can be a flawed overreaction. In native tissue matrices it is more likely to be a consumption, not a transport effect. Hence, cell density (cell-rich/matrix-rich) and cell type are critical. Neither is this a piece of academic hair-splitting. Put simply, if mass transport is generally as good as it is likely to get, then heroic efforts to improve it are going to make only a small difference to the 3D cell experience. On the other hand, applying our thinking to the **consumption** side of the equation can have a much greater impact. For example, what *are* all those cells needed for? Do they all have to work *so* hard, in such a dense mass?

So, are we getting unnecessarily worried about cells in 3D culture by fears of hypoxia and cell death? More than likely, the answer is *yes*, if we do not *measure* and *understand* the system we are using (system = material-density; path-lengths; cell type and density). This is made starkly obvious by the *final* part of the Figure 3.13 story. This oxygen transport-consumption example was measured in response to the 'obvious problem' that core fibroblasts were more than 1 mm from the surface, and so were '*sure to die*'.

As we can see, they did not die (and, in fact, they thrived for five weeks at the core!), because they did not consume enough oxygen to deplete below tissue levels. However, by that time a system had been developed for inserting many fine channels (of

blood capillary diameter) through the length of the collagen rod. Even though these contain only static culture fluid, not flowing blood, the core O_2 levels were pushed back up to those of normoxia. The 'non-problem' had been 'over-fixed'. In effect, the channels just minimize the number of consuming cells acting on any one region (the core) by providing a tube full of undepleted medium *with no cells in it*.

The take-home moral of this fable is that in biological tissues (and good mimics), it is cell *consumption* of oxygen and nutrients that we should consider (and even worry about) as the potential wolf of the 3D cell experience. Certainly, we are unwise to allocate 'bad-guy' status to imaginary diffusion 'barriers' until the transport characteristics of our 3D system have been *measured*. This is a major Extreme Tissue Engineering lesson because, figuratively, it saves us from either taking long detours out of our way or investing in serious breathing and climbing gear, when 'Mount Doom' is really just a 300 metre high family ramble trail*.



3.6 Matrix-rich, cell-rich and pseudo-3D cell cultures

In considering the theme of this chapter, the cell 'experience' in 3D culture, it is instructive to lift the lid on another key assumption which was embedded in the preceding discussion. This is the matter of biomaterials design and cell perfusion as they link to 3D space (see Chapter 5). A largely unintended consequence has emerged from the development of 3D cell support biomaterials/scaffolds with multi-micron diameter pores (i.e. greater than cell diameter).

The need for large pore scaffolds was a linchpin of early attempts to engineer tissues. It was based on the perceived need (a) for cells to be seeded deep into the material and (b) for 'rapid nutrient exchange' with the culture medium – i.e. mass transport). To maximize the direct contact of resident cells with culture medium, this class of scaffold materials was also designed with high interconnectivity between the pores. This structure means that the pores and cell-supporting struts which form the '3D culture surface' are many times larger than the cells they carry. The spatial relationship between attached cells and the walls of such porous materials, as opposed to conventional flat-plastic culture plates, is illustrated diagrammatically in Figures 3.14a and 3.14b.

However, this normally means that attached cells will experience an environment closer to that of monolayer ('2D') culture. Such cells at low, typical seeding densities will be anchored only at one (ventral) surface, as they are in monolayer culture (Figure 3.14a), with all that this implies. In effect, cells cannot easily distinguish between flat and slightly curved surfaces, which effectively eliminates the key distinguishing feature of 3D culture (Figure 3.14b), namely the shift from a strongly asymmetrical ('2D') to a more symmetrical cell space. As a result, systems where cells are seeded into large-pore materials have been termed 'pseudo-3D cultures'. In reality, this probably gives spatial cues to cells which are closer to conventional monolayer culture.

This situation will not change until cell proliferation begins to increase the cell density. Cell density in the pores increases to the point where lack of surface area forces cells to grow as multi-layers. This is where a new form of 3D culture gradually and locally develops (Figure 3.14c). Similar structures can also be seen in types of cells where they reach super-confluent densities and go from mono- to multi-layers.

