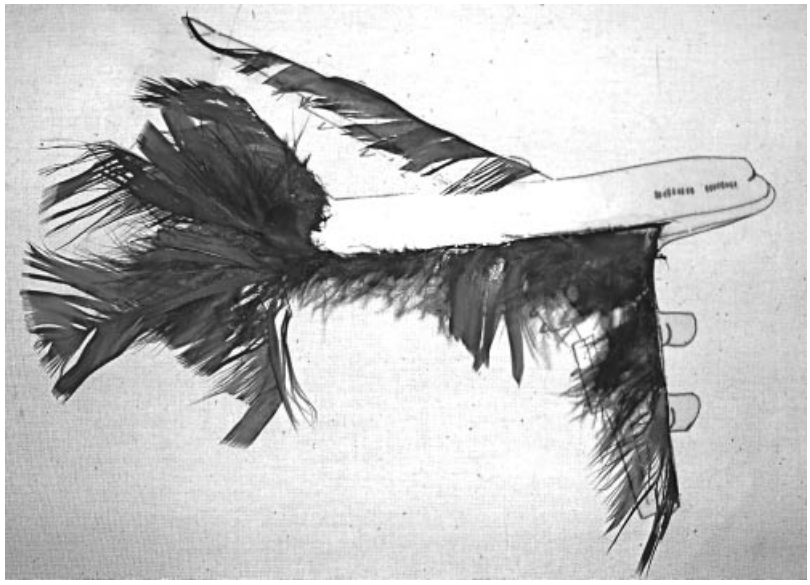


The Bio-Boeing-with feathers and flapping (see Figure 9.14)

9 Towards 4D Fabrication: Time, Monitoring, Function and Process Dynamics

The last chapter ended on the somewhat problematical issue of the 'great bioreactor project'. In this chapter, we shall examine why there are, in fact, plenty of reasons to be cheerful – even optimistic – about the future for extreme tissue bioreactor technologies. However, it is clear that 'next generation concepts' will be essential for *controlled, stepwise fabrication* processes, including bioreactor culture stages. In effect, we would hope that multi-step controlled processing will replace the current, less effective, one-step (neo-agricultural) processes discussed in Chapter 8[21].

[21] At the risk of over-simplification, we are entering the era where we re-introduce the engineering into tissue engineering. This can take many forms, but the Trojan Horse approach here will use the theme of 'process dynamics and monitoring'.

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

The first stop in this guest duet of 'dynamics and monitoring' will be process dynamics. Put simply, this refers to the changes that occur to our tissue construct as time progresses and (hopefully) as the process generates the structures we require. In fact, we are all pretty familiar with the *basics* of process dynamics. We normally think of these, for example, as increases *over time* in:

(a) resident cell numbers or differentiation state;
(b) changes in mechanical strength and stiffness;
(c) overall physical dimensions; or
(d) extracellular matrix material complexity and stability.

Respectively, we would see these expressed as changes in this-or-that unit of:

(a) live (viable) cell number;
(b) break-stress/stiffness modulus;
(c) μm of wall thickness/mm diameter; or
(d) protein concentration/cross-link density.

But in *all* cases, this should be expressed as 'per unit of *time*'.

Where our processing is rapid, ultra-rapid or just optimistic, this will be per hour, per minute or per second. Most likely, the processes measured over these short time scales would be non-biological fabrication and assembly. Processes which are monitored over periods of days, weeks or months are more likely to be biological, cell and culture-based. Over those time periods, it is increasingly unlikely that processes will be economically viable, except for specialist, high-value applications.

In other words, in this chapter, we are allowing *time* to become our master (as opposed to 3D structure or tissue bulk and composition). We have, then, reached the fourth dimension of extreme tissue engineering.

9.1 Controlling the dynamics of what we make: what *can* we control?

As we have seen, the dynamics of processes which are *predominantly biological*, such as cell culture, tissue bioreactor operation and cattle farming, are different from those in *engineering processing*. In particular, the level and type of controls in biology-based culture processes normally have a lighter touch. They tend to be lower resolution, operating with less detailed control, often using basic function outcomes, relatively wide tolerance ranges or qualitative measures (Text Box 9.1). The more remote

Text Box 9.1 How we make 'things' changes with the way we measure the making process (Figure 9.1)

Examples of how we measure the making process include:

- cell density would be monitored by actual counting of nuclei;
- break strength would be measured by physical clamping and stretching;
- meat quality (Figure 9.1) would be measured by weighing the meat : fat : gristle ratio.

In contrast, if the latter example were possible using an engineering process, we might expect to make more indirect, extrapolated measurements. For instance, cell density would be derived from real-time monitoring of CO_2 production, tensile properties from optical scanning of fibre content and meat quality deduced from an ultrasound scan of the cow's thigh. However, for these to 'work', we need to have such a high level of confidence in the process detail that it becomes possible to extrapolate *reliably* from remote (implicit rather than explicit) markers of what is happening.

By analogy, a school teacher taking the register *could* just count the empty desks or the coats in the cloakroom, or weigh the used milk cartons, if the children all behaved and dressed predictably. Needless to say, though, teaching children is a highly bio-social process, and wise teachers will go as far as positive facial recognition to exclude extremes due to truancy, kidnap or sibling-substitution. The result of this is that current monitoring tends to be occasional-slow-direct, rather than real-time and rapid using implicit or indirect markers. 'Indirect' monitoring is simply too unreliable for current, soft-control systems, where we do not know in sufficient detail just what is going on.

these measured parameters are from the final function, the more likely it is that they will correlate only weakly with that final function we want. As a result, there is a pressure to make the acceptable limits of these control tolerances ever wider – in other words we accept greater variability. In the case of free-range Limousin cattle, grazing on a mix of sunny and shady, flat and sloping pastures, we can see that measuring their tail diameter or leg length might be quick and easy to do, but it will give us only the roughest estimate of muscle : fat ratio or rump steak quality.

So, these *direct explicit*, end-function measures are characteristic of biological-based cultivation/agricultural processes. In contrast, those controls which are realistically available tend to be *indirect, implicit* and almost 'passive' in nature. This seems reasonable and normal, as so many aspects of biological cultivation are complex and dependent on the cells/tissues or organism involved. The processes being measured, after all, are subject to biological variance and uncertainty – indeed, this can be their defining feature. For example, where and when bioengineering and biotechnology processes develop 'engineering-like' forms of control, they cease to look like biological-cultivation and they start to resemble engineering-type fabrication and assembly processes, but using biological components.

For example, surgical implants can be made of (non-cellular) natural collagen protein sponges. These are made from natural components, but are purified, refined, structured and assembled using closely definable engineering processes – they are not grown. However, it is often not cost-effective to apply engineering-type levels of process control to biological cultivation processes. These light-touch control bio-cultivation systems commonly work quite well enough for what is needed.

To illustrate this, we can build analogies with the obvious dynamics that we find in the process of cattle rearing (Figure 9.1). Most of the basic, detailed controls and feedback processes for growing a Limousin cow to maturity (beef status) are inherent in the breed (genetics) and its habits/tolerances. This is a key part of the breed (gene content) that is built into the cattle. The farming process has developed to make the most of these and optimize how they operate *as a whole*. Farmers might monitor rates of growth by weight or physical dimension, muscle to bone ratios, rate of calving, bacterial load. In response, they can make adjustments (sometimes delicate) to the process to modify how these measures change. They might administer antibiotics, provide extra feed or supplements in winter, fend off parasites or kill off any toxic plants growing in the field.

Figure 9.1 Limousin cattle grow themselves in a nutrient-filled bioreactor (i.e. a field), through a dynamic protein production process. The process engineer, or farmer in this case, monitors and adjusts the process in gross terms (food and shelter in the snow, antibiotics for infections and birth assistance) and generally balances the supply-removal of start material and end products.

However, it is important not to confuse peripheral, enabling controls with those which are primal and fundamental. Note that the cow grows on its own. This is hopefully optimal, converting grass into muscle proteins at a rate and in a shape (meat-cut) which is inherent to the breed. More particularly, it remains a Limousin cow in shape, composition and growth characteristics. These are the parameters that the cow controls through its genome. The farmer does not need, in fact cannot, do much to change these. Much as the beef farmer may wish it, the cow will only *ever* produce two hind legs and one rump. There is no room (and critically, no process-programme) for an extra set of prime rump muscles.

The point here is that for bio-production systems, from cell culture to farming, we generally do

not even contemplate the detailed process controls which would be essential in a standard engineering processes. System complexity, and in many cases its inbuilt biological efficiency, make this impossible and/or unnecessary. The farmer never wakes up with the idea that he will change his process and produce Limousin cattle with 50 per cent more muscle in the rump region (though he may *dream* of five-legged cows). Cell culture labs might aspire to higher level of detailed process control but, unless they have the resources for decades of top level research into basic cell biology, their aims still tend to focus on optimizing what the biology offers (e.g. mineral content of the local grass species). They would rarely/never consider meddling with cell-division rates in the bone growth plates, average transcription rates or endoplasmic reticulum protein output in a major muscle block. Fiddling with the nuts-and-bolts of biological processes is simply not an option.

The contrast with the directors and managers of Toyota cars or Nokia telephones could not be greater. True, they are still limited by the laws of physics and economics, but these are basically very well understood (OK, except for the economics). Also, they still occupy much of their time 'optimizing' process dynamics to make sure the production lines do not run short of control pedals or batteries. But (and this is the *big* 'but'!) Toyota and Nokia have their names on the products; they design and fabricate all the parts in their products, from the most basic components to the box the product is delivered in (i.e. they work bottom-up).

If there is suddenly a need to make a cheaper, lighter mobile phone (Figure 9.2), a miniature computer-communicator or a pink electric car, it is in their power to produce it, so long as the bottom-up knowledge is in place.

Hence, the basic operating concepts of these two process types are poles apart. When we fabricate engineered devices, we *expect* to get right down to the minute details and to control every aspect of production – because we can. In contrast, when we grow biological products or devices, we *expect not* to have to control much of the nitty-gritty detail. The 'bio-' part (plus 500 million years of evolution) does it all for us.

As we have seen, this is a pefectly legitimate, reasonable approach when dealing with biological growth and *cultivation* systems, with their inbuilt bio-controls and feedbacks. However (and this is a big 'however' which some readers may have already spotted lumbering over the extreme tissue engineering horizon), this returns us to the key bioreactor fault line from the last chapter. – namely, the realization that we are not culturing/growing a complete *bio-system* in our bioreactors. The problem is that *isolated* cells are not playing with a full deck of cards. They cannot have the full system

(a) (b) (c)

Figure 9.2 Nokia have processes for fabricating mobile phones. The big difference, though, between this and cattle production, is that if Nokia wants to change that process to make a different phone, they can completely redesign the phone *and* the process, with intimate control of the detail. In this example, it shifts from one mobile format, a heavy, chunky structure (a) to a lightweight web phone (b), by altering (c) some basic parts.

Text Box 9.2 Designing for what we want or what we can get

The distinctions between designing what we *want* and optimizing what we can *get* appear to be blurring as they move further into biology. For example, genetic engineering research now allows us to shift the habits and structures of cultivated animals and plants, such that we are acquiring greater, more basic levels of process control. Similarly, and more recently, advances in stem cell (re-)programming are beginning to offer similar shifts in cell-based culture systems.

This is particularly clear in the idea of inducible pluripotent stem (IPS) cells. In this case, differentiated cells, committed to a specific function (e.g. skin), are de-restricted such that they return to being multi-potent 'stem' cells. The intention would then be to re-programme such IPS cells into something else (e.g. nerve cells). This is, in part, a result of the catch-up of detailed 'bottom-up knowledge', which is essential.

While these are exciting new ways to take control of the growth processes, they retain elements of the familiar biological top-down approach. In this case, we are restructuring the pre-existing cell unit. After all, it is difficult, even undesirable, to break out of the effects of complexity which come from 500 million years of evolution. It is almost as if *top-down* bio-cultivation processes have to operate within 'someone else's' (Darwinian) rules. In contrast, *bottom-up* engineering processes use the simplest feasible components and processes to fabricate objects from scratch.

Although in snapshot these can look similar – even convergent – they may actually be moving in opposite directions.

of controls and information needed for building spatially defined 3D tissues.

