Report on a problem studied at the UK Mathematics-in-Medicine Study Group Nottingham 2006
< http://www.maths-in-medicine.org/uk/2006/cell-passage/ >

Identification of point of passage for cell culture

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Mathematics in Medicine Study Group 2006

Abstract

There are many possibilities within tissue engineering and regenerative medicine for producing new and exciting treatments for a whole range of disease through the culture of tissue in the laboratory. In order to meet the demands of such regenerative therapies it is necessary to develop high throughput systems. One obstacle in this process is determining the point at which to divide the growing cells into more flasks. In this Study Group report a number of models of growing cell populations are considered. We use these models to identify the optimum point of passage.

1 Introduction

Over the past few years there has been much publicity about the possibilities of tissue engineering and regenerative medicine producing new and exciting treatments for a whole range of diseases and injuries. Examples include artificial skin, used to treat burn victims, and the repair of cartilage damage from sporting injuries. However, despite encouraging early results many would agree that for expectations to be met there are still numerous obstacles that must be overcome. One difficulty is scaling up laboratory based cell and tissue culture into high throughput systems that could meet the demands of regenerative therapies.

There is currently much interest in developing automated production systems for the mass manufacture of tissue engineered products. Such systems currently exist for manufacture of well behaved, well defined cell lines with application in the screening of pharmaceutical products. For the manufacture of tissue engineered products, however, cells are typically harvested from a donor or the patient's own body and then cultured. These cells are

of variable quality and their growth characteristics are unknown. Thus, it is difficult to predict the behaviour of the cell culture.

One of the main purposes of cell culture is to produce appropriate quantities of cells for therapy. In practice, there are only a certain number of cells that can be effectively grown in a given flask before they are no longer functional. Thus, once a flask has reached its capacity its cell population is split into multiple flasks and sub-cultured. This process is called passaging and it is important that passaging occurs when the cells are in their most proliferative state.

Currently the point of passage is determined very subjectively. On a laboratory scale culture flasks are visually inspected to assess the area of the plate covered, cell connectivity and cell distribution. In order to move this culture process for uncharacterised cell lines into an automated system it is necessary to have a more quantitative way of determining the correct point of passage.

Current tissue engineering protocol suggests passaging at $70 - 80\%$ confluence. The term confluence describes the cell culture in terms of the area of the flask covered, the cell connectivity and cell distribution and the size of the cells. It is also noted that the maximum number of passages to achieve the required number of cells is limited, as cells loose their functionality if over passaged. A rough estimate of 6 passages was given.

The Study Group developed a number of mathematical models of growing cell populations to examine the effect of the point of passage on achieving the cell numbers required by regenerative medicine. In particular, we aimed to answer the following questions:

1. How do we mathematically define confluence?
2. At what confluence should passage take place in order to minimise the time taken to obtain the required number of cells?
3. How sensitive is the culture process to the point of passage?

The report structure is as follows. In section 2 we introduce a logistic growth model for a single population of cells and determine the optimum point of passage. Section 3 further develops this model to include a population of multiple cell type. We introduce a spatial model in section 4 and summarise our findings and discuss potential further work in section 5. All those who contributed to this Study Group are acknowledged in section 6.

2 Logistic growth model with one cell type

As a first modelling approach we considered a logistic growth model. Let $\Gamma(t)$ be the gross cell density (total number of cells in a flask over the area of a flask), t the time in hours and Γ^* to be the carrying capacity of a flask. Then Γ satisfies,

$$\frac{\mathrm{d}\Gamma}{\mathrm{d}t} = k\Gamma\left(1 - \frac{\Gamma}{\Gamma^*}\right) - \alpha\Gamma, \tag{1}$$

with k the birth rate and α the death rate. We impose the initial condition $\Gamma\left(0\right) = \Gamma_0$.

We non-dimensionalise equation (1) using the scalings

$$t = \frac{1}{k-\alpha}\tilde{t}, \qquad \Gamma = \frac{(k-\alpha)\Gamma^*}{k}\tilde{\Gamma}, \qquad \theta = \frac{k-\alpha}{k}\tilde{\theta}, \tag{2}$$

and, dropping tildes, obtain

$$\frac{\mathrm{d}\Gamma}{\mathrm{d}t} = \Gamma - \Gamma^2, \tag{3}$$

with $\Gamma(0) = k\Gamma_0/(k-\alpha)\Gamma^* = \gamma_0$.