Unfortunately, a central design feature of many porous scaffolds is a huge surface area for cell growth. This requires much cell division for complete coating, and there is no good reason why this cell saturation should occur evenly throughout. Hence, transitions from pseudo-3D configuration to true, cell-rich '3D' cultures will tend to be slow, local and ill-defined. Indeed, such culture systems

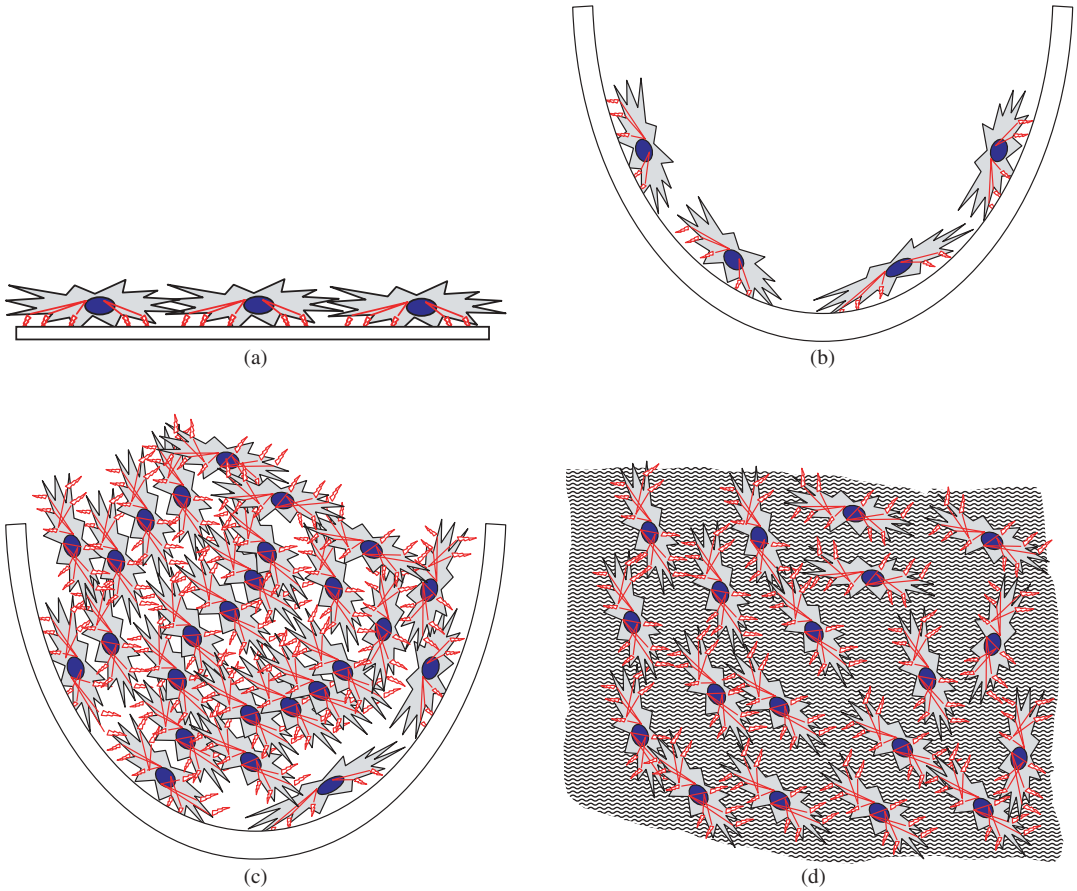


Figure 3.14 (a) Classic monolayer or '2D' culture of cells on a flat culture surface: basal attachment and cell skeleton (red). (b) 'Pseudo-3D' cells attached (basal surface only) onto the non-flat surface of large pores in a deep biomaterial scaffold. (c) Cell division in pseudo-3D culture eventually produces cell-rich true 3D culture within the large-pore (>100 μm) biomaterial. Cell interaction is predominantly cell-cell, tending to cell-matrix with further culture time required for matrix deposition. (d) 3D matrix-rich culture without the pseudo-3D stage, in which cells attach mainly to matrix on all surfaces from the start (t_0); e.g. collagen gels, see Chapter 4.

would be *expected* to generate big differences in cell growth in deep and superficial zones, as we shall see later (see Chapters 5 and 8), forming unintended tissue layers.

By definition, the point at which such an indirect spatial transition occurs will be dependent on culture time, cell density, average pore surface area and cell type. Cell type, in particular, is critical here. It seems reasonable, then, to be cautious in suggesting that such configurations provide spatial cell cues resembling native tissues, as resident cells will only *gradually* receive 3D signals from their matrix and neighbours. Even then, this configuration is predicted to develop at different rates in local patches throughout the substrate.