And why ever *should* we expect a group of isolated cells to 'know' how, and in what shape, to make a knee joint? Real knees, after all, are made as an integral part of a single, coordinated, *time-based* process for making a whole leg, attached to a hip, attached to a spine, etc. as per the song. This means that strategies which leave process timing and dynamics to be decided later deserve a closer reality check. After all, leaving key steps till later, as a *refinement stage*, is only reasonable where we can be pretty certain that the process will get there, and will work at all.

A second danger sign lurks within the last chapter (under the heading 'Sterility (and scale-up)' – see Section 8.3.1). As a basic rule of tissue engineering, *it is never too soon to consider scale-up*. This rule is written into the obituaries of many biotech companies from the 1990s. It must argue very strongly that leaving process analysis as a downstream task (i.e. for someone else to do) is a luxury – an indulgence, even – that we should be very, very cautious of allowing ourselves to take.

Consequently, to conclude this section, it is still common in tissue enginering circles to tacitly consider that the dynamic and the time course of tissue fabrication are *matters for the future*. They can be left as 'someone else's problems' (the SEP principle[22]). After all, we are told, when it is difficult to get *anything* functional to grow, surely it is a reasonable strategy to first get *something* working, *then* to tackle the question of how long the process takes. Hopefully, the analysis here has highlighted the self-perpetuating danger built into this approach (Text Box 9.2).

Our analysis has gradually shifted from cattle rearing (indirect) to telephone manufacture (direct) – or from bioreactor cultures to tissue assembly processes. In each case, however, we have seen that the strategic landscape is *completely different* as we travel along the two tracks. We are forced to analyze how achievable our process controls will (ever) be and how we can objectively

[22] Source note: Arguably the first, but certainly the most famous description of the concept that 'people do not take any notice of a problem that can be assigned to someone else' came from the late Douglas Adams in his *Hitch Hiker's Guide to the Galaxy* series (Heinemann Press, London). *This proposes that someone else's problem (SEPs) are effectively invisible.*

Text Box 9.3 Direct and indirect tissue engineering

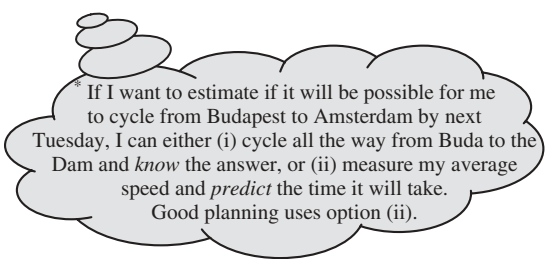
As we have seen previously, it is possible to distinguish between two general types of production process – *direct* and *indirect* – represented here by cultivation- and industrial-type fabrication processes. Clearly, the same distinction can be made between direct and indirect engineering of tissues. We can generate a working definition for direct (DTE) and indirect (ITE) tissue engineering (all good concepts need an acronym). This is based on the subjective judgment of which processes are largely under direct human control, against those where production is essentially under the control of non-human living systems (isolated cells, tissues or whole organisms). This is an imperfect division, as processes such as fermentation are sufficiently closely controlled to be thought of as direct, even though they are based on yeast synthetic processes.

In general, ITE systems work at developing better and better biological and cell-culture controls. DTE is where we devise fabrication and assembly processes for 3D tissue support materials which rely predominantly on human control processes, independent of cell activity.

However, in the special case of tissue engineering, there is a second characteristic which makes the processes doubly indirect. The use of temporary, normally synthetic 'scaffolds' to support the cells adds a further 'indirect' element, as the fabricated element is an extra pre-stage not found in nature. This is similar to the indirectness of fabricating a bronze statuette by a process of mould preparation (e.g. using a lost-wax technology), as opposed to directly cutting the shapes out of bronze. The corresponding ITE equivalent of this is to make an intermediate non-native cell support material (a 'scaffold' in the shape of the tissue), to be eventually replaced under cell action. In contrast, direct tissue engineering would aim to assemble the base parts of the tissue under human control with minimal cell involvement, at least in the first place.

This concept is helpful where we need to critically analyze our favourite process and bioreactor strategies.

measure (monitor) how fast we are moving towards our target.* The good news is that making this



analysis *and* monitoring its progress transforms the tissue engineering vista completely.

In effect, the target is no longer dominated by such a weak question (i.e. 'Can we get *anything* to grow?'), but rather it is elevated to the more challenging question, 'Does the rate of progress provide a reasonable tissue construct in a reasonable time period (with numerical estimates of what 'reasonable' might be)? With this, we move into the fourth dimension of tissue fabrication: the full use of *time* and *sequence* for engineering tissues.

9.2 Can we make tissue bioreactor processes work – another way forward?

So, can we use this analysis (see Chapter 8 and Text Box 9.3) to identify another more favourable model for engineering of tissues, directly: a new way forward? One available approach is to use the fourth dimension intelligently, to design dynamic/sequential processes which are hybrids of our two options. This is a pragmatic mix of direct and indirect tissue fabrication and processing – in other words, processing which would use the best of both *in sequence*.

For example, this might use fabrication (bottom-up engineering) in the early time stages, but shift in later stages of the sequence to biological cultivation of cells. This sequence could be used to generate levels of bio-complexity which are beyond our current technologies to generate directly.

Text Box 9.4 Process sequence: order, disorder and chaos

Sequences, as some people insist, 'are *everything*!' The picture on the left of Figure 9.3 shows a large building site in Chengdu, SW China (such sweeping-scale projects are more common in China than in Europe). An old, run-down housing area is being redeveloped (see Chapter 8, Section 8.25) to make way for a much needed sports and medical facilities. The former residents are getting new homes away from the busy city ring road which runs nearby. The sports and medical centre will go onto the red oval footprint (red line) and the planned new access road (black) is shown in the right hand picture.

From Chapter 7, we can recognize this as a good analogy of natural soft tissue growth remodelling cycles, where insertion of the new elements is linked with remodelling of existing structures to accommodate the structure changes. In natural processes, we barely notice the key element of sequence which is obvious here. In this building site, the previous occupants have already been moved to their new suburban homes and the site is being cleared of buildings. Traces of the new road are visible and being used by lorries to move the rubble away, with the foundations surveyed and marked into the levelled ground. The sequence of planning and process operations is clear to see. The quality of this essential sequence detail can be described, for both tissue building and urban development, as one of three levels: 'Ordered', 'Disordered' or 'Chaotic'.

The point here is that in making both cities and tissues, it is simple to distinguish (dis)order from chaos (one works; one does not). Identifying the difference between good order and less good order (disorder), however, is tricky. The difference is one of degrees; for example, as progress rate, number of hold-ups or final cost. In the end, the efficiency of our processes depends on where we find ourselves relative to this blurred line.

To illustrate, it is simple to see that arranging for the bulldozers, trucks and demolition cranes to arrive three weeks before the residents have moved out would cause *chaos*. Similarly, trying to lay the access road before the old flats had been moved away, or building on top of uncleared rubble, would also cause chaos. The process would stop dead. However, booking the roofers to arrive on the same date as the foundations are being dug would be just as inefficient as the roofers would be sitting around for three months, drinking tea in their cabins. Equally, late delivery of the drainpipes to the site will lead to sludge and delay every time it rains, with trucks and Town Council inspectors sinking into the bog that was once the access road.

To the casual or occasional observer, these sequences can look much the same (generally 'ordered'), but the whole project becomes a bit longer and there are more wasted materials. These are the differences between good sequence (order) and poor sequence (disorder). They translate to efficiency and inefficiency, and potentially success or failure for tissue production. We can either discover the difference (expensively) in hindsight, or employ or collaborate with process experts from the start.

The moral of the 'sequence' caveat, then, is that most non-experts, from any building speciality or tissue engineering tribe, can see chaos when it hits them, but improving disorder into order needs collaboration with specialists.

Figure 9.3

9.2.1 *Blending the process systems: balancing the Yin and the Yang*

Our new option, then, becomes one of balancing the 'Yin' of direct fabrication with the 'Yang' of cell cultivation. We have immediately avoided the 'either-or' extreme and are now focusing on making the best of each system by managing their *sequence* (Text Box 9.4). To develop such Yin-Yang process strategies, it is necessary to step back from the 'whole-process' and do an ILAS:

(i) IDENTIFY which are the important stages and sub-stages of production.
(ii) LIST the various possible timings and sequences.
(iii) ANALYZE options for how each stage could be integrated into a process sequence.
(iv) SELECT and prioritize the most promising process sequences for testing.

In other words, we are starting to divide into production *stages*, asking the question, 'Which can be done better by fabrication or cultivation?'

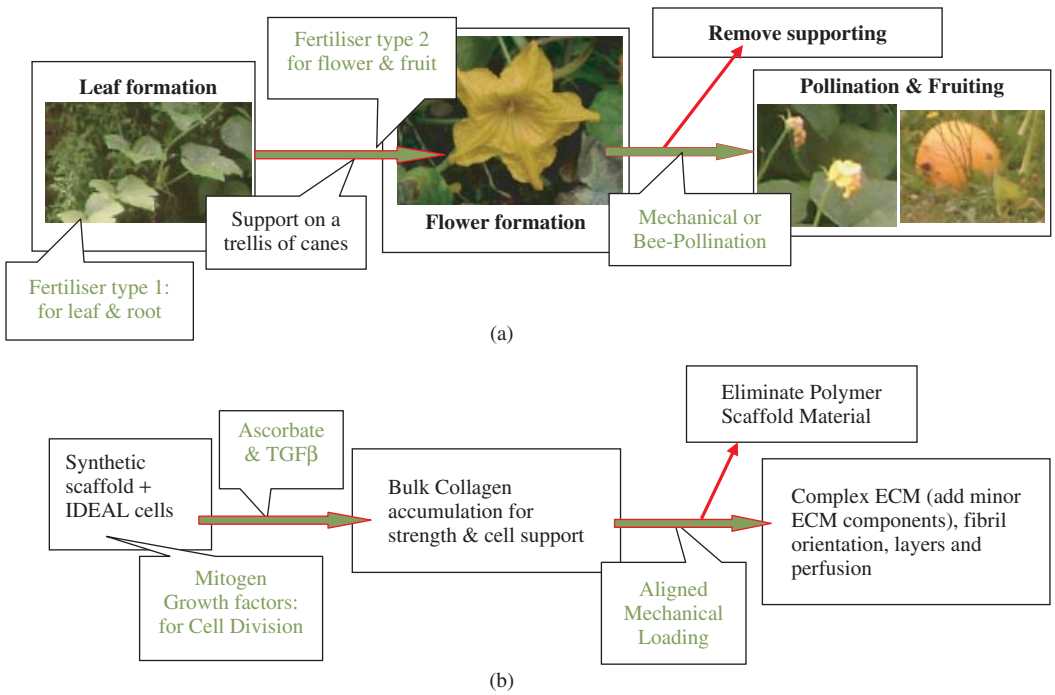


Figure 9.4 Translation of the pumpkin bioreactor analogy into a plausible scheme for bioreactor production of skin equivalent, using comparable 'trigger-and-growth' process. (a) Pumpkin bioreactor analogy: basic cultivation conditions are maintained throughout the process, with a sequence of staged inputs or triggering cues which work with and assist the growth process. For example, as the young plant is establishing, the fertilizer content promotes leaf and root development. As the stems get larger, some are mechanically supported for even illumination or wind protection. At a predetermined point (monitored trigger point), the fertilizer content might be changed to that which promotes flower and, later, fruit formation. In the meantime, flowers are pollinated and, as fruit forms, the supports are removed, allowing the pumpkins to lie on the ground and grow bigger. (b) A comparable sequence of triggers and supports can be envisaged for the cell production of skin-like tissue. Between each stage, the cells themselves are being required to perform their innate, programmed tasks of tissue synthesis and deposition. Key to both of these is the *sequence* in which the process supplies controls and enabling factors and then, in effect, sits back and waits for the natural cultivated 'growth stage' to complete. The control points can either initiate the next stage in the process, or speed up the rate at which it is completed. Importantly, in the case of biological production, these controls are unlikely to substantially alter the sequence of stages, as these are largely inbuilt to the growth process itself.

Is there then, an optimal sequence for these stages, and how and when can we move the construct from stage to stage? Let us first examine how this happens with familiar cultivation-dominated examples of tissue growth.