Solving equation (3) we obtain,

$$\frac{1}{\Gamma} = e^{-t}\left(\frac{1}{\Gamma(0)} - 1\right) + 1. \tag{4}$$

When cells are seeded onto a surface there is a delay time, t_s, during which the cells are adhering to the surface and no proliferation takes place. We incorporate this delay into equation (4) giving

$$\frac{1}{\Gamma} = e^{-(t-t_s)}\left(\frac{1}{\Gamma(0)} - 1\right) + 1. \tag{5}$$

It is noted that this delay time also includes the negligible time that it takes to carry out the passaging process.

Once the cells have adhered to the surface they start to proliferate. When the cells reach the desired confluence $\Gamma(t) = \theta$, where $0 \leq \theta \leq k/(k-\alpha)$, the cells must be detached, and divided in to n_f flasks. The final condition

of one passage gives rise to the initial condition for the next passage. We define confluence to be the percentage surface area of the flask covered. The process of passaging cells from one flask to another is an inefficient one and some cells do not survive the process. It is noted that the efficiency decreases with number of cells. We assume that the proportion of cells which survive passaging is given by $\Lambda(\theta)$, and for our model define $\Lambda(\theta) = \lambda_0\theta$.

We assume that the passaging process starts with just one flask and that the passaging confluence remains constant throughout the process that is, same θ for each passage. We wish to calculate the time taken to reach the required number of cells, Γ_{max}. First we calculate the time until first passage, t_{p1}, that is $\Gamma(t_{p1}) = \theta$. Using (5) we find

$$t_{p1} = \ln\left(\frac{1}{\gamma_0} - 1\right) - \ln\left(\frac{1}{\theta} - 1\right) + t_s, \tag{6}$$

and the time between subsequent passages to be

$$t_{p2} = \ln\left(\frac{n_f}{F_l\gamma_0^2} - 1\right) - \ln\left(\frac{1}{\theta} - 1\right) + t_s, \tag{7}$$

where $F_l = \lambda_0 k/(k - \alpha)$ is the non-dimensional Fletcher constant.

We wish to determine the passaging confluence, θ, such that the time to reach the required cell number, Γ_{max}, is minimised. We reach the required number of cells when $\sum_i \Gamma \geq \Gamma_{max}$, with i equal to the number of flasks at the end of the experiment, i.e. $i = n_f^{n_p}$. The total time taken, T, is given by

$$T = t_{p1} + n_p t_{p2}, \tag{8}$$

where n_p is the number of passages performed and must take an integer value.

We define Λ_{end} to be the final efficiency of removing the cells from the flasks and obtain the total number of cells at the end of the experiment to be

$$\sum_i \Gamma = \theta i \Lambda_{end} \geq \Gamma_{max}. \tag{9}$$

Using equation (9) we find the number of passages required to reach Γ_{max} to be

$$n_p = \min_{j \in \mathbb{N}} \left\{ j \geq \frac{\ln\left(\frac{E_w}{F_l \theta}\right)}{\ln\left(n_f\right)} \right\}, \tag{10}$$

where $E_w = \lambda_0 \Gamma_{max} / \Lambda_{end} \Gamma^*$ is the non-dimensional Eastburn-Walter constant.

We now use n_p to calculate $T(\theta)$ and minimise T with respect to θ.

2.1 Results

In figure 1(a) we see that as the growth rate, k, increases ,the total time taken, T, to obtain the required number of cells, Γ_{max}, decreases exponentially. For $k \ll 1$ we see that the required number of cells can never be obtained as the total time tends to infinity.

Figures 1(b) and (c) show the effect of the growth rate on the optimal passaging confluence, θ, and the required number of passages, n_p, respectively. It is noted that the observed discontinuity in both is caused by the restriction on the number of passages taking an integer value. In our discussion of these figures we neglect $k \ll 1$ as it is not possible to reach the required number of cells in this region.