As discussed in the previous section, such cell masses tend to be characterized by cell-cell adhesions (dependent on cadherin membrane receptors). Only when cells within this mass start to secrete and accumulate a collagen-rich, extracellular matrix will this construct begin to resemble a native connective tissue. As shown also in Figure 3.10, spatial cues in connective tissues are dominated by symmetric interaction with protein fibres, i.e. with predominantly cell-matrix adhesion

receptors (integrins), with all that this implies for cell signalling (Figure 3.14d).

Understanding the basis of this spatial transition, from pseudo-3D to cell-rich and eventually to matrix-rich 3D culture, is critical in the field of cell-scale spatial cuing. Its key contribution is in highlighting the difference between growing cells *on* large pore synthetic support materials, as opposed to *within* nano-fibrous meshes. The latter provides spatial and receptor-based cues which immediately resemble those of native connective tissues (Figure 3.14d). The former systems more closely resemble high-surface-area monolayer cultures in the first instance, giving way gradually to matrix-rich 3D system in a highly cell-dependent manner (Figures 3.14a–c).

3.7 4D cultures – or cultures with a 4th dimension?

It should be clear, from the many references so far to 'cell adaptation', that this is a significant factor in considering spatial cues in culture. Unfortunately, cell adaptation represents one of the most complex and least understood aspects of cell biology. Particularly in recent years, there have emerged new tracts of biological understanding which have revealed the extensive levels of cell plasticity possible both *in vivo* and in culture.

Some of these are better described than others, such as the dramatic shift towards fibroblastic synthetic characteristics when chondrocytes (cartilage cells) are grown for long periods in monolayer culture. In this classical example of adaptation to culture conditions, chondrocytes, in monolayer for one or two passages, shift from synthesizing cartilage-specific to skin/tendon collagen types (types II to I), with reduced proteoglycan synthesis. This can be prevented by growing in suspension or agarose gel culture, or by using a micro-mass culture with ultra-high chondrocyte seeding densities to give cell-rich 3D conditions. This chondrocyte to fibroblast shift is at least partly reversible. Cell phenotypic shifts such as this are often sensitive to poorly understood environmental cues, making them major problems rather than opportunities for tissue engineering processes.

Unlike the characteristics of differentiated cell types present at time-zero, such adaptations – by definition – take time to develop. This emphasizes the role of 'time', or culture period, as a key factor in spatial control systems – and so we reach the place of 4D culture systems.

As we have discussed already (Figure 3.14), systems set up at t_0 as monolayer ('2D') or pseudo-3D cultures will gradually develop characteristics of true 3D with the loss of basal-only attachment. With more time still, the right cells, under the right conditions, may go on to deposit significant amounts of intercellular collagen, mimicking connective tissue 3D biology. This is the basis of a whole branch of (scaffold-free) engineered connective tissues.

However, even this short description of the potential difficulties of *cell adaptive changes* hints at the potential for control problems, where we cannot control or understand this aspect over time. In general, then, the tendency is for this time-dependence to introduce non-ideal conditions unless the dynamics, rate constants and efficiencies of such adaptations can be worked out. This certainly tends to be the case for systems using highly plastic stem/progenitor cells in 3D bioreactors, and where the aim is to deposit lots of extracellular matrix over extended time periods.

We can illustrate just how uncertain this effect can be, and thus just how much undesirable variability it can generate, by expanding on our previous 3D culture-progression (Figure 3.15). There is absolutely no good reason we should expect that any two or three cell types will progress *at the same rate* from pseudo-3D to cell-rich 3D (Figure 3.14a,b). The same is true for the next transition, from cell-rich to matrix-rich 3D culture – (b) to (c). The trouble is, these transitions are governed by two very different cell behaviours.