We can develop this (as seen in Figure 9.4a) as our familiar aspiration of pumpkin cultivation, drawn from the last chapter, with the process extended to highlight its parallels in a conventional tissue engineering plan for growing a skin equivalent (Figure 9.4b). As we have seen in the case of the pumpkin, the basic biological machinery of leaf and flower production is supported through both chemical and mechanical promoters (fertilizers to promote fruit formation and canes to support the leaves). New tissue formation is initiated, nurtured, accelerated and ripened in the case of the fruit, to the point of its harvest. This reflects surprisingly well the general plan for formation of a tissue such as skin using bio-culture (Figure 9.4b). It includes assembly of the chief biological and support components, amd provision of both chemical and mechanical factors which nurture and guide early tissue formation.

At the stage when accumulation of *useable tissue bulk* takes over, the support structures (scaffold) and growth additives are removed to promote maturation of the final tissue product. This final step sees the development of tissue/ECM (extracellular matrix) complexity. Both of these processes can be viewed as essentially a *sequence* of cell/tissue-based growth stages, each initiated or enabled by a series of external process triggers. The process supplies the triggering or enabling conditions. The plant (pumpkin) or cell-mass (skin) then performs its next programmed production task.

As we have established previously, such a strategy means that much of the fabrication process relies on the innate action of the cells under cultivation. We can now move our 'Yin-Yang' analysis along simply by asking which stages of the process our cells (i.e. currently the best available) can do *adequately*, and which stages are slow or problematic. Such weak points can be where the tissue output is poor (i.e. functional deficiencies), or where it is excessively slow to produce even minimal function. In other words, this tells us where the dependence on cell cultivation is leading to a log-jam point in the process.

Therefore in order to progress we still need:

(i) to set down criteria for minimal tissue properties; and
(ii) to identify effective ways of measuring them, *within* the process (i.e. without stopping the process or damaging the construct).

This is directly analogous to our example of a planned Budapest-Amsterdam cycle trip (see Footnote 3). While 'Just do it!' may be a good catchphrase for sports or for advertising a male fragrance, it is not a sound tissue engineering philosophy where we do not yet know how, or even if, the task will be possible.

In the example we have here, of skin engineering, it is clear that the generation of shape and complex 3D μ-structure is exceedingly difficult to get right outside the embryo. In biological terms, this is morphogenesis, a subject of serious scientific uncertainty (SSU). The subject of poor micro-architecture, characteristic of scars formed in most tissues during natural tissue repair, has been discussed extensively in previous chapters. It is clearly a limitation, given the *overwhelmingly* modest successes of engineering natural morphogenesis, even after 20 years of trying.

A second log-jam, identified previously, is the very limited ability of cell culture systems to generate and grow the *bulk material* part of connective tissues. Producing bulk ECM, needed for good functional mechanical properties, is a slow and energy-hungry process for cells in culture. The location of these two key log-jam points (μ-structured template and bulk native ECM), are shown in Figure 9.5. This flow diagram (derived from Figure 8.16 in Chapter 8) breaks down a typical tissue construct fabrication sequence into four stages, with the log-jam points marked in stages (*) and (**).

To summarize this section, a key (sometimes misunderstood) distinction is made in Figure 9.5 between having a cell-free scaffold which then must be cell-seeded, and having the cells pre-incorporated into the 3D support material as it aggregates around them. The latter is a 'passive' process involving no

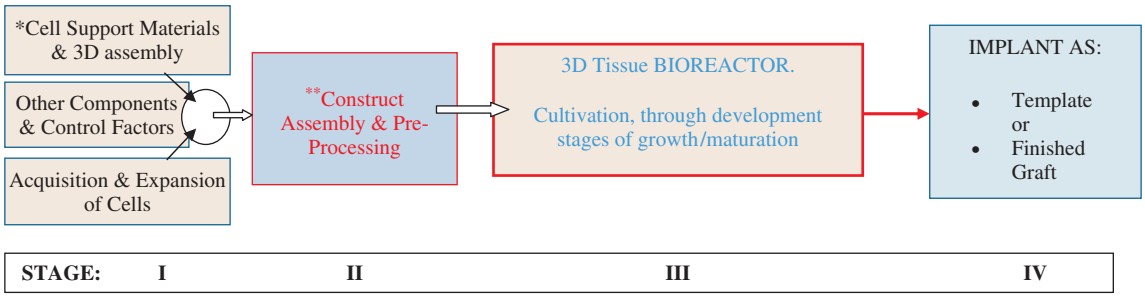


Figure 9.5 New scheme, evolved from Figure 9.4 (and see Chapter 8) for tissue bio-processing with a bioreactor stage in the process sequence. A strategy can now be drawn out which allocates the fabrication of parts to the tasks where it is best suited. In turn, the (cell-dependent) biological cultivation stages remain in the process where they cannot in practice be replaced. The first, *fabrication* stage can be described as production of components, including the 3D material that will support the cell content – in effect, the 'extracellular matrix' of the engineered tissue (*). This can be any biodegradable material but, as we have discussed previously, the closer it is to a native protein ECM, the closer the process is to direct tissue engineering. The second fabrication stage is seen in tissue template *assembly* (**), where the key basic tissue parts are put together to form a 3D equivalent of the eventual structure. It is assumed that this will be a simplified version, but how simple can we get away with? Sufficient 3D authenticity is needed for the eventual required tissue function. This is the point where the tissue is *constructed*, in this case without cell reliance, though cells must be introduced as the living component. The biological, cell-dependent processing comes after this assembly, in stage III. It involves all the bio-processes expected for growth and maturation to a functional tissue, performed through cell action in a cultivation bioreactor stage. The first fabrication and assembly stages (I & II) produce and fit together the 3D template, more familiar to embryologists as morphogenesis. The second, bioreactor stage is expansion and stabilisation, recognizable as tissue growth and maturation.

additional effort on the part of humans or cells. Cells are simply enmeshed in the structure of the biomaterial (in this case the ECM) as it aggregates, without any input from them at all – passively, to labour the point. Such constructs are clearly not cell-free, but neither would we consider that their fabrication is 'cell-dependent'. Therefore, such pre-seeded support materials clearly save us one complete stage. In effect, we have lost the drag of cell-seeding.

9.2.2 Making the most of hybrid strategies: refining the timing and sequence

This hybrid strategy, then, represents a new and potentially powerful approach which places the engineering (i.e. the direct fabrication technology) into those parts of the process scheme where they are most needed. These are the stages where cultivation technology is least effective. Previous chapters have examined mechanisms and materials which could be used for:

(i) direct fabrication of the initial cell support material;
(ii) direct assembly of the component parts into complex 3D tissue templates.

Current examples of these include:

(i) the use of directly assembled native fibrillar collagen supports (final ECM material of many tissues);
(ii) assembly of prefabricated matrix or cell layers into multi-layered 3D 'tissues' (i.e. layer engineering).

Examples of these direct fabrication processes are provided in previous chapters (Chapters 6 and 8), based on the example of collagen plastic compression and layer engineering. Other technologies to achieve these ends are also possible, and are being developed. At present, the detail of how direct tissue fabrication is achieved is less important than the fact that it *can* be achieved. It is the *availability* and *practicality* of such direct processes which allows us to design and analyze hybrid processing at all.

In fact, the process outline in Figure 9.5 is, by necessity, an over-simplification. In particular, Stage I is a catch-all for assembling the basic components for the process, cells, cell-support/3D scaffold and other controls such as growth factors. In practice, this would be likely to involve one or more

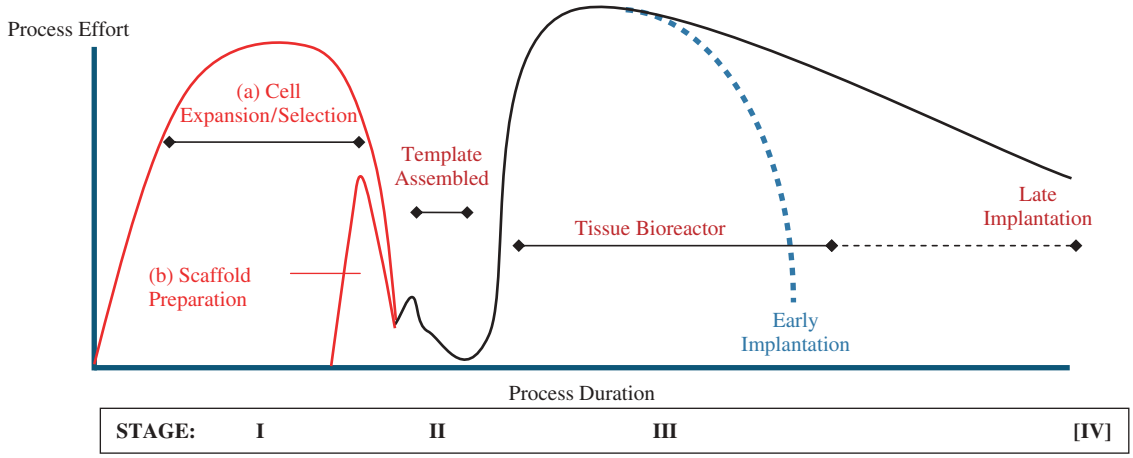


Figure 9.6 Process-Design graph (nominal axes) typically expected for indirect tissue engineering (ITE), based on a biodegradable polymer scaffold. This shows how the various stages might contribute to, and interconnect, during a conventional bio-cultivation process (derived from Figure 9.5). Typically, the first stage (red curves) would have two major components requiring process time and effort: (a) acquisition of the cells to be seeded; and (b) preparation of the polymer cell-support scaffold. Stage II is that part of the process where all of the components (cells, scaffold, adhesion and growth factors) are put together, and with a culture period to allow cell distribution, etc. Stage III takes the construct to some form of 3D tissue bioreactor with the purpose of getting the cells to deposit a biomimetic, collagen-rich ECM under the culture conditions provided. The aim here is to largely replace the biodegradable polymer scaffold. This may involve complex mechanical and/or growth factor cues, progressive perfusion and constant medium replenishment over prolonged periods. As indicated, this is likely to be the dominant stage in terms of time and, most likely, effort and cost. Early implantation of the construct (as a template tissue – blue dotted plot) would shorten this stage. However, where the requirement is for a functional graft-like tissue, this culture currently requires weeks or months for significant replacement of polymer scaffold function.

stages of cell acquisition, preparation, purification and validation. Traditionally, this could involve extended periods, particularly for time-dependent cell expansion in culture.

The refinement of our hybrid example can be illustrated in a different format, this time as a concept-graph of predicted 'process effort' versus 'time', in Figure 9.6. This is an evolution of the scheme shown in Figure 9.5. In this, our conventional process for producing a skin equivalent would probably use an *off-the-shelf* biodegradable polymer scaffold (therefore very short 'scaffold' preparation times). It would, though, need a significant and variable time for polymer surface treatment, cell seeding and (again) culture, in order to establish a useable cell density throughout the depth of the material.*

* Indeed, these remain largely unsolved topics of research and development for many tissue applications.

Only then would the construct really be ready for bioreactor culture, to grow a natural ECM with gradually developing mechanical properties.

If all went well, this cell-derived ECM would eventually replace the polymer scaffold: degradation rate of the polymer is key. The bioreactor stage could be very long (potentially months) for production of mature, mechanically strong, graft-like tissues. Shorter periods (days/weeks) are possible if the aim is to implant immature, limited-function templates or pre-tissues, which would mature *in vivo*. This form of design-plot provides a useful framework around which to:

- assess process sequence;
- predict (even to quantify) where problem stages are;
- identify opportunities to speed up the overall plan;
- design new sequences and envisage the linkages between stages.

In other words, even though such plots can be simple or qualitative, they are also invaluable tools

for rational design, analysis and improvement of both content and sequence of our processes.

In the example analysis in Figure 9.6, we can predict that cell expansion and bioreactor culture are likely to be rate-limiting steps in the process. Cell expansion (part of Stage I) can be reduced by designing constructs to use:

- low cell seeding densities;
- immature epithelial sheets;
- rapidly dividing cell types/stages (immature progenitors, growth factor and gene activation); or
- allogeneic donor cells able to be pre-cultured en mass (so taking 'expansion' out of the process).

Currently, the bioreactor culture stage, particularly for connective tissues, which requires deposition of a dense, collagen-rich and mechanically functional ECM, will be long and costly. In this conventional process, there are few alternatives available to reduce the impact of Stage III, particularly where a stiff, synthetic polymer scaffold must be replaced. Indeed, the later periods are predicted to generate increasing problems (and hence complexities and delays), because cell synthetic activity will need to be high. This places corresponding demands on deep layer nutrient/oxygen delivery due to consumption and changing diffusion coefficient of the new ECM (see Chapter 8). If ECM synthesis and deposition are not to decline, then deeper zones must be perfused, perhaps by channelling.

