Collective cell migration assays

An extensive overview of currently available assays and tips on how to select the most suitable assay for your research

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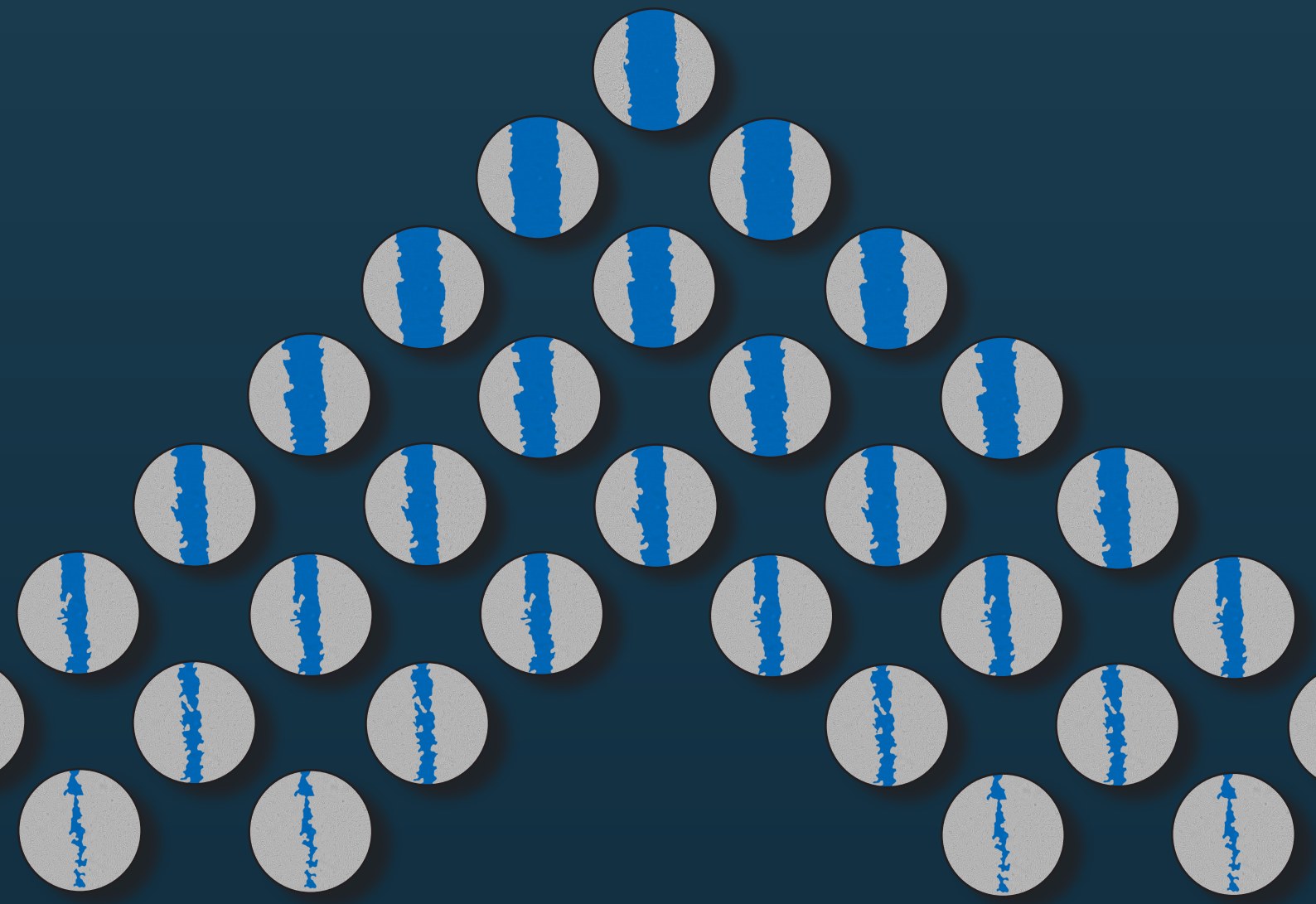

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Chapter 1. General introduction

1.1. Introduction

Cell migration is an essential process involved in important physiological processes such as embryogenesis[1], angiogenesis[2], wound healing[3] and the immune response[4]. Unfortunately, cell migration is also involved in many pathologies, including cancer metastasis[5] and inflammation[6]. In each of these physiological and pathological processes, the basic process of cell migration, i.e. translocation along or through a tissue substrate, is the same. However, cells migrate in different modes depending on the type and function of the cells. Leukocytes, for example, migrate the majority of their life span as single cells within virtually any tissue in the body.[4] Many other cell types only move at specific situations to place, shape or repair tissue. Most of these cells move in groups that are loosely or closely associated. This type of migration is called collective cell migration.