The driver for cell-rich 3D cultures (notional function 1 in Figure 3.15) is clearly cell density, so determined by proliferation and cell-death rates. But transition to matrix-rich 3D culture must be dependent on cell deposition of a collagen extracellular matrix – function 2. In other words, two

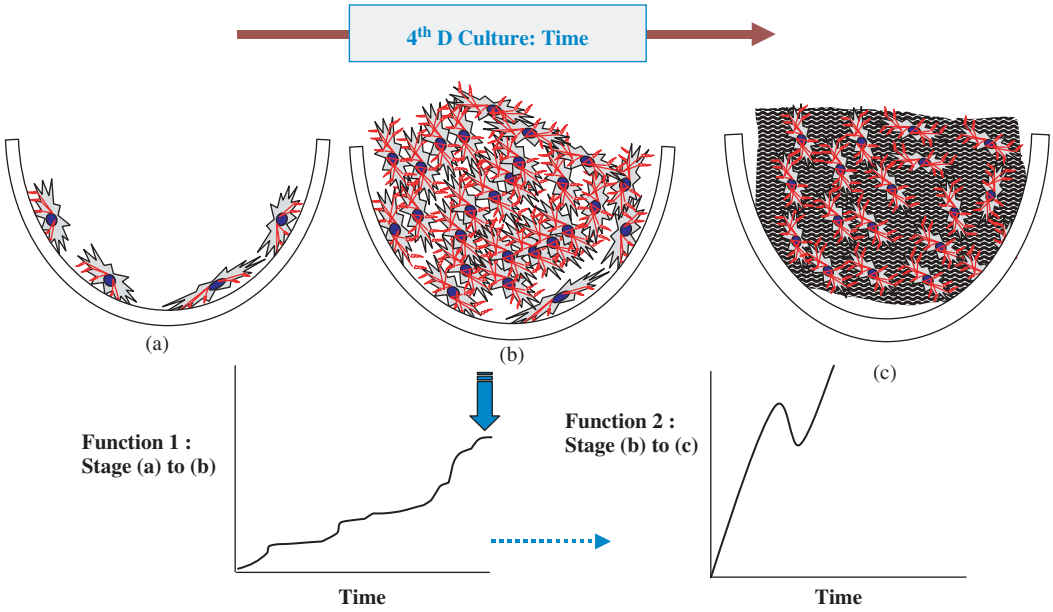


Figure 3.15 Example of '4D' culture, with transition stages, arrow (a to b and b to c), that trigger the start of very different but important functions (in this example, function 1 = cell division; function 2 = extracellular matrix deposition).

consequential processes are needed to give the 3D tissue structure: first, cell division; second, synthesis/deposition of a bulk matrix. This combination needs to be well understood and closely controlled, in order to prevent the system generating spatially variable heterogeneities. In other words, unexpected patchy tissue structure can be due to faster proliferation or matrix deposition rates in some, rather than other, scaffold pores. Such local rate differences, over just a few hundred µm in radius, will be almost inevitable in the absence of fine-scale control of mass transfer and of the 3D support material structure, as discussed above. Without this, increasing culture time will generate structural randomness in the final construct.

Uncontrolled variability of structure and composition of the newly deposited tissue has the potential to cause serious disappointments in the dream of fabricating tissues, and it leaves us with a less than ideal target. We are now left hoping only that constructs will be predictably 'average' in performance, over wider spatial volumes. This implies that some zones of our constructs will perform differently from others, in a manner beyond our control.

It is very doubtful if this remarkably modest (and diluted) aim was even in the finest of fine print of the tissue engineering dream contract we bought into. Certainly, it would be hard to believe that the prospect of making 'metabolically active, average-function-tissue-blobs' would have set alight 1990s biotech as it did!

Given the nature of the troublesome fine print we have been looking at here, it is tempting to wonder if the original thinking did not go this far because it was hoped that '*the cells*' would take care of their own 3D organisation and micro-environment. The current hunt for 'special (stem) cells' then looks suspiciously like the same aversion to tackling the big problems which were always in the small print anyway. In fact, maybe it was not even a 'small print' issue. Perhaps we just missed the *neon-sign-obvious* part of the contract – the word 'engineering'. This is an idea we return to repeatedly on the track of extreme tissue engineering.

The take-home conclusion from this example is that it is critical to identify, analyze and minimize the consequences of 4D culture on the spatial behaviour of cells. At the moment, this means either:

(i) minimizing the culture time; or
(ii) reducing the tendency for our cells to adapt to the spatial cues provided.

This situation improves in relation to our ability to design, engineer and monitor tight control of the cell space (at the 100 µm scale) over time (see Chapter 9). It seems unlikely that it will be possible to eliminate cell-matrix adaptation from many of the biological systems we design. This makes it all the more important to mitigate their uncertainties.