In the first region, $0.08 < k < 0.1$, figure 1(b) shows that a small change in growth rate has no effect on the optimal passaging confluence, $\theta = 0.14$. It is in this region that the most passages are taken to reach the required number of cells, $n_p = 6$, as shown in figure 1(c). This is because the delay time, t_s, that is incurred after passaging, is small compared to the slow turnover time of the cells.

In the second region, $0.1 < k < 0.16$, figure 1(b) shows the optimal passaging confluence varies with the growth rate. This is explained in more detail in figure 2(b). Figure 1(c) shows that fewer passages are required to reach the required number of cells when the growth rate is larger, $n_p = 5$.

In the final region, $k > 0.16$, figure 1(b) shows that the optimal passaging confluence, $\theta = 0.56$, remains constant as the growth rate varies. It is in this region that the smallest number of passages is optimal, $n_p = 4$. This is because the delay time incurred after passaging is large compared to the quick turnover time of the cells.

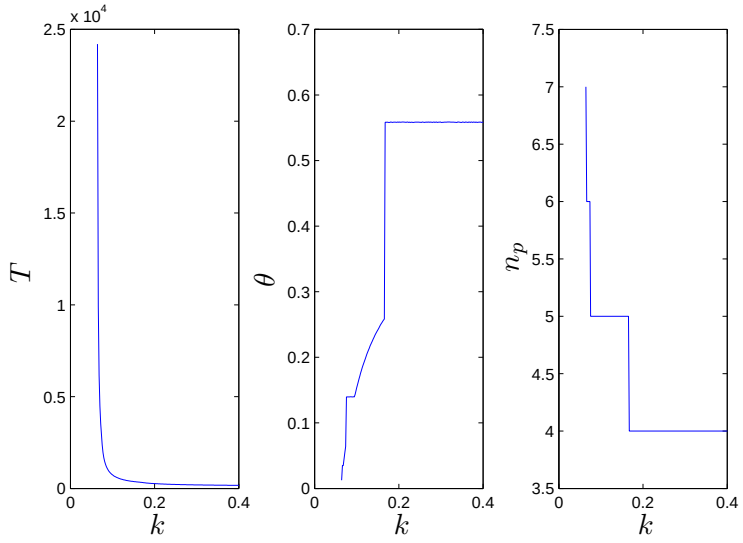

Figure 1: Plot showing how the total time, T, optimum passaging confluence, θ and number of passages, n_p, vary with growth rate, k. Dimensional parameter values are $n_f = 4,\ \alpha = 1/16,\ t_s = 24,\ \Lambda_{end} = 0.7,\ \lambda_0 = 0.95,\ \Gamma_0 = 1/(0.05^2\pi),\ \Gamma^* = 100/(0.05^2\pi),\ \Gamma_{max} = 10000/(0.05^2\pi)$.

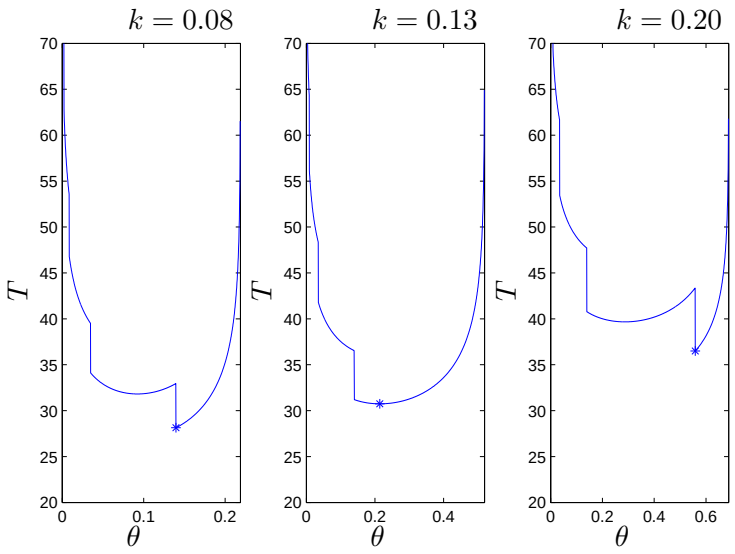

Figure 2: Plot showing how the total time taken, T, to reach the required number of cells, Γ_{max}, varies with the level of passaging, θ. Point of minimum time marked by $*$. Dimensional parameter values are $n_f = 4,\ \alpha = 1/16,\ t_s = 24,\ \Lambda_{end} = 0.7,\ \lambda_0 = 0.95,\ \Gamma_0 = 1/(0.05^2\pi),\ \Gamma^* = 100/(0.05^2\pi),\ \Gamma_{max} = 10000/(0.05^2\pi)$