1.2. Rationale and outline

In this e-book, we aim to provide tools to help you find the most suitable experimental setup and corresponding analysis method to investigate collective cell migration. In order to do this, first the different modes of both single and collective cell migration are explained in “Chapter 2. Modes of cell migration”. An overview of currently available single-cell migration assays is given in “Chapter 3. Assays to invenstigate single-cell migration”. The most common forms of 2D collective cell migration assays: cell removal assays, cell exclusion assays and cell outgrowth assays, are discussed in “Chapter 4. Comparision of 2D sheet migration assays”. In “Chapter 5. Guidelines to select the most suitable collective cell migration assay”, guidelines for selecting a suitable collective cell migration assay are presented. In “Chapter 6. Data acquisition & analysis”, image acquisition and analysis will be discussed.

Chapter 2. Modes of cell migration

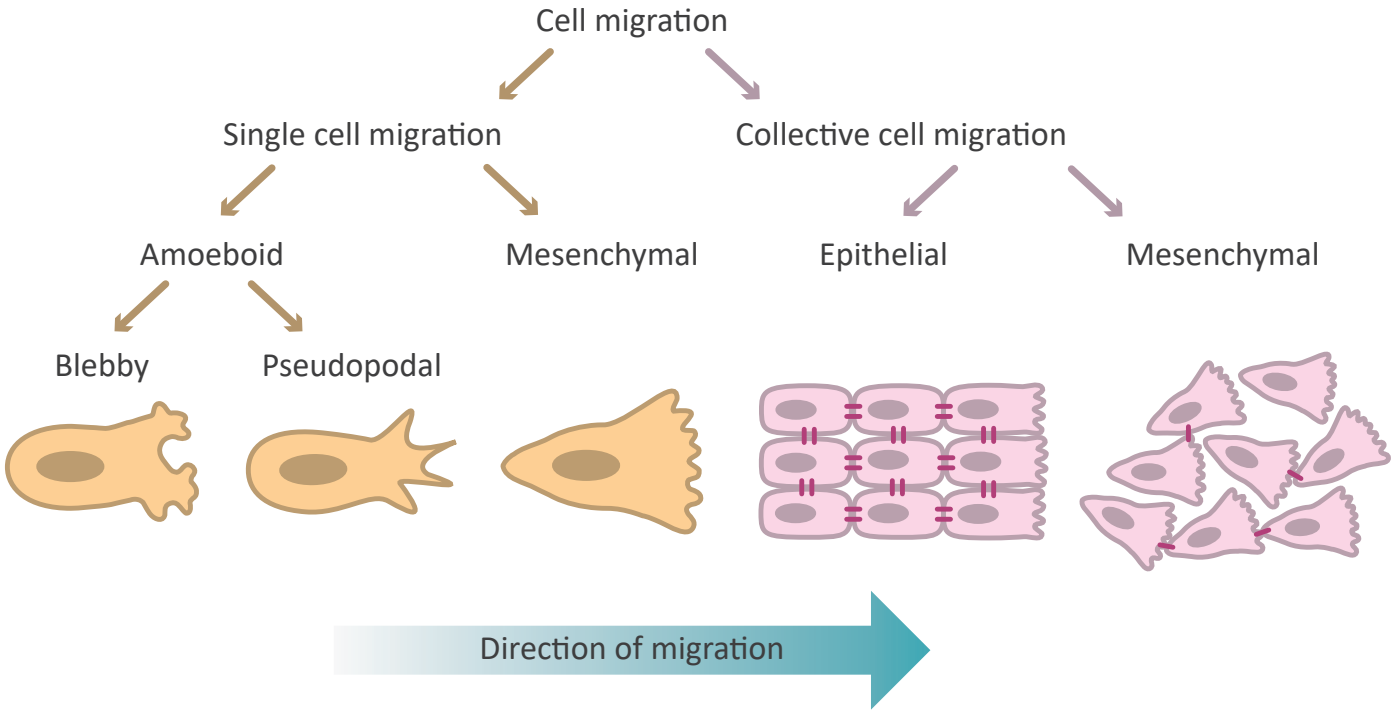
2.1. Introduction

In general, the basic process of cell migration all starts with cells having a directional polarity, with a leading edge and a trailing end. Subsequently, actomyosin mