



Reproducibility of scratch assays is affected by the initial degree of confluence: Experiments, modelling and model selection



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HIGHLIGHTS

- Scratch assays with different initial cell densities are performed.
- Rate of re-colonisation is very sensitive to the initial density.
- Calibrating the Fisher–Kolmogorov model implies that the cell diffusivity, D , & proliferation rate λ , appear to depend on initial density.
- Calibrating the Porous Fisher model suggests a reduced dependence of D & λ on the initial density.
- In general, our approach suggests that the Porous Fisher model is better suited to our experiments than the Fisher–Kolmogorov model.

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ABSTRACT

Scratch assays are difficult to reproduce. Here we identify a previously overlooked source of variability which could partially explain this difficulty. We analyse a suite of scratch assays in which we vary the initial degree of confluence (initial cell density). Our results indicate that the rate of re-colonisation is very sensitive to the initial density. To quantify the relative roles of cell migration and proliferation, we calibrate the solution of the Fisher–Kolmogorov model to cell density profiles to provide estimates of the cell diffusivity, D , and the cell proliferation rate, λ . This procedure indicates that the estimates of D and λ are very sensitive to the initial density. This dependence suggests that the Fisher–Kolmogorov model does not accurately represent the details of the collective cell spreading process, since this model assumes that D and λ are constants that ought to be independent of the initial density. Since higher initial cell density leads to enhanced spreading, we also calibrate the solution of the Porous–Fisher model to the data as this model assumes that the cell flux is an increasing function of the cell density. Estimates of D and λ associated with the Porous–Fisher model are less sensitive to the initial density, suggesting that the Porous–Fisher model provides a better description of the experiments.

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1. Introduction

Two-dimensional *in vitro* cell migration assays are routinely used to investigate the ability of cell populations to re-colonise an initially-vacant region. The most common type of *in vitro* cell migration assay is called a scratch assay, which is performed by: (i) growing a cell monolayer; (ii) removing a region of the monolayer by scratching it with a sharp-tipped instrument; and (iii) making observations of the re-colonisation of the initially-vacant, scratched region (Ashby and Zijlstra, 2012; Kramer

et al., 2013). Comparing the rate of scratch closure in an experiment where cells are exposed to a chemical stimulus to the rate of closure in a control assay provides insight into the roles of growth factors and putative drug treatments relevant to malignant spreading and tissue repair (Ashby and Zijlstra, 2012; Kramer et al., 2013).

Although scratch assays remain popular, various alternative *in vitro* assays have been proposed. These alternatives, including circular barrier assays (Vo et al., 2015), circular invasion assays (Kam et al., 2008) and IncuCyte ZOOM™ assays (EssenBioScience, 2015), are often claimed to be superior because of issues associated with scratch assay reproducibility (Gough et al., 2011). The purpose of these alternative assays is to reduce some source of

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variability in the experimental design. For example, standard scratch assays can be performed with various types of instruments (e.g. pipette tip, and razor blade) and with varying degrees of pressure. Each of these variables is thought to have the potential to affect the results in some way. To address these limitations, the IncuCyte ZOOM™ real time live cell imaging assays have been developed (EssenBioScience, 2015). IncuCyte ZOOM™ assays use a mechanical tool, called a WoundMaker™, to create 96 identically-sized scratches in each well of a 96-well tissue culture plate. Each WoundMaker™ scratch has the same dimensions, and is created with the same amount of pressure.

In this work we explore a previously overlooked source of variability that has the potential to impact the interpretation of various types of cell migration assays. While standard experimental procedures for many cell migration assays require that a sufficient amount of time is allowed for the population to become confluent before the experiment is initiated (Ashby and Zijlstra, 2012; Kramer et al., 2013), most experiments do not report any quantitative measurements of the initial degree of confluence (Gujral et al., 2014; Kam et al., 2008; Maini et al. 2004a,b; Sherratt and Murray, 1990). Many experimental protocols simply state that the monolayer is either fully confluent (Kam et al., 2008) or 80% confluent (Bryant et al., 2010) prior to making a scratch. These reports of the *degree of confluence* are typically based on a qualitative judgment rather than quantitative measurements. To investigate the significance of this, we perform a suite of IncuCyte ZOOM™ assays, using PC-3 prostate cancer cells (Kaighn et al., 1979), in which we systematically vary the initial density. A qualitative comparison of the experimental images suggests that the rate of scratch closure is extremely sensitive to the initial density. This dependence on the initial density is important since experimental results are almost always reported without any quantitative measurement of the initial density. This could explain why scratch assays are difficult to reproduce.

We make quantitative measurements of the IncuCyte ZOOM™ assays by extracting cell density profiles and calibrating the solution of the Fisher–Kolmogorov model (Fisher, 1937; Kolmogorov et al., 1937), to that data. This procedure provides an estimate of the cell diffusivity, D , and the cell proliferation rate, λ , for each initial density considered. This procedure is standard; however, typical approaches deal with just one initial density (Cai et al., 2007; Habbal et al., 2014; Maini et al. 2004a,b; Savla et al., 2004; Sengers et al., 2007; Sherratt and Murray, 1990). The appropriately calibrated solutions of the Fisher–Kolmogorov equation match our experimental observations, for each initial density, very well. However, our estimates of D and λ appear to depend upon the initial cell density, and our estimates of D are extremely sensitive. This result has two implications. First, additional mechanisms, unaccounted for in the Fisher–Kolmogorov model, are likely to be acting in the experimental system. To explore this possibility we also examine the suitability of some potential extensions of the Fisher–Kolmogorov model, such as the Porous–Fisher equation (Sherratt and Murray, 1990; Sengers et al., 2007; Simpson et al., 2011; Witelski, 1994,1995). Second, our results suggest that previously reported procedures for estimating D and λ by calibrating the solution of the Fisher–Kolmogorov equation could provide misleading results.

2. Methods

We perform monolayer scratch assays using the IncuCyte ZOOM™ system (Essen BioScience, MI USA). All experiments are performed using the PC-3 prostate cancer cell line (Kaighn et al., 1979) from the American Type Culture Collection (ATCC, Manassas, USA). Cells are propagated in RPMI1640 medium (Life

Technologies, Australia) in 10% foetal calf serum (Sigma-Aldrich, Australia), with 100 U/mL penicillin, 100 µg/mL streptomycin (Life Technologies), in plastic flasks (Corning Life Sciences, Asia Pacific) in 5% CO₂ and 95% air in a Panasonic incubator (VWR International) at 37 °C. Cells are regularly screened for *Mycoplasma* (Nested PCR using primers from Sigma-Aldrich).

Cells grown to approximately 80% confluence are removed from the plastic flask using TrypLE™ (Life Technologies) in phosphate buffered saline (pH 7.4), resuspended in medium and seeded at various densities in 96-well ImageLock plates (Essen BioScience). Cells are distributed in the wells as uniformly as possible. We report results for initial cell densities of 10,000, 12,000, 14,000, 16,000, 18,000 and 20,000 cells per well. After seeding, cells are grown overnight to allow for attachment and some growth. We use a WoundMaker™ (Essen BioScience) to create uniform, reproducible scratches in all the wells of a 96-well plate. To ensure that as many cells are removed from the wound region as possible, we modify the manufacturer's protocol by repeating the scratch action 20 times before lifting the WoundMaker™. After creating the scratch, the medium is aspirated and the wells are washed twice with fresh medium to remove cells from the scratched area. Following the washes, 100 µL of fresh medium is added to each well and the plate is placed into the IncuCyte ZOOM™ apparatus. Images of the collective cell spreading are recorded every two hours, for 48 hours. For each different cell density, we perform three identically prepared experimental replicates ($n=3$).

We obtain numerical solutions to various parabolic reaction–diffusion models using a finite difference method (Morton and Mayers, 2005). The spatial domain, $0 < x < L_x$, is uniformly discretised with grid spacing δx , and the spatial derivatives are approximated using a central-difference approximation. Some of the models we consider involve a nonlinear diffusion term, which is discretised with an arithmetically averaged inter-node diffusivity. For all models considered, spatial discretisation leads to a system of coupled nonlinear ordinary differential equations that are integrated through time using a backward-Euler approximation with constant time steps of duration δt (Morton and Mayers, 2005). The systems of coupled nonlinear algebraic equations are linearised using Picard (fixed-point) iteration, with absolute convergence tolerance ϵ , and solved using the Thomas algorithm (Morton and Mayers, 2005). For all results we choose δx , δt and ϵ so that our algorithm produces grid-independent results.

3. Results and discussion

3.1. Qualitative assessment of experiments

A subset of the experimental images are presented in Fig. 1 for the experiments initiated with 12,000, 16,000 and 20,000 cells per well. Images in Fig. 1(a), (f) and (k) show that each experiment is initiated with a clean, and sharp scratch. The initial difference in cell density is visually distinct in the regions well behind the position of the scratch. The temporal progression of each experiment is shown in the columns of Fig. 1. In each case we see evidence of combined cell migration and cell proliferation. Cells located near the edge of the scratched region move into the vacant region over time. Cells are also proliferating since we see the cell density behind the location of the scratch increasing with time. Interestingly, if we compare the final images of each experiment, in Fig. 1(e), (j) and (o), a large portion of the initially-vacant wound space in Fig. 1(e) remains uncolonised, whereas the total area imaged in Fig. 1(o) appears to be colonised by $t=48$ h.

Our visual interpretation of these images indicates that the ability of PC-3 cells to re-colonise the wound space is very

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