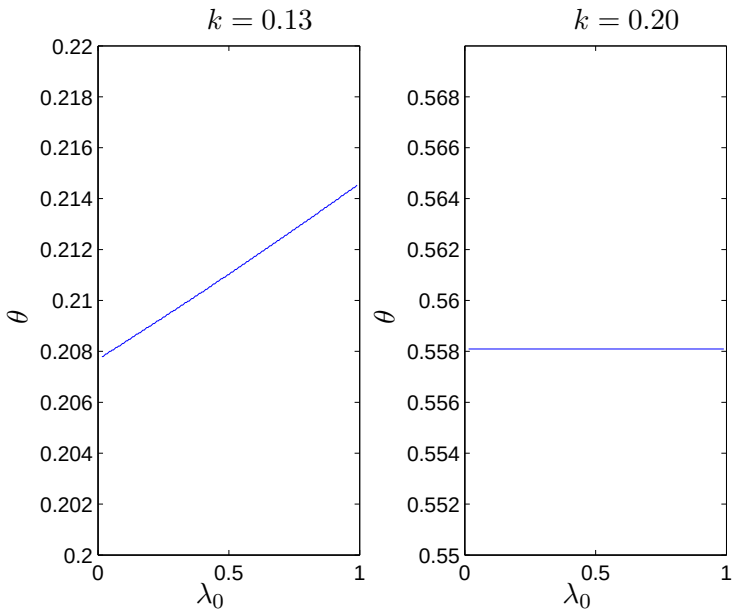

Figure 3: Plot showing how the optimum level of passaging, θ, varies with passaging efficiency, λ_0, for different growth rates, k. Dimensional parameter values $n_f = 4, \alpha = 1/16, \quad t_s = 24, \ \Lambda_{end} = 0.7, \ \Gamma_0 = 1/(0.05^2\pi), \ \Gamma^* = 100/(0.05^2\pi), \ \Gamma_{max} = 10000/(0.05^2\pi)$.

In figure 2 we illustrate how, for fixed growth rate k, the total time taken, T, to reach the number of required cells, Γ_{max}, varies with the passaging confluence, θ. In all cases shown two asymptotes exist. The first when θ approaches 0, if the passaging confluence is too small, the required number of cells, Γ_{max}, will never be reached. The second exists for the steady state of the system, $\theta = (k - \alpha)\Gamma^*/k$. In this case it is not possible for the population of cells in the flask to reach the required confluence and so passaging never takes place and the required number of cells is not obtained. The second feature of note is the discontinuities in the plots. As the passaging confluence, θ, is reduced it becomes necessary to increase the number of passages, n_p, if the required number of cells are to be obtained. This increase in number of passages results in a vertical discontinuity that captures the delay time, t_s, incurred when a further passage is carried out.

Figure 2(a) shows $k = 0.08$ which lies in the first region discussed in figure 1. Here we see the minimum of the curve corresponds to a point where reducing θ any further would require a further passage to be carried out. Figure 2(b) shows $k = 0.13$, which corresponds to the second region discussed in figure 1, here we see that the minimum passaging confluence, θ, is not limited by the penalisation for passaging, and so an increase in k in this region moves the

minimum along the curve. Finally figure 2(c) shows $k = 0.20$, corresponding to the final region discussed in figure 1. The minimum of the plot here is limited by the time penalisation of another passage, as for figure 2(a).

Finally we show figure 3. This illustrates that efficiency of passaging is only of importance for growth rates in the region $0.1 < k < 0.16$. The efficiency of passaging effects the optimal passaging confluence only when k lies in this region. This can be explained because this is the only region for which the minimum total time taken, T, is not limited by the time penalisation of a further passage.