If and/or when the process can be progressed to completion against rate-limiting factors, the result would be a connective tissue of relatively high, but as yet unknown, density. We cannot yet estimate, in practice, where or when our resident cells will simply stop depositing more or stronger matrix as a result of their inbuilt feedback mechanisms. We do know for sure that such negative feedbacks will operate at some point. This point seems, sadly, to be reached at disappointingly low levels of matrix density (often, many fold below tissue levels). While technical innovation in bioreactors will improve this situation, the gap is quantitatively very large, even using culture periods which we know are far too long. Even so, our plan still falls short of promising to generate local tissue-like μ-structure or compositional features (zones/layers) similar to those of native tissues. Producing these is again likely to increase the culture periods required.

Consider, then, how dramatically the process could be changed if we switch to *direct* engineering of the tissue, even if there were no change in the cell acquisition or expansion phases. In a direct engineering system, maximum effort would be invested into assembly of cell-material templates. As many as possible of the *most basic* structures and compositional features of the target tissue would be pre-fabricated at this stage. This is shifting the fabrication effort away from the cells within the construct and onto our engineering ingenuity. In other words, such a shift to direct engineering takes process-effort out of the (rate-limiting) bioreactor culture stage (III) and moves it into the construct assembly stage (II).

There are as many ways to achieve the detail of this new approach as there are possible versions of the 3D tissue templates we want to fabricate. However, in the present example, it is possible to identify that the most basic, important tissue mimetic

Text Box 9.5 Some tissues can, and some tissues can definitely not: early implantation

In practice, commercial bioreactors, including those for skin equivalents (such as Apligraf® or Dermograft® use relatively short bioreactor stages and follow a process-time plot resembling the dotted line in Figure 9.6. In other words, these processes are designed to lead to early implantation. As a consequence, at the time of implantation, constructs are either mechanically weak (with short survival times) or structurally dependent on the original polymer scaffold.

This is feasible for some tissues (e.g. skin) where function can develop slowly, but not for others, such as blood vessels or heart valves, where '*now*' is essential and non-negotiable.

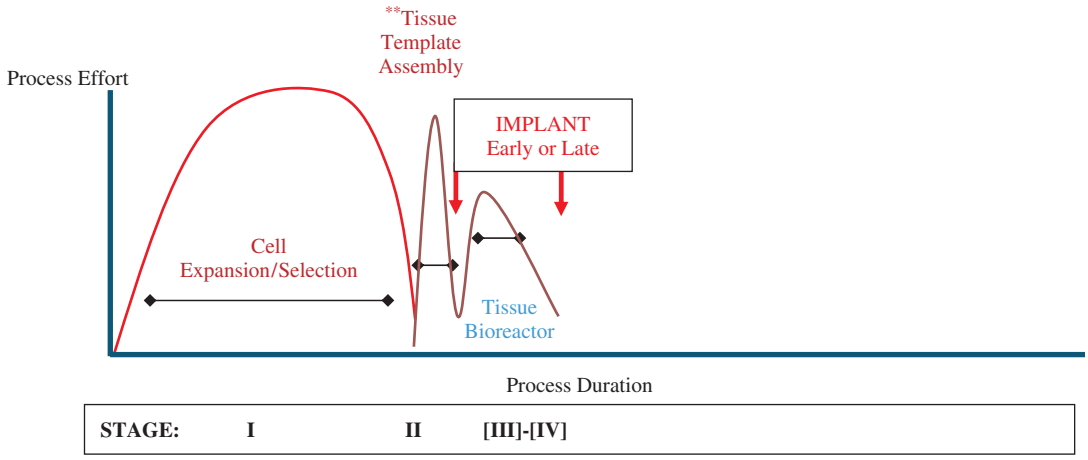


Figure 9.7 Process design graph (nominal axes) typically expected for direct tissue engineering (DTE), based on a native extra cellular matrix (natural polymer) material. Derived from Figure 9.6, in this process the cell acquisition and expansion (I) remains essentially unchanged, but these cells go directly into a tissue replica or template, prefabricated from native matrix proteins (e.g. by collagen, fibrin or, perhaps, polysaccharide material engineering). As a result, a simple but native 'tissue' has been assembled by the end of stage (II), ready to implant as a living graft. Two important differences are clear: (a) since (II) in this case is an assembly stage, rather than a cultivation stage, it is likely to be very rapid (minutes or hours at most); and (b) since the matrix is native, this represents a living tissue graft from the start, not a cell-seeded prosthesis, and so it can *immediately* act as a template to guide local natural tissue remodelling. The need for a distinct bioreactor stage (III) is now questionable, and it is only required to add cell-based complexity.

feature we introduce is the embedding of our cells (fibroblasts here) inside a dense, nano-fibrous network of native collagen fibrils. This example is the cells-embedded-in-collagen-gel system described previously. Ideally, constructs would be made as anisotropic/asymmetric layers, perforated by many μ-channels to improve perfusion (see Chapter 6). While the change from indirect to direct tissue fabrication would require new knowledge in some areas (e.g. in engineering native protein aggregates: collagen engineering), once achieved it would completely alter the process design graph, as shown in the shift from Figure 9.6 to Figure 9.7.

The new (direct engineering) process design, in Figure 9.7, still retains the cell handling and culture stage (I) for cell-seeding into the construct (Text Box 9.5), but cells are now produced as 'one of the starting components', as if they were a reagent rather than the primary producers. Indeed, it may be that this shift alone will generate new approaches in time. If we are going to ask these cells to perform other functions (now downstream remodelling rather than building the initial tissue bulk), it may be that cell acquisition will be less difficult. Similarly, the stage of cell differentiation may be less critical and total numbers less demanding (Text Box 9.6).

However, the changes after cell acquisition show that the effort invested has shifted from cultivation to fabrication of the tissue bulk material, to completely alter the overall strategy. Recent examples of this new DTE approach – and particularly the use of a rapid cell-matrix assembly (stage II) – can make bioreactor culture completely unnecessary. In effect, the directly fabricated construct is a simple tissue in its own right, ready to be implanted as a living (if simple, immature) graft.

Such constructs, comprising living cells embedded in dense, native ECM, qualify as basic tissues, just as those which are produced by the end of conventional indirect engineering using 3D bioreactors, in Figure 9.6. As a result, the *direct engineering* process design can completely remove, or at least dramatically shorten, this major rate-limiting stage. At the same time, the implanted

Text Box 9.6 What do we need so many cells for, anyway?

There is an interesting question here, though it is outside the immediate topic of this chapter, namely, 'If we are shifting the process plan and the requirements of the bioreactor-culture, do we still need the same number or type of cells in our constructs?' Clearly, a lower cell-seeding number could translate into much shorter times for Phase I. Assumptions about the cell type, stage and the number of cells required to engineer any given tissue have tended to be aimed at the rate-limiting step of 'making tissue bulk'. The new, *direct* process plan shifts this emphasis and, with it, our expectation of how many cells will be needed to complete the plan.

For example, if we can *directly* fabricate dense collagen matrices for skin engineering, it seems likely that we will not need fibroblasts to lay down the high densities of collagen that make up the bulky dermal component. Rather, it would then be important to use smaller numbers of specialist calls to introduce cross-links, elastin or matrix-swelling components such as proteoglycans. Alternatively, non-fibroblasts might be able to introduce μ-channels or blood vessels to improves deep perfusion. In other words, the assumption that the cell-expansion stage (I) will remain the same, while stage (II) undergoes radical change, is probably not correct.

So – could 'less cell effort' also mean lower construct cell density, therefore less hypoxia/nutrient depletion, and so much faster cell expansion?

graft is immediately able to participate in local host cell-based remodelling. Hence, they can act as true tissue templates, in the same way as tissue grafts can be remodelled by the surrounding tissues. This is very different to the situation using synthetic polymer-based and prosthesis-like devices, where natural remodelling is, at best, delayed.

A second possibility is suggested by the two points at which implantation may take place, shown in Figure 9.6 – early and late. This suggests that where a bioreactor culture stage is retained (albeit a much reduced time period), it would have a very different purpose. The aim here is to increase the structural or compositional complexity of the construct. For example, resident cells might be encouraged to deposit elastin and minor collagen types for blood vessel walls, add collagen cross-links for strength in fascia implants or proteoglycans and calcium deposits for cartilage and bone, respectively. Such subtle bioreactor functions were largely not envisaged in Figure 9.6, where the first target is to replace the polymer scaffold with a bulk of ECM. In fact, in this 'new' bioreactor role, resident cells would be used to develop the construct complexity well beyond those envisaged in Figure 9.6. Consequently, we would not only expect dramatically increased process throughput, but also much more advanced tissue structure than first envisaged

9.2.3 A real example of making tissues directly

The example so far has been provided to demonstrate how it is possible to analyze a complete tissue engineering process. That analysis has allowed us to identify the weak and the rate-limiting stages, to highlight where the critical problems lie and to redesign the process sequence and timing accordingly. For completeness, it is important to explain that this was not a Utopian example which is implausible and so would never be of any practical value. In this example, the key change in Stage II (construct assembly) led to an increase in the process rate and allowed for removal of the problem Stage (III). This is not an imaginary ideal, but in fact has been demonstrated as feasible, indeed practical, as illustrated in Figure 9.8.

The tissue fabrication and assembly prototype described in this figure (produced as a proof of concept device) would effectively assemble and fabricate a simple collagen tissue in minutes. This device (and now others in commercial production) achieves the new Stage (II) of simple tissue template assembly by directly fabricating the bulk matrix, out of native collagen, around the required cells. *This collagen is aggregated and 'engineered' much as if it were a synthetic polymer* support, to give a living tissue-equivalent template – but in minutes.

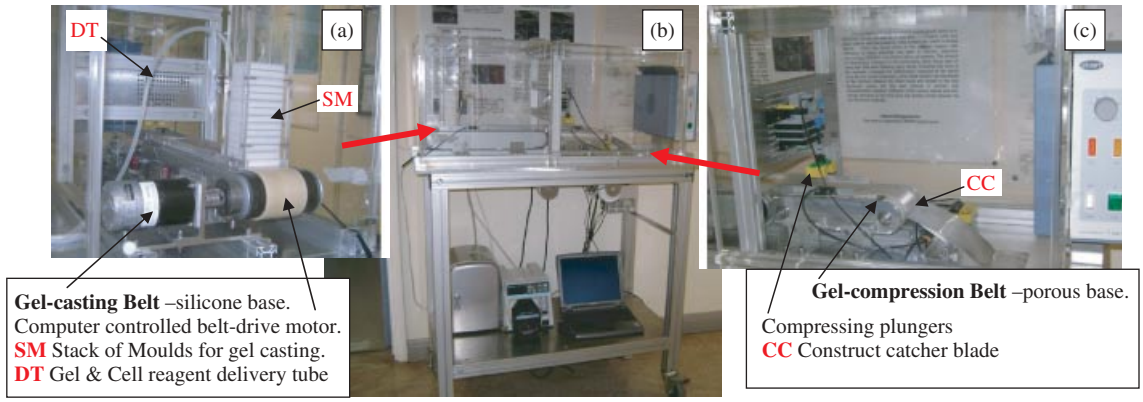


Figure 9.8 Photo of WG* machine, designed to carry semi-automated **living tissue assembly** shown in Figure 9.7. (a) shows a detail of the construct gelling belt with fresh gel moulds in a stack hopper, ready to drop and be dragged onto the silicone belt for filling (computer-controlled delivery of collagen, cells, particles, etc). Gels set (in about 30 min) as they warm on this belt and are transferred to a second, porous surface 'compression belt' (c). Once positioned, the plungers push down into the mould, compressing out controlled amounts of fluid (<5 mins) into the absorbent below. After compression, the now dense layers of living tissue construct are peeled off and stacked in the sequence to give multi-layer complex structures (the moulds are recycled). Timers and position-sensors, switches and motor drive feedback to the control computer (b) to regulate the content and structure of the layers. Importantly, many more collagen/cell/layering controls and components can be added into this base process (including perfusion channels), for fully customized end-tissues. The finished tissue emerges in minutes, as a predefined series of layers ready for use or culture. (*WG-device working nickname: *Wallace & Gromit machine*).

In the WG device design, the entire fabrication device (Figure 9.8b) is housed in a controlled chamber at 37°C, to promote collagen gelling. It comprises two processing conveyer belts. Belt number 1 (Figure 9.8a) is where gels are assembled in their moulds and set, in the sequence they will be layered together; on belt number 2 (Figure 9.8c), the gels are compressed, by plungers to give the required fluid removal. 100 µm thick layers are delivered from the second belt to form stacks of any predetermined sequence, to produce the required tissue construct, by repetition and stacking of layers in sequence.

As with most modern engineering fabrication, it is a *repetitive sequence* of many small sub-processes. In reality, this is repetitious to the point of mind-numbing tedium. And this is the key message which we can learn from the example. The processes and sub-processes are simplified to the point where they can be *fully defined* (and in this case made to work by non-specialists using Lego!). However, because we can *define*, precisely and reliably, *all* of the timings, speeds, durations and volumes involved, we can then build the complexity back up through controlled repetition and sequence.

This is the reality of *directly* engineering tissues. The new speed of its operation means that some readjustment of expectation and planning is needed on our behalf. Two front-runner options open up:

(a) The '**bedside graft delivery**' concept now has a tangible tissue-fabrication-device as an exemplar. The effect of this might be imagined sitting next to the patient, delivering custom-made constructs for a surgeon to implant for minor reconstructions *as they are needed*. In addition to the 'as-and-when' attraction, there is the potential for huge product tissue versatility (in fact, reflecting the enormous variability in the detail of tissues needed by any given patient). In effect, it now becomes a feasible aspiration for the surgeon to dial up the detailed structure of each construct, customized to the patient's

needs. This includes tailoring to the needs of the injury or disease (hand trauma, tumour resection, cartilage-bone degeneration) and to the patient's age, sex, ethnicity and cosmetic needs (e.g. between eyelid, cheek or foot skin).

(b) Alternatively, there is a '**mass production**' mirror-image logic which envisages a very large fabrication machine operating at a remote factory site, making, packing and shipping literally thousands of *identical constructs*. Where the cells do not need to come from the patient themselves (e.g. allogeneic cells), this would meet demand for off-the-shelf tissues (e.g. skin grafts for burns, major trauma or leg ulcers). This includes new 3D model tissue applications for animal replacement test kits for pharmaceuticals, cosmetics, chemical toxicity, hospital diagnostics, forensics and research (discussed in Chapters 4 and 5).

The gatekeeper step which opens these new (extreme) horizons is the minimizing/elimination of slow cultivation stages. This must reduce costs, with dumbing down (computer automation) of operations and applying a rocket to 'reproducibility'. So, examination of time and sequence can be the key to locating 'the box' we need to think outside.

To conclude, in those areas where strategies rely on biological controls (tissues in the *in vivo* bioreactor and cells in culture-rich bioreactors) we can take advantage of the faster, simpler and cheaper nature of bio-growth and cultivation, **where the products meet our needs**. However, because these are under rather limited human control, our options are limited where the results are not so good (e.g. slow, poor tissue quality, etc). The real step forward, then, comes where we replace cell-dependent production stages with equivalents which use cell-independent engineering approaches.

9.3 The 4th dimension applied to bioreactor design

9.3.1 Change, change, change!

So, picture the most successful bioreactor design you can imagine, carefully assembled to purr along, producing superbly functional tissue slices. With time, in culture, we would hope that these slices gradually increase in strength and complexity until the surgeon cannot tell where they came from. Importantly, though, where your bioreactor has achieved this happy end-point, there is one trick that, by definition, it *must* be managing to do. That essential element is *continuous change*.

As the construct changes from 'just assembled' to 'tissue-like', it must change its matrix properties (diffusion, mechanical, physical dimensions). It must change its cell properties (density, distribution, synthetic activity, perfusion level) and its fluid content (from high to low, protein poor to protein rich, oxygenated to hypoxic). So, in order to keep the bioreactor process functioning through the inescapable biological sequence in which new tissue develops, its running conditions must also change. These changes can either be in response to changes in the construct or, in some cases, they will *predict* and even *drive* construct development.

This is where we reach another crunch-point. How can our bioreactor conditions be designed to change together with, or ahead of, the needs of its cargo? Disappointingly, the simple answer is: **it depends**.

However, there is another more useful (if equally obvious) catch-all answer: **when the bioreactor sensors tell it to change!**

This sounds pretty trivial, but used correctly it is hugely helpful. At least a little humorous triviality here ensures that we *remember* to get the basics right. The simple secret is that there is at least as much philosophy as technology in good monitoring systems. In other words, the key decision of what to measure (and so how to measure it) needs cold logic and careful analysis*. This is not *necessarily*

* A wobbly basic selection generally keeps on wobbling all the way downstream. Also, contrary to common optimism in biological sciences, two wobbly basic logics do not cancel out; rather, they square the downstream wobbliness.

the same as buying the most cutting-edge, laser-flashing devices, or expensive, emotionally satisfying marker systems – though it can be.

The problem is that the monitoring component has normally not been an integrated part of the philosophy of your treasured bioreactor, built in with steely forethought from the start of the process. More commonly, it is a near-panic-led afterthought to correct major process shortcomings, as and when they arrive. Good monitoring can make a mediocre process work and a good one excellent – but poor monitoring can allow the very best process to *fail*.

The aim here is *not* to list and critique all the possible monitoring systems available. Not only is this impractical and boring, but that information is already easily available. It is more important for you (as a future process designer) to understand the logic which can lead you to just the right technologies, literatures and equipment manuals. More ambitiously, it would lead you to understand *when* to install the monitoring system.

So, by way of a conclusion, two questions:

- When is change not an issue? (*A: When you switch off the bioreactor*).
- What sort of changes do we measure? (*A: changes of rate, magnitude, frequency and direction*).

9.3.2 For bioreactor monitoring, what are we really *talking about?*

The first thing is to demonstrate where this idea of measuring, sensing, monitoring comes in, and why. This can best be done at the same time as dissecting out exactly what should make a good bioreactor work well. In this case, it can be useful to generalize, since so many biological processes operate in distinct sequences and stages. They have easily identifiable start-and-stop cellular and molecular events. Figure 9.9 illustrates how this can translate into a tissue bioreactor sequence, where each stage is 'triggered'. Once a new stage is initiated, the bioreactor continues to 'cultivate' under its new conditions until this stage is, in its turn, completed and the next stage-trigger is activated. The simplest version of this would be the increase of tissue dimensions, physical stability and/or complexity, over and above those of our initial assembled template. Such an 'expansion-stabilization' stage in bio-engineering corresponds to 'growth-maturation' in biology.

Where these stages overlap, or have indistinct beginnings and ends, then the process has to compromise in its identification of the 'trigger point. This involves locating the *least damaging or problematic* value of the monitored parameter for *both* the previous and the approaching stages (i.e. already triggered and about to be triggered shortly). For example, a cell density of between 1 and 5×10^6 per ml might be good for promoting an ideal cell differentiation during Stage 2 of the process. The next one, though (Stage 3: matrix deposition), turns out to work best at cell densities above 8×10^6 per ml. A good compromise 'Stage-2-to-3 trigger' value would then be in the region of 6×10^6 cells per ml.

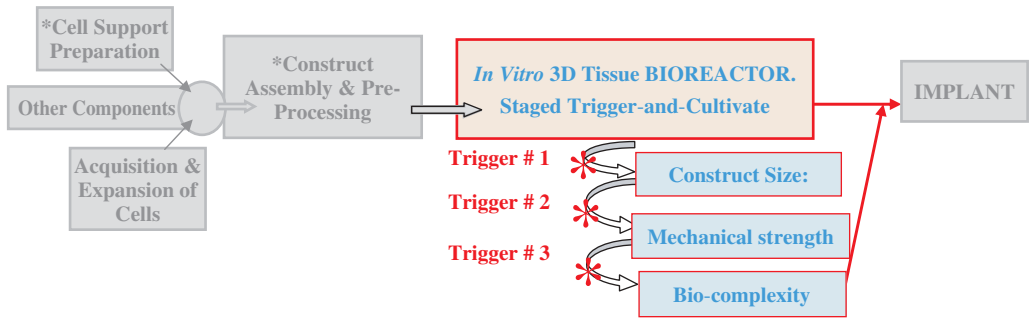


Figure 9.9 This diagram has evolved from Figure 9.5, focusing on and adding detail to the bioreactor stage, illustrating the 'trigger and cultivate' logic. In this example, changes in bioreactor conditions are triggered where: (1) the construct reaches a predetermined diameter or thickness; (2) its mechanical properties exceed a given value; (3) it gains a level of bio-complexity marked by the expression of one or more new proteins.

'Trigger-and-cultivate' processing is perhaps the most common we are likely to meet in cell systems. The construct goes through stages of growth or development, so the bioreactor needs to add factors or change parameters (e.g. mixing, temperature, pH, oxygen tension) as each stage is reached. This mirrors how natural systems develop, sometimes referred to as 'cascades' or 'cycles'. In effect, we must detect when that stage has been reached in order that the change in conditions can be triggered.

The example illustrated in Figure 9.9 has a relatively simple set of three trigger points using three measureable parameters in the construct, overall size, mechanics and marker protein expression. In this simple case, the system uses sharp step changes in bioreactor conditions (perhaps addition of a new growth factor or application of intermittent cyclic loading).

Where the control mechanisms are understood at a more subtle level, it might be better to grade one set of conditions into the next. For example, core O_2 perfusion might be gradually increased by graded fluid flows to meet the needs of greater cell consumption or longer diffusion path-lengths as the construct grows.

So, to summarize, monitoring at its core is essential to identify where a critical parameter has reached its trigger-point, and feedback is then needed to change the bioreactor conditions. This is the point where 3D tissue bioreactors show themselves to be either effective and well designed, or just likely to run out of steam. 'Running out of steam' is a term more colourful than precise; rather, what we see where the bioreactor needs better monitoring and feedback is a gradual decrease in the rate of change (development) of the tissue. In fact, the cells have their own feedback, so the construct rarely 'dies'; it just stops progressing! Better and more frequent tuning of conditions keeps change happening.

9.3.3 Monitoring and processes – chickens and eggs: which come first?

The big (or just easy to ask) questions here are how do we identify the key parameter(s) and how do we monitor them? In detail, of course, what should be measured depends on what is being engineered. Monitoring of the interior colour coordination in a dumper truck factory-production-line is going to be a lowish priority. Corrosion protection will be the winning factor for truck makers, though less so for manufacturers of city-shopper cars. We can, however, ask ourselves a few questions which will lead to a helpful design philosophy, whatever the nature of the tissue system.

1. What are the three or four main **sources of the variation** in the process (and do they interact)? This question tells us where we are on the spectrum of 'reasons why we are monitoring'. Processes with inherently variable or unpredictable outputs (e.g. bio-cultivation of human tissues) need wide tolerance ranges with reliable systems to *find and reject* the extremes.
2. What are the most important **functional** factors governing target tissue performance?
3. What would be the most **damaging features** if they developed (and what is the risk of this)? This 'global' question identifies how the final tissue construct really *must* and *must not* perform to be useful; and then, if the most important of these **functions** can be measured *directly*, or must be deduced from *indirect* measurements.
4. What are the most *prominent stages* in the development of the desired tissue?
5. Are there any *graded changes* and which could be candidates for measuring rates of change? Here we are considering the finer detail of 'bioreactor events', perhaps at the day-to-day level (and potentially minute-to-minute for some events). The simpler logic deals with these as parameter-switches, in effect go/no go, or keep/reject markers of good and poor constructs. Parameters measured here must be *clearly* either in or out of a pre-set range. The more sophisticated logic (when it is appropriate) leads towards measuring parameters which gradually change (e.g. fast or slow), so that the process can be tuned as it progresses.

The more analytical reader will notice that these questions represent a series with a rapidly narrowing

focus, but along a single logic track. In more personal terms, it might run from 'Why am I here?' to 'What's for dinner?' via 'Who won the football?'

The first question determines where we sit on the most basic spectrum – namely, are we discussing monitoring a process which is *fundamentally variable* or one which is *defined and predictable*? A good parallel can be found in ceramics and pottery. In this case, we can be making machine-made tiles for the walls of hotels and banks, or individually hand-thrown mugs and bowls for Christmas gifts. The analogy here is that the ceramic tile producer and the hand potter both have to identify and understand just what their main user wants *most*.

Large areas of wall tiles build up into neat, symmetric patterns based on long (white grouted) straight lines between the tiles. This gives them (and so the customer's building) their grand beauty. Our eyes are exquisitely tuned to appreciate the perfect straightness of the lines, especially over very long distances. The concept here is that:

$$[\Omega(\text{Length of uninterrupted tiled wall}) + \Phi(\text{Number of grout} - \text{lines})] \times \gamma(\text{price per tile}) = W \text{ or } L$$

where W = wealth of the bank and L = luxury of the hotel.

The task of the tiler who constructs these patterns is relatively easy, provided the tiles themselves are all 'perfect'. Perfect in this instance means identical in dimensions, angles and thickness, *at all points and in all planes*. Even tiny inter-tile variations, fractions of a millimetre in length or degrees of corner angle, can make the job difficult or impossible. In turn, the customers/users are distinctly disappointed when the scale or precision of this pattern is degraded, reducing both W and L to all (the putative clients) who view the structure.

On the other extreme, customers of trendy gift shops and boutiques want, above all, a gift that is distinctive and unique. The happy recipient of such gifts feel especially honoured to own items as individual as they feel themselves to be. The contrast with 'corporate tiling' could not be clearer. But what does this teach us about process monitoring? After all, the Mega-Ceramics tile fabrication machine makes perfect tiles and the craftsman potter makes quirky, individual cups and saucers.

The interesting, even surprising, point is that both industries need to be rigorous in their process monitoring. Surprise comes as the tilers might not understand why the potters bother, and the potters wonder what the tilers have to measure. The answer is both simple and illuminating (Figure 9.10). Even where the product is varied and 'random' (variation being a merit), some randomness can be damaging to the basic function. On the other hand, quaint shapes and paint patterns are good in a cup, but having the handle or bottom drop off is definitely not. There are functional limits even to quaint variation.

So, in the case of variable products, the processes must be monitored for *basic* functionality and how long that function lasts. In contrast, the tile manufacturer's machine allows very little measureable variation to develop, but the consequences of just a few rogue tile shapes or pattern colours are so damaging that careful monitoring of *machine* performance is essential. In the tiler's case, the real value of monitoring comes with the certificate of quality given to the customer and, more subtly, the very visible sale of cut-price reject tiles to 'less discerning' users. These two factors keep up the price that the tiles can demand for perfect consistency. In the potter's case, the value comes from fewer discussions with lawyers who specialize in scald injuries.

This analogy illustrates the important tissue engineering spectrum of 'inherent variability'. Many processes, such as those based in hand-crafts, biology and cultivation, tend to have relatively variable outcomes. This is especially true of processes dependent on human cells, which vary hugely. This hugeness then gets even bigger when the cell come from sick or injured people – in other words, patients. Our control over these processing variations is often minimal (which can explain the proportional and progressive disengagement, of our colleagues from the 'engineering tribes').

At the far end of this spectrum of process variability, monitoring is often set up to ensure that

(a) (b)

Figure 9.10 Identical floor tiles and variable cups. (a) The need for identical, non-varied tiles is clear from the perfect patterns and long straight lines of grout which make up the desirable wall and floor effects. (b) On the other hand, the distinctiveness of individually made cups and bowls is central to their value, with visible, quirky differences being deliberately introduced to each item.

key *functions* are fulfilled. They check that the construct performs within fairly wide tolerances and does not fail catastrophically. The monitoring, in this case, may be designed to measure a selected 'performance indicator' in the finished constructs. When the value of that performance parameter is above a predetermined cut-off, the pot (or tissue construct) is accepted and boxed ready for sale. However, immediately it falls below the triggering threshold, it is rejected and sent to be smashed (right hand side of Figure 9.10). In engineered tissues, this type of pass/fail monitoring might involve histological examination of the tissue structure, the number of living cells (as opposed to dead areas) or the ability to hold sutures during surgery. So we see the parallels with the accept/reject system which operates in making hand-crafted cups. In this case, variability is not a problem (in fact, it is a benefit), so long as the handles stay attached and tea does not dribble into the saucer.

As the detailed mechanisms of the process become better understood, there is a natural shift to the right, in Figure 9.11, in the types of monitoring used and the way in which results are applied to the process (i.e. process feedback). This is because greater process understanding opens the possibility of predicting events and intervening *before* the product is complete. In other words, the aim is to tune the process as it is running, not after the event. This might, for example, involve speeding up or slowing down one of the process stages, making the constructs thicker or thinner or inserting more/less cells.

Clearly, there are considerable advantages where we go down this route. No longer does *every* product item, be it a tissue construct or a cup, have to be functionally tested (not a good situation – see below), but neither do all defective constructs *have* to go through the whole process before rejection. More importantly, this form of predictive monitoring allows us to introduce feedback changes to 'correct' or adjust conditions *during* the process. Process correction implies that we can also establish bands of acceptable construct performance, as opposed to the previous sharp 'fail/pass' line.

This evolution of how we aim to monitor the process is commonly based on increasing levels of understanding of the process itself. Interestingly, this it is more apparent in top-down processes such as in cultivation of cells or whole organisms, from farming to tissue engineering. Where it becomes possible to use low-variability components, and where the process becomes increasingly sophisticated, we can see that it is possible to

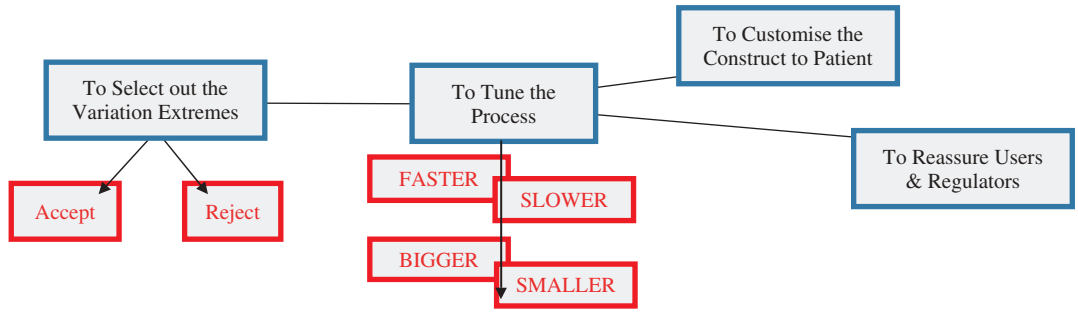


Figure 9.11 Diagram to illustrate the spectrum of targets for process monitoring. This demonstrates the tension which exists between 'simple' reject/accept of useful end products, as opposed to constant fine tuning of the process, depending on the level of 'inherent variability' in the process. The left of the scheme shows the approach of selecting on the basis of simple end-function competence. On the right, the logic is to feed the responses of our monitoring back into the process (e.g. bioreactor conditions), to fine tune it as it is running. Monitoring in the latter case will use parameters which are indirectly related to primary function and can be used to modify the process conditions. Though it is more complex to set up, process tuning leads to customization.

move further to the right of the spectrum in Figure 9.11.

It is possible to view this as moving up the process 'food-chain', in that the process itself can be monitored as the construct grows and matures. In tissue engineering terms, this can go hand in hand with the ability to:

(i) move away from dependance on autologous (patients own) cells, perhaps using pooled donor cells or re-programmed stem cell;.
(ii) use well-defined fabrication processing wherever possible, with less dependence on cultivation and biological production.

However, it also tends, in the case of tissue engineering, to take us further into the future.

The features of cell behaviour utilized here need to be understood to the level where rates of division/death can be predicted across a range of conditions and over time. Parameters such as percentage differentiation and response to reduced oxygen, for example, can be defined before cells enter the process, along with confidence limits. Where these parameters can be measured, they act as benchmarks for comparing constancy or variance of successive 'runs' or process cycles.

Process control tends to move further to the right in Figure 9.11, where we consider bottom-up engineering processes such as mobile phone or automobile production. Where this happens in engineered tissues, our monitoring control also takes on a special value and new uses. The upper branch in Figure 9.11 indicates the opportunity to replace the broad ranges of 'acceptable function' with *fine subdivisions* which lie within an acceptable range. This is important, as it moves us towards designing the final construct to *meet precisely* the patient needs, i.e. customized tissues. Where we reach this level, we have raised our target to well above the current aspiration, which presently aims to produce average, general or lowest common denominator tissues.

For example, we currently aspire to make skin grafts which are biomimetic yet *average*. This combination is at the same time both correct and disappointing, because the level of target biomimesis is so modest. 'Biomimetic' here means having two layers, one with fibroblasts in collagen, the other a surface covering of multilayered keratinocytes. Clearly, this very general definition of 'skin' can describe pretty well any part of the human body, so it is also 'average'. In contrast, the equivalent *customized* skin would be mimetic of a particular site, and definitely *not* average – resembling eyelid, forearm, back, palm or facial skin. It would be different for a child, a boxing champion or a pensioner! You have your own example of a skin to test out here. How many different 'types of skin' can you find over your body? We are looking for

elasticity, orientation, stiffness, thickness, colour, hairiness (and other fluffy bits), pimples, dimples and creases.

Once we can monitor and fine tune the process, in ways that are common in conventional manufacturing, customization ceases to be a dream. By analogy, few of us would now expect to say to our bathroom fitter, just get me 'a toilet' (Figure 9.12). We think nothing of trawling through hundreds of types, sizes, colours, shapes and levels of embellishment, depending on our pocket-depth, mood and bathroom-location. It is truly surprising, then, that our aspiration has for so long stopped at 'just skin'. Perhaps an unexpected and happy side effect of sequence analysis in extreme tissue engineering will be new level of immodesty, as we see what is possible.

Finally, the last, far right consequence of process tuning, in Figure 9.11, is the extra value which comes with the tightest (and most expensive) monitoring (Text Box 9.7). In this case, the most sophisticated forms of monitoring and control data can be used *as an output in itself*. This is where the availability of such in process data can be used to reassure the product user (in our case the surgeons, patients or government regulators).

Mobile phones and precision lab equipment often come with data sheets declaring the accuracy and performance of that batch, or even that individual instrument. For the mobile phone, this might be the battery life or range of signal detection. For lab liquid handling pipettes, a data sheet would give tolerances for the volumes dispensed (accuracy) and reproducibility over many operations (though sadly we still await a coefficient of 'resistance to students'). Where the process is designed for pharmaceutical production, government regulators will keenly scrutinize the chemical purity of the drug, the content of the pill casings and the accuracy of the active ingredients in each tablet. At this level, the user reassurance that comes with such data means that process monitoring has, in effect, become a central part of the product (i.e. the tissue construct) itself. While much of tissue engineering is not yet at that stage, it would seem prudent to plan for its arrival.

9.4 What sort of monitoring: how do we do it?

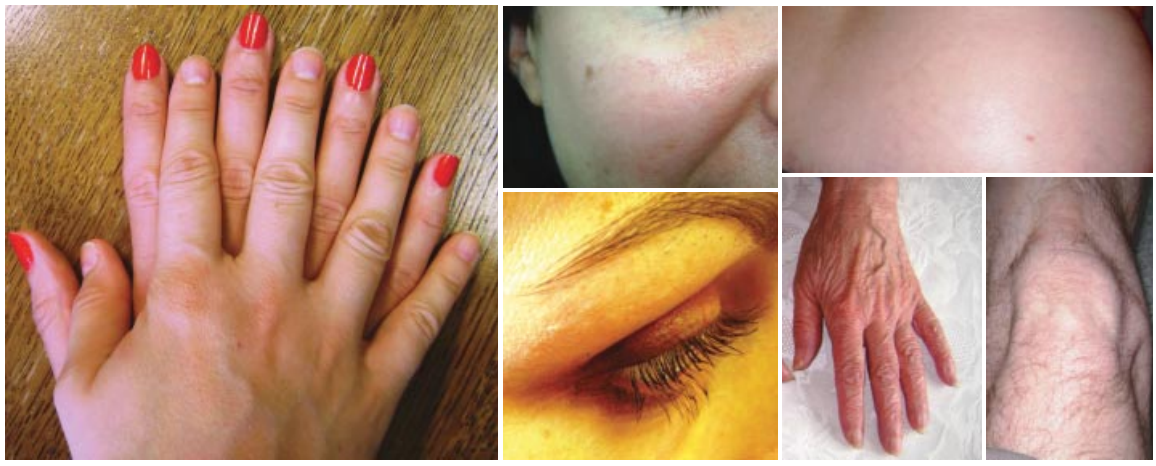
Parameter selection, and the measurement of how parameters change *over time*, are just as important for successful bioreactor operation as they are for monitoring performance of an implant once it is in the patient. By now, the reader should be familiar with the habit in this book of *not* providing lists of what to do or not to do. True to form, we are not going to end with tables of how to measure this or that feature of your skin, bone or blood vessel construct. Rather, the theme of 'extreme tissue engineering' is to identify and analyze the concepts behind monitoring, so that the reader can tailor his/her *own* design rationally to the needs of their *specific* construct and its *particular* disease/injury application and anatomical site.

Listing these possibilities is too large a task for this volume, and it would be dangerously restrictive even if it were possible. As with the London Underground, it is only rational to work hard at understanding the platform and escalator signposts or route-planner – there are just too many track and train permutations to give people an instruction manual or SOP of how to use it.

9.4.1 Selecting parameters to be monitored

The previous discussion has concentrated on theoretical aspects; now we should look to the practicalities. What parameter(s) or characteristic(s) *should* we measure, and in what priority? Answer, of course is simple but again seems unhelpful; it depends on the function that the construct is designed to carry out. Skin must be water-proof and tough; nerve-repair guides must carry axon re-growth *fast* and in one direction. Conversely, it can be just as important to monitor for things that the construct definitely must *not* do:

- Thombus formation in small-bore blood vessels is a *major* 'no-no'.



(a)

(b)

Figure 9.12 Of toilets and skin types: customization of variables and fine-tuning to function is already a default requirement in society. The question is, are we aiming too low with the notion of making a 'one-size-fits-all' or 'average' skin, any more than we would be in expecting an *average* toilet to suit all our homes and businesses? In the end, we need tissues which function *and* match their recipient. An octogenarian might dream of a *whole* new skin, but might not appreciate having a patch of 20-year-old tissue grafted into their 'old' hand. (a) shows a few skin types. From left, clockwise: young adult back of hand; cheek skin; mid-life back skin; hairy (non-footballer) knee skin; older person's hand skin; female eyelid. (b) shows a few of the forms of toilet in 'common' use. From bottom left, clockwise: typical modern, minimalist male urinal; traditional ornate (Spanish) urinals; modern US-style WC (China); classic (high cistern) early 20th century WC; high-tech, electrically heated combined WC and bidet (Japan).

Text Box 9.7 Evolving of process monitoring in top-down systems: milk

In the 19th century, a dairy farmer might reasonably have developed a milk production process in which the cow converted grass into milk in his smallholding or farm. He periodically extracted this cow-juice, bottled and sold it. Unfortunately, milk and cows being what they are, many people became ill from drinking this product. The situation was made worse because mothers fed cow's milk to their infants, thinking it better than their own (some things never change . . .). Clinical infections such as bovine tuberculosis, undulant fever or brucellosis were frequent and lethal. Poisonings such as 'milk sickness' (from cows eating poisonous weeds) or due to 'swill milk' (where cows were fed on distillery waste) were also seen. Early farmers may have fed batches of milk to the farm cat before selling to their best customers, to check for (and reject) the very worst batches. This is a gross, pass/fail test for acute toxic or infective defects, *assuming the cat is susceptible*.

As time went by and the infective nature was better understood, suspect batches of milk might be sent to the local dairy centre for culture on bacterial plates, identifying the infection type and titre (load or density). This made it possible to assess the milk quantitatively, based on tables of human tolerance to this or that level of each microorganism. Interestingly, at this point, it may have become possible to effect crude full-process tuning – or feedback control – by testing *the cows* for that infection and putting down those which tested positive (which is still the practice for foot-and-mouth disease).

With yet further understanding and technical investment, it is now conceivable to test the milk online for biochemical markers of the worst (i.e. key-marker) infections or for known poisonous contaminants. At this stage, we might expect problem cows to be immunised or given antibiotics, rather than destroyed. This represents a progressive track back from direct, crude functional testing, through reductive bacterial culture testing and finally to indirect or implied, molecular testing. In addition to progressively improving the process, the milk-products and the herd, this is great news for the dairy cat.

Plausible as this scenario is, such fables often do not play out so perfectly in practice. The reality is that the improved understanding which develops along with better monitoring can provide simpler and cheaper alternatives to the *process* itself. In our milk example, the insertion of a Pasteurisation stage to the process largely eliminated bacterial infections, although microbiological testing developed in parallel for other reasons. Also, fencing off the cows from toxic weeds and using healthy foodstuffs prevented poisonous milk. Indeed, better process knowledge though development of monitoring systems commonly leads to major process changes which are, in fact, *less* complex than the original.

Interestingly, we *still* rely on the cow as the core self-monitoring, self-tuning and economic bulk grass-to-milk converter-machine. Where the cow can/will not meet the key-marker output measures (frequently because of infection), we still shoot it and start again. However, this economy-driven simplification of the monitor-feedback loop is only available to whole-organism culture processes. Delegation to the cow is not a luxury available to the tissue engineer. We must take on the mantle of the 'constant tailor', perpetually measuring and adjusting the process. So, as in other fields the constant tailor not only develops how and what he measures, but also what it means to the *process*.

- Urate crystal seeding into our urothelial constructs spells 'seriously uncool pain-in-the-bladder-region'.
- Immunogenic reactions to our favourite skin equivalent is rejection in any language.

This all sounds a bit glib, but notice it refers to 'what *the construct* does or does not *do*'. It is not based on what target tissue it is *supposed to be*, nor what it *looks* like. But the answer will be different for each implant (note: 'implant', not 'tissue') we may choose to engineer in the future. As a result, this is probably the most specific answer we can hope to get at this stage.

The take-home-message is that we really must aim, at the earliest stage, to establish which parameter(s) are absolute 'must-haves' specifically for our new implant and its application. Clearly, once identified, these will help to define the one or two monitoring systems which need to be built into the process from the start.

9.4.2 What is so special about our particular 'glass slipper'?

If you are into making glass slippers, it is sensible to make *absolutely* certain they are a perfect fit for the feet of the wearer. So, let us plot out the logic we need (right from the start) to identify this special fingerprint analysis which will link our 'glass-slipper' engineered tissue to its unique implant target.

It is not easy to determine the real 'Number 1' where the range of possibilities is so very large. After all, this not only depends on the tissue itself but its eventual anatomical location, the state of the patient and his/her local tissue bed. A tendon can operate a pianist's finger by transferring feather-like loads with smooth precision; while in the foot, the same kind of tissue, in the form of the Achilles tendon, can drive a sprinter's full body weight forwards at speed. A nerve can serve facial muscles at one extreme, or sprout out from of the spinal roots at the other. A leg vein in a 60 year old patient and a pulmonary artery for a child may have many basic structures in common, but their primary demands are different. The age, injury and disease type – even the drug regime of the patient – can determine the primary must-have function. In the example above, reconstructive surgery of flexor tendons in the hand requires *relatively* modest mechanical loading, but tendons *must* glide freely or the hand cannot work at all. As a result, it is a primary requirement *not* to form fibrous adhesions. Achilles tendons must carry *very* large loads; but the problem of adhesions is minor.[23]

It is, therefore, not enough to go to classical anatomy or histological textbooks (Text Box 9.8) to identify the must-haves, because they:

(a) normally deal with mature (end point) tissues, while we need to measure the stages on the way to maturity;
(b) mostly describe static structures – but people move, so implants need to be dynamic;
(c) describe (in the main) healthy structures, while implants are for injuries, and patients take drugs.

Rather, the information we need comes from discussions with surgeons, pharmacologists, wound repair biologists and engineering collaborators – simple for any good tissue engineering team.

The detail of this *primary* must-have function still depends on a range of factors which are specific for each implant type, as shown in Table 9.1. Clearly, it is not always practical to select monitoring parameters and systems on a rigorous case-by-case basis, as implied by this table – there are just too many variables. However, it is not difficult to balance the general of the production and the specific of the implant needs. This involves compromise between practical process needs and efficient function of the construct. For example, it is simple to group the tissues and injury sites such that a platform process and monitoring system can be designed (e.g. for general nerve-guide implants). However, this can have the

[23] Note the examples have an extra layer. Contrast what the pianist needs as opposed to, say, a tyre-fitter. A face and a leg nerve reconstruction may be provided for David Beckham and Michael Caine – but which gets which matters to the result needed.

Text Box 9.8 Key tip: the Jumbo Jet principle

We are 'engineering' tissues here and it is not always important for the construct to 'look' exactly like its native bio-equivalent. After all, clinical needs can include performance of temporary or partial support of a body function. For example, an extracorporeal liver would support between transplants, and cardiac-assist muscles will supplement heart outputs. We sometimes just want to guide the natural repair process (nerve regeneration in the hand), and sometimes (e.g. in spinal injuries) we need to make tissues which never existed naturally. So, 'looking like the structure' of the native equivalent is often *not such a good monitoring target*.

After all, when we *functionally* fly like a bird to New York or Beijing, we are only too happy that the Jumbo Jet has *no* feathers and *minimal* wing-flap (see front piece p. 216).

Table 9.1 This illustrates the divergence of the 'absolutely-must-have' parameters for a few example engineered implants. These turn out to be a little like the needs of the distinctive hand-made pots.

Tissue type	Body location	Patient age	Example injury	Probable primary need
Tendon	Hand	45	Sharp trauma	No adhesion
Tendon	Achilles	25	Sports injury	Tensile strength
Nerve	Face (to mouth)	25	Surgical accident	Fast re-growth/cosmetic
Nerve	Spinal root	25	Road accident	*Any* nerve re-growth
Blood vessel	Leg vein	65	Disease	Non-thrombotic
Blood vessel	Pulmonary artery	12	Congenital	Mechanical strength
Skin	Cheek	18	Resected melanoma	Cosmetic match
Skin	Leg	65	Chronic wound	Strength and stability
Cornea	Corneal epithelium	28	Chemical burn	Maintain stem cells
Cornea	Full depth cornea	58	Cornea endothelial failure	Functional endothelium

potential to produce sub-groups of constructs for different patient groups and applications.

So, to summarize, precision in this early selection stage is a pivotal point in process design, where sound intellectual analysis leads to selection of the critical parameters. This early 'brain-work' will determine success or failure for years to come.

It is helpful here to understand the various categories of analytical approach which are available, and to practise balancing their advantages and drawbacks.

(i) Explicit/implicit, direct/indirect

Explicit/direct monitoring systems go straight for the functional focus, determining the *actual* key function. For example, this could be direct, explicit assessment of the lumen thrombogenicity of a blood vessel construct while it is being produced. For this, we might introduce whole blood (containing fibrinogen and platelets) and measure how much fibrin clot forms on the walls in unit time. This is as direct an assay as you could get, although it is difficult to implement repeatedly over time without damaging the construct.

An indirect/implicit test might monitor the production, over time, of endothelial cell markers such as anti-thrombogenic compounds or thrombolytic enzymes (e.g. heparin-like molecules to block coagulation and plasmin to digest clots when they form). Such biochemical assays would be far simpler to design for real-time data collection, and much easier to repeat or re-analyze. More particularly, our excellent understanding of coagulation biochemistry, and the good correlation between such markers and function, would make interpretation of the output data pretty robust.

(ii) Destructive versus non-destructive testing

Where the aim is to engineer, for example, a tendon, it would seem reasonable to follow the development of an aligned fibrillar structure or acquisition of uniaxial mechanical strength. In this way, the effectiveness of the process, and its stage of development, could be judged and adjustments made to the cell culture conditions. However, the classical (gold-standard) method of determining tissue structure is through histology: fixation, embedding, thin slicing and staining of the tissue for microscopy. Similarly, a functional break-stress test involves clamping and loading the construct until it breaks in the middle. Obviously, while both of these would be excellent for giving direct measures of functional success, widely accepted in biology, the construct is destroyed, and so our knowledge is 'past tense'.

Destruction of the test material is a major problem for *in vitro* processing, as it is wasteful and not real-time, so not helpful for process tuning. Its results are indirect, and data must be extrapolated to constructs which are *not* destroyed. For clinical use, where constructs are grown within the body, or for assessment of construct progress post-implantation, it is normally out of the question. Try to imagine the reaction of a surgical patient if the implant team were to demand that they must remove the tendon six months after implantation, to make sure it was up to scratch!

While destructive testing is just about acceptable during the research phase, even here it is expensive, it is time-consuming, and it gives indirect data. As with other aspects of process design, it is good practice to plan for this early. In other words, the aim is for destructive testing to be minimized as an early requirement in the design (Text Box 9.9).