Time-based monitoring of cultures, then, becomes a key requirement. This suggests that the appearance of functional stages in the culture will need to be monitored *in real time*, with corrective control steps as required. The time dimension of the 3D culture hinges on the use of minimally invasive, real-time monitoring of culture progression in a way which is far less critical than for '2D' cultures.

It should now be clear to the reader that time/sequence (culture period) is at least as important as more traditional parameters in controlling 3D space. **Real-time monitoring is, therefore, a major future requirement**.

3.8 Building our own personal understanding of cell position in its 3D space

The main take-home message of this chapter is that, for 3D cultures, it is critical to understand the cell space from a cell perspective. To do this effectively, it is essential to get right down (mentally at least) to the size of individual cells to understand just what that space consists of. Hand in hand with this is the need to understand which types of information are most important to each cell type (i.e. for any given tissue engineering application). In human terms, this translates to 'what *really* matters' to your circumstances and needs? These two principles work as an inseparable pair and are best applied together in constructing hypotheses to explain how cells will grow in any system, or in designing culture system modifications.

Again, let us try this dangerous trick of humanising cells. Take a look at Figure 3.16. This is a photograph of commuters travelling on a crowded London underground, the Tube, during the rush hour. If you *can* ride the Tube for real, do have a go; if not, then use your imagination. First, close your eyes and then stand, holding onto a secure position, and take in all the information that you can from the surroundings. Work out *how you know* what the train is doing as it moves. As you travel and get thrown about by the train, try to identify where you get your spatial information from. After a while, ask yourself how can you tell which is up and down, left and right? Is the train moving, or at a station, accelerating, slowing down or going round a bend?

It should become clear that many of your reference points come from your basic body asymmetry (see Chapter 6), coupled with an intimate knowledge of that asymmetry. You are standing upright, balanced against gravity, so 'up' and 'down' come from any tendencies you have to fall over against

Figure 3.16 Photograph of commuters travelling on the London Underground (the Tube) in rush hour. First, close your eyes and work out where you get your spatial information from? Second, what is important about that information to *you*? Reproduced with permission © Ryo Hirosawa.

the surfaces you are touching. These might be other passengers, seats, support poles or doors. More actively, these may be the uprights, straps and handles that you have selected as your full-time anchorage points (i.e. you are 'holding on tightly' as the guard recommends!). Similarly, you have a well-developed understanding of your left-right asymmetries. Again, as you lean one way or the other or start to pull harder on one of your anchor points, you can immediately calculate which way you are falling.

Once the train moves off and gathers speed between stations, it lurches and sways, turns and brakes, and these movements will throw you against soft or hard supports. Once again, you will lean on your anchor points or (by now) your adjacent fellow passengers. This simultaneously provides you with both for support (minimizing the embarrassment factor) and as sources of information. By deduction, you almost instantly compute that you are being moved by acceleration/deceleration and changes in direction/momentum, which you can relate to the movements of the train almost without thinking.

However, if you are to be able to use these as reliable sources of information, you must *already* know (or more dangerously, have made assumptions about) the mechanical properties of the structures now support you. This you might consciously consider as 'their ability' to resist your movements. For example, a lurch to the left may push you into contact with an apparently compliant, retreating surface. But is this a handrail, moving away from you *even faster* than you are leaning towards it, or is it just a shy passenger? The data ambiguity in this case is particularly stark as it could be suggesting two equally plausible interpretations; both dangerous in very different ways:

(i) The train is turning over: panic now? . . . or . . .
(ii) Careful, are you ready for a serious socio-legal incident?

Each requires more information (and quickly) to clarify the ambiguity. Similarly; is that a leather hand-strap you just grabbed for, or another passenger's hat? To compute the most likely meaning of these ambiguities, you will need a clear understanding of the material properties of these 3D support structures – whether they are standard anchor points or parts of fellow passengers' anatomy.

Secondly, to understand the various ways that different people interpret this stream of positional information (i.e. 'individual sensitivities'), it is also important to understand how you routinely filter the significance of the data. We can see this as comparable with the adaptations of different cell-types. For example, people unfamiliar with big city habits – those who are comfortable with more personal distance, such as older people or those who are just vulnerable souls and fear muggers and nasty infections – will treat this monitoring system quiet differently from hardened city commuters and New Year revellers. Just like cells, then, we are *adapted*, and this makes a major difference to the assumptions through which we interpret the spatial monitoring data we collect.

The translation of these human-centric observations to understand how cells *might* use cues about space and position is clear, though we should be cautious about over-extrapolation. For example, it seems unlikely that gravity is a major *direct* cue to most cells, as their own mass is so small. However, this will also tend to be true for our commuter who is packed tightly between other passengers. We can modify our analogy here to imagine what would happen if we lost the gravity-cue, perhaps under the sardine-packing conditions of the Tokyo underground. In this case, gravity stops being too important as our weight is supported by the mass of passengers squashed between the carriage walls. However, cells (and commuters) can compensate for this by using (monitoring) the change of momentum, during sharp turns or accelerations, of the whole mass of passengers to which they are attached. For the commuter, this is a people-mass; for cells, it might be the large mass of extracellular matrix (e.g. dermis) into which they are meshed. In either case, the inertia of the relatively large masses involved can provide detectable strains on individual commuters or cells, indicating how the whole train is moving.

3.9 Conclusion

Different forms of cells in culture inevitably collect and use cues from their 3D space in very different ways. The major differences in spatial behaviour between epithelial and stromal cells (e.g. skin keratinocytes and dermal fibroblasts) form an unambiguous example of this.

However, we must expect to find a spectrum of smaller, less obvious or transient adaptations to spatial monitoring in the many different cell types employed to engineer tissues.

Further reading

1. Hawking, S. W. (1988). The Unification of Physics. In: *A Brief History of Time*, pp. 155–169. Transworld Ltd, London.
   [Check-out the 'Hawking' (basic physics) spin on what 2D means to biology–demonstrated using a dog.]
2. McGrouther, D. A. & Higgins, P. O. (1997). (CD-ROM) Primal Pictures Ltd, London.
   [Visual storm of images and explanations of the Dynamic, 3D structure of this limb: interactive CD, surgeon's-eye view illustration of our spatial challenge.]
3. Hay, E. D. (2010). EMT (epithelial-mesenchymal transition) Concept and Examples from the Vertebrate Embryo. In: Savagner, S. (ed.) *Rise and Fall of Epithelial Phenotype*, pp. 1–11. Landes Bioscience, Georgetown, TX.
   [Embryologist's view of epithelial sheets and their relationship with stroma – from the veteran leader of the field.]
4. Yamada, K. M., Pankov, R. & Cukierman, E. (2003). Dimensions and dynamics in integrin function (a review). *Brazilian Journal of Medical and Biological Research* **36**, 959–966.
   [Understanding what holds cells down to their matrix, integrins, from a prolific authority of the cell-matrix domain.]
5. Sands, R. W. & Mooney, D. J. (2007). Polymers to direct cell fate by controlling the microenvironment. *Current Opinion In Biotechnology* **18**, 448–453.
6. Yannas, I. V., Tzeranis, D. S., Harley, B. A. & So, P. T. (2010). Biologically active collagen-based scaffolds: advances in processing and characterization. *Philosophical Transactions, Series A, Mathematical, Physical, and Engineering Sciences* **368**, 2123–2139.
   [References 5 & 6: Reviewing trends in polymer scaffolds and natural protein sponges to mimic 3D culture for cells.]
7. Reddi, A. H. (2000). Morphogenesis and tissue engineering of bone and cartilage: inductive signals, stem cells, and biomimetic biomaterials. *Tissue Engineering* **6**, 351–359.
   [The case for deriving 3D signalling from natural embryo development: developmental biologist's strategies for engineering tissues.]
8. Novosel, E. C., Kleinhans, C. & Kluger, P. F. (2011). Vascularisation is the key challenge in tissue engineering. *Advanced Drug Delivery Reviews* **63**, 300–311.
   [Case for the importance of vascularisation: but is it tackling Mount Doom or a measured afternoon ascent?]
9. Hadjipanyi, E., Mudera, V., Deng, D., Liu, W., Cheema, U. & Brown, R. A. (2011). First implantable device for hypoxia-induced angiogenic engineering. *Journal of Controlled Release* **153**, 217–224.
   [Paper explaining the practical use of cell hypoxia in 3D culture to engineer accelerated blood vessel integration.]