3 Logistic growth model with different cell types

In some tissue engineering and regenerative medicine applications stem cells are used to culture a cell population. These stem cells divide maintaining themselves and producing a daughter cell. This daughter cell differentiates with each subsequent division until it is a fully differentiated cell. We further develop the logistic growth model to include cells of different generations in an attempt to capture this process of differentiation and analyse its effect upon the optimal passaging confluence. Figure 4 shows this process for 3 generations.

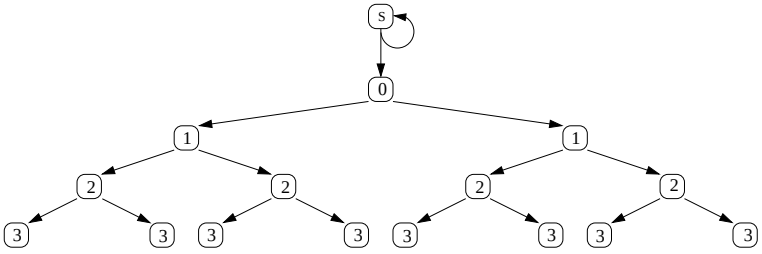

Figure 4: Family tree for three generations. S = a stem cell, i = a generation i cell for $i = 0, ..., 3$.

We note that a stem cell's growth rate is typically twice that of other cells.

Let $N_i(t)$ be the number of cells of generation i, $N_s(t)$ be the number of stem cells and $N_d(t)$ be the number of final generation cells where d is the number of generations. Birth rates are denoted k_i and death rates α_i. N^* is the capacity of the flask, θ is the passaging confluence, $\lambda_0\theta$ captures the efficiency of the passaging process and Λ_{end} the efficiency of the final collection process. The governing equations for the number of cells in each generation is then given by

$$\begin{aligned}
\frac{dN_s}{dt} &= -\alpha_s N_s \\
\frac{dN_0}{dt} &= k_s N_s \left(1 - \frac{\sum N_j}{N^*}\right) - k_0 N_0 \left(1 - \frac{\sum N_j}{N^*}\right) - \alpha_0 N_0 \\
\frac{dN_i}{dt} &= 2k_{i-1} N_{i-1} \left(1 - \frac{\sum N_j}{N^*}\right) - k_i N_i \left(1 - \frac{\sum N_j}{N^*}\right) - \alpha_i N_i \\
\frac{dN_d}{dt} &= 2k_{d-1} N_{d-1} \left(1 - \frac{\sum N_j}{N^*}\right) - \alpha_d N_d, \qquad (11)
\end{aligned}$$

where $\sum N_j = N_s(t) + \sum_{j=0}^{d} N_j(t)$ and with initial conditions $N_s(0) = N_{s_0}$ and $N_i(0) = N_{i_0}$ for $i = 1, ..., d$

Letting $t = k_s \tilde{t}$, and dropping tildes, we obtain

$$\begin{aligned}
\frac{dN_s}{dt} &= -\frac{\alpha_s}{k_s} N_s \\
\frac{dN_0}{dt} &= \left(N_s - \frac{k_0}{k_s} N_0\right) \left(1 - \frac{\sum N_j}{N^*}\right) - \frac{\alpha_0}{k_s} N_0 \\
\frac{dN_i}{dt} &= \left(2\frac{k_{i-1}}{k_s} N_{i-1} - \frac{k_i}{k_s} N_i\right) \left(1 - \frac{\sum N_j}{N^*}\right) - \frac{\alpha_i}{k_s} N_i \\
\frac{dN_d}{dt} &= 2\frac{k_{d-1}}{k_s} N_{d-1} \left(1 - \frac{\sum N_j}{N^*}\right) - \frac{\alpha_d}{k_s} N_d. \qquad (12)
\end{aligned}$$

As for the previous model a delay time, t_s, is introduced to capture the time taken for cells to adhere after passaging and passaging takes place when the cells reach the desired confluence, θN^*, where $0 < \theta < 1$. Again the final condition of one passage gives rise to the initial condition for the next passage.

We solve this problem numerically, minimising the total time taken, T, to reach the required number of cells, N_{max}, with respect to the passaging confluence, θ, to obtain the optimal passaging confluence. The simulation is run with, $d = 3$, giving a system of 5 ordinary differential equations with initial conditions, $N_s(0) = N_i(0) = 10$ for $i = 0, ..., 3$.

3.1 Results

In figure 5(a) we see that as the passaging confluence, θ, increases, the number of passages, n_p, necessary to reach the required number of cells,

N_{max}, decreases. This is discontinuous as the number of passages must take an integer value. It is noted that the number of passages required for the parameter values used in this simulation are higher than the recommended value, $n_p = 6$.

Figure 5(b) we see that as the passaging confluence increase the total time taken, T, to obtain the required number of cells, N_{max}, varies discontinuously, as seen in figure 2. Again, the vertical jumps correspond to the delay time, t_s, incurred when a further passage is carried out. The optimal passaging confluence for these simulation values is $\theta = 0.19$, with a total of 8 passages being needed to obtain the required number of cells. For this simulation the minimum number of passages possible to reach the required number of cells is $n_p = 7$, for this number of passages the optimal passaging confluence is $\theta = 0.76$, which gives good agreement with the current protocol used within tissue engineering laboratories. It is important to minimise both the number of passages taken to reach the required number of cells as well as the total time taken.

Time did not permit further investigation into this model within the course of the Study Group.

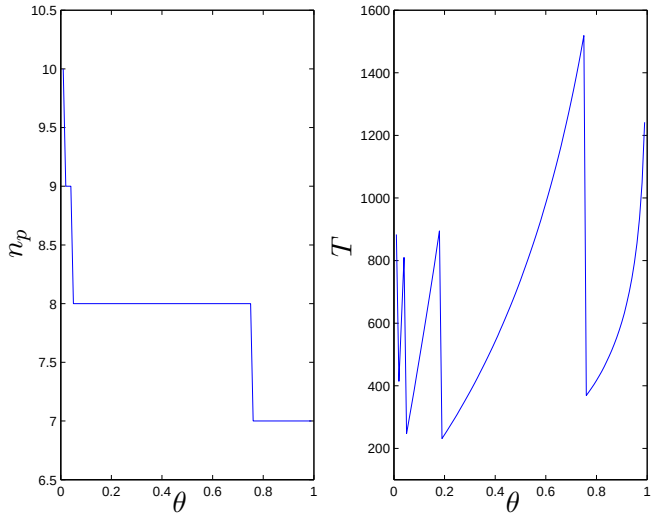

Figure 5: Plot showing how the number of passages, n_p and the total time taken, T, to reach the required number of cells, N_{max}, varies with the passaging confluence, θ. Non-dimensional parameter values are $n_f = 4$, $k_s = 16$, $k_i = 8$ for $i = 0, ..., 3$, $\alpha_i = 0$ for $i = s, 0, ..., 3$, $t_s = 24/8$, $\Lambda_{end} = 0.7$, $\lambda_0 = 0.9$, $N^* = 10$, $N_{max} = 10000$

4 Spatial model - Brewardotaxis

The effect of including cell movement into the model was also considered. Let $\Gamma(\mathbf{x}, t)$ be the local number density of cells on the surface of the flask, which for simplicity was modelled by the two-dimensional disc $\Omega = \{\mathbf{x} \in \mathbb{R}^2 : \|\mathbf{x}\| \leq R\}$. It is known that above a critical local cell density Γ_h, cells move to spread out and reduce cell packing. This was modelled using a non-constant diffusion coefficient as follows. Let $\Gamma(\mathbf{x}, t)$ be the local number density of cells, then Γ satisfies

$$\frac{\partial \Gamma}{\partial t} + \boldsymbol{\nabla} \cdot (\Gamma \mathbf{v}) = k\Gamma \left(1 - \frac{\Gamma}{\Gamma^*}\right) - \alpha\Gamma, \tag{13}$$

where

$$\mathbf{v} = \begin{cases} D\left(1 - \dfrac{\Gamma}{\Gamma^*}\right) \boldsymbol{\nabla}\Gamma & \Gamma > \Gamma_h, \\ \\ 0, & \Gamma < \Gamma_h, \end{cases}$$

with D representing the diffusion coefficient.