Adjusting to the technical need to find new, non-destructive monitoring approaches is, yet again, a matter of shifting our tissue engineering tribal thinking into 'extreme tissue engineering' mode. Where our aim continues to be to impress our biological tribe members and elders that we truly *have* made a tendon, skin, cartilage, blood vessel or muscle, we are likely to cling to the familiar (semi)-destructive methods. It is only when we try to sell this to team members from surgical or engineering tribes, and experience their gentle laughter and tough questions, that we wish we had thought ahead. The truth is, this is one of the *diagnostic points* distinguishing the naïve and the newcomer groups with limited inter-tribal mixing from experienced, habitual collaborators.

(iii) Invasive versus minimally invasive monitoring

This distinction parallels that of *destructive/non-destructive* monitoring. In the latter case, the sample may suffer damage and destruction, while in the former it is the patient who is not damaged. The less invasive the information-gathering step is to the patient (or the bioreactor), the more often and more easily data can be collected. However, the more invasive and the more destructive the test, the less ambiguous, more clear-cut is the meaning of the test.[24]

In both cases, then, the quality of the test method commonly has to be balanced against the damage done in getting that information. Procedures needing open surgery to collect large lumps (biopsies)

[24]The tension between these data collection modes mirrors the tension between surgeons and pathologists. The surgeon has the opportunity to do everything to save the patient, but often can't know what the problem really is. The pathologist knows the problem, *exactly* – but can't do a thing about it.

Text Box 9.9 Case study 1

We clearly *must* develop non-destructive monitoring techniques suitable to measure the primary functions at *some stage* in the tissue engineering process. Therefore, logically, this should be in the early process developmental stages, where they can be integrated and adapted most flexibly. In the tendon example, there are alternatives, such as minimally invasive optical fibre scattering measures. Quantitative fibril density and alignment data from such analyses can be used to follow structural development changes in real time.

This cannot deliver the mass of detailed information, especially around cell distribution, that is provided by histology. However, this point is exactly the message of our case study. We have already established the importance of *early* identification of the primary 'must have' parameter to be monitored. For tendon, this would be collagen density and alignment, *not* cell distribution, which might be a distant third or fourth in ranking.

Equally, our basic material-mechanics knowledge allows *excellent* extrapolation from such material-fibril parameters to stiffness and break strength without having to break the construct every time. In short, non-destructive testing of functional measures is frequently possible and is an early requirement.

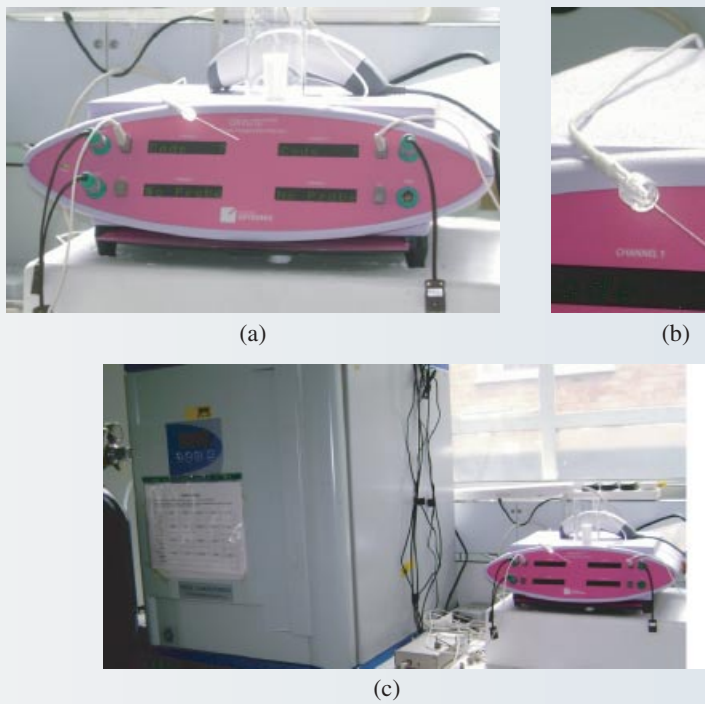
of tissue are invasive. Minimally invasive collection would be performed down fine needles with a minimal scale of surgical intervention. As a general rule, the more an anaesthetic is needed (the bigger the volume of patient anaesthetized), the more invasive the test. Non-invasive techniques such as ultra-sound, optical or MRI imaging involve no physical entry at all into the patient's body.

Key, then, is that process monitoring is designed strictly to balance these tensions, rather than on grounds of tradition or familiarity for the host tribe.

Text Box 9.10 Case study 2

An example of real-time data collection would be the measurement of changing, real-time oxygen levels in the core of our cell rich skin construct. This can be monitored directly using a 300 µm diameter fibre optic probe (Figure 9.13), with minimal O_2 consumption. Together with data on the changing matrix density over time, and the sensitivity of your particular cells to hypoxia, it becomes possible to determine when and where cell stress or death *is about to occur* in the construct. In other words, core O_2 levels are converted by a simple computer model, into predictions of cell viability in space (3D location) and time (in the future).

This off-the-shelf technology would clearly transform our traditional 20th century tissue fermentor into a 21st century 3D bio-process system. In the former (20th century) approach, we establish when/where cells *had* died as the basis of accepting or rejecting the skin construct *at the end* of production (just as, in the niche potting industry, excessively wobbly mugs are smashed). In the 21st century approach, the O_2 sensor and computer model automatically feed data and its conclusions back to change the perfusion conditions in the culture chamber *before* cell damage or unwanted changes occur. In this case, functionally constant constructs emerge with their certificate of quality (just as precision tiles leave the ceramics factory).



(a) (b) (c)

Figure 9.13 Real-time oxygen monitoring by optical fibre (Oxford Optronix, Oxylite 4000) (a). The fine fibre probe (b) is placed inside the construct to the monitor inside the incubator (c).

(iv) Real-time versus end-stage

Finally, many techniques, especially those used in research phases, are designed to sample constructs recovered at the end of the procedure. These often equate to destructive or invasive tests, and again translate poorly to processing or clinical implant monitoring. In this case, real-time monitoring must be our ideal as it provides many data measures over relatively short periods. It also identifies changes/rates of change *as they happen*. The value of this is enormous, feeding again into the all-important time dimension of the process (Text Box 9.10). First, in-process data allows for feedback and correction, or tuning of the process before it is complete (i.e. too late to change the result). Second, real-time (RT) data streams can be used to generate rates and trends which, in turn, can hugely improve the interpretation of indirect analyses. This can be the case where the trend or rate-of-change equations are pre-fed into computer models to predict the meaning of our indirect parameter measurements in terms of the function we want to know about (Text Box 9.11).

9.5 The take-home message

The last two chapters have taken us from Hippocrates to Limousin beef farming and jumbo jets. But their take-home messages can be rolled together into a relatively simple whole. This suggests that the evolution of extreme tissue engineering bioreactors is starting to give us the confidence to wrestle control of our processes away from the cells and into our own hands.

Logic tells us that we *must* eventually do this, and new technologies tell us we *can*. This control helps us to re-think bioreactor design and, more radically, why and when we need them. The slavishly nurturing of our cells does not need to be a first priority. We can radically re-shape the time courses of our processes to design the fourth dimension and, with it, we can generate higher targets for our constructs and the way we monitor them.

Speed, reproducibility and the possibility of customization can revolutionize our ideas on how we employ even simple fabricated tissues. These new uses can range from mass-produced model tissues for replacing animal tests, to tailor-made bedside tissue implants. In fact, this 'new paradigm' was always embedded in the original tissue engineering idea. However, by thinking outside the cell-cultivation box (which we *must* do anyway), we can now explore ***direct tissue fabrication*** processes which can lift our targets.

In the same way, the driving concepts for human flight in the early 1900s moved away from images of Icarus and feathery-winged angels towards those

Figure 9.14 'Flying with feathers'. When a technology is new and our concepts are based mainly in the natural 'bio-world', it is inevitable that we are slow to appreciate how we might use non-natural, engineering equivalents. A good historic example of this is in heavier-than-air flight, where it is possible to plot the gradual disappearance of the shapes, flapping and feathers which characterize bird-flight. The real pioneer moments must have come where inventors and thinkers suddenly glimpsed how this or that aspect of a flying machine could be made to a *human design* or with *human-type materials*. This is where we are with extreme tissue engineering.

Text Box 9.11

Explain in one sentence, supported by a short analytical essay, why the evolution of process monitoring (from simple, direct and reactive to indirect and predictive) is more apparent in top-down bio-cultivation processes rather than bottom-up engineering and assembly processes. Use your own process examples and flow diagrams to illustrate the case.

which now allow us to construct shiny metal jumbo jets (Figure 9.14).

Further reading

1. Jones, S. (2005). On Darwin airlines. In: *The Single Helix: a turn around the world of science*, pp. 3–5. Little Brown, London.
   [Withering demolition of Creationist teaching in the UK using the tongue-in-cheek analogy of a naturally evolved airliner, flapping into its airport: in other words, *a feathery Boeing*.]
2. Vunjak-Novakovic, G. (2006). Tissue engineering: basic considerations. In: Vunjak-Novakovic, G., Freshney, R. I. (eds.) *Culture of Cells for Tissue Engineering*, pp 131–155. John Wiley, New Jersey.
   [Nice, basic entry into process thinking and tissue production sequences. Best used only as an illustration when it comes to detailed methodologies which use 'non-extreme', 1st generation technologies.]
3. Freed, L. E. & Vunjak-Novakovic, G. (2002). Spaceflight bioreactor studies of cells and tissues (review). *Advances in Space Biology and Medicine* **8**, 177–195.
   [Comprehensive analysis of the parameters which can affect bioreactors (at least at time zero), as needed to understand the effects of spaceflight.]
4. Freed, L. E., Guilak, F., Guo, X. E., Gray, M. L., Tranquillo, R., Holmes, J. W., Radisic, M., Sefton, M. V., Kaplan, D. & Vunjak-Novakovic, G. (2006). Advanced tools for tissue engineering: scaffolds, bioreactors, and signalling (review). *Tissue Engineering* **12**, 3285–3305.
   [Comprehensive if conventional analysis of bioreactor processing: leading to more recent . . .]
5. Haj, A. J., Hampson, K. & Gogniat, G. (2009). Bioreactors for connective tissue engineering: design and monitoring innovations (review). *Advances In Biochemical Engineering/Biotechnology* **112**, 81–93.
   [. . . advanced discussion, including the need for real-time and progressive analysis.]
6. Rice, M. A., Waters, K. R. & Anseth, K. S. (2009). Ultrasound monitoring of cartilaginous matrix evolution in degradable PEG hydrogels. *Acta Biomaterialia* **5**, 152–161.
   [Example of advanced non-destructive analysis: ultrasound-based.]
7. Mason, C., Markusen, J. F., Town, M. A., Dunnill, P. & Wang, R. K. (2004). Doppler optical coherence tomography for measuring flow in engineered tissue (review). *Biosensors and Bioelectronics* **20**, 414–423.
   [Example of advanced non-destructive analysis: optical-Doppler measurement of flow.]
8. Liu, J., Barradas, A., Fernandes, H., Janssen, F., Papenburg, B., Stamatialis, D., Martens, A., van Blitterswijk, C. & de Boer, J. (2010). *In vitro* and *in vivo* bioluminescent imaging of hypoxia in tissue-engineered grafts. *Tissue Engineering Part C, Methods* **16**, 479–485.
   [Example of indirect, image-based monitoring of cell oxygenation: contrasting with . . .
9. 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.
   [. . . example of direct, real-time, quantification of oxygen tension over time and position.]
10. Ziegelmueller, J. A., Zaenkert, E. K., Schams, R., Lackermair, S., Schmitz, C., Reichart, B. & Sodian, R. (2010). Optical monitoring during bioreactor conditioning of tissue-engineered heart valves. *ASAIO Journal* **56**, 228–231.
    [Example of imaged-based monitoring of cardio-vascular tissue growth, in contrast to . . .]
11. Syedain, Z. H., Meier, L. A., Bjork, J. W., Lee, A. & Tranquillo, R. T. (2011). Implantable arterial grafts from human fibroblasts and fibrin using a multi-graft pulsed flow-stretch bioreactor with noninvasive strength monitoring. *Biomaterials* **32**, 714–722.
    [. . . example of real-time monitoring of the development of vascular construct mechanics: dynamics over about two months' growth in culture.]