It should be noted that this model ignores short range cell attraction. We assume the same passaging procedure as before, except that since the passaging condition involves the total number of cells, we must integrate Γ over the whole disc. Hence the times to passage t_{p_i} are defined by

$$\iint_{\Omega} \Gamma(\mathbf{x}, t_{p_i}) \, \mathrm{d}x \, \mathrm{d}y = \theta \pi R^2 \Gamma^*, \qquad i = 1, 2. \tag{14}$$

Note that simpler monolayer models could also be used.

A model in which cells secrete a diffusible chemoattractant was also considered. In this model, cells diffuse with constant diffusion coefficient D_Γ and actively move up gradients of the chemoattractant, as well as proliferating as in previous models. We assume that the chemoattractant diffuses with constant diffusion coefficient D_C, is secreted by cells at a constant rate β and degrades naturally at a constant rate γ. The local number density of cells $\Gamma(\mathbf{x}, t)$ and chemoattractant concentration $C(\mathbf{x}, t)$ therefore satisfy the equations

$$\frac{\partial \Gamma}{\partial t} = D_\Gamma \nabla^2 \Gamma + k\Gamma\left(1 - \frac{\Gamma}{\Gamma^*}\right) - \alpha\Gamma - \chi \boldsymbol{\nabla} \cdot (\Gamma \boldsymbol{\nabla} C),$$
$$\frac{\partial C}{\partial t} = D_C \nabla^2 C + \beta\Gamma - \gamma C. \tag{15}$$

Letting

$$\mathbf{x} = R\tilde{\mathbf{x}}, \quad t = \frac{L^2}{D_\Gamma}\tilde{t}, \quad \Gamma = \frac{(k-\alpha)\Gamma^*}{k}\tilde{\Gamma}, \quad C = \frac{D_C}{\chi}\tilde{C}, \tag{16}$$

we obtain non-dimensionalised equations

$$\frac{\partial \tilde{\Gamma}}{\partial \tilde{t}} = \nabla^2 \tilde{\Gamma} + \mu\tilde{\Gamma}(1 - \tilde{\Gamma}) - \boldsymbol{\nabla} \cdot (\tilde{\Gamma} \boldsymbol{\nabla} \tilde{C}),$$
$$\frac{\partial \tilde{C}}{\partial \tilde{t}} = D\nabla^2 \tilde{C} + \lambda\Gamma - \sigma C, \tag{17}$$

where

$$\mu = \frac{R^2(k-\alpha)}{D_\Gamma}, \quad D = \frac{D_C}{D_\Gamma}, \quad \lambda = \frac{R^2\beta\chi(k-\alpha)\Gamma^*}{D_\Gamma k}, \quad \sigma = \frac{\gamma R^2}{D_\Gamma}. \tag{18}$$

These equations are to be supplemented with suitable initial and boundary conditions.

Unfortunately there was insufficient time to solve these models numerically during the Study Group.

5 Conclusions

In this Study Group we focused on a number of different aspects of the biological problem. In section 2 we studied a logistic growth model for a single cell type. We found that for small growth rates, k, it is possible to passage at lower passaging confluences, as the delay time, t_s, is small in comparison to the growth time. For larger growth rates it is necessary to passage as few times as possible, because the delay time is large in comparison to the growth time. Therefore, it is optimal to passage at higher confluence.

In section 3 we presented a logistic growth model for multiple cell types. Here we noted that we should minimise the number of passages, n_p, as well as the time taken, T, to obtain the required number of cells, N_{max}. For the simulation run we found good agreement with current tissue engineering protocol. Further work could be carried out on this model, with consideration given to how the results shown vary for different growth rates, k.

Finally we introduced a spatial model which we were unable to investigate within the scope of the Study Group. Further work on this model obviously exists and would be of interest to investigate.

6 Participants

The following people were involved with this problem during the Study Group: Chris Breward, Sophie Buck, Helen Byrne, Sarah Eastburn, Alexander Fletcher, John King, Melissa Mather, Alex Walter, Sarah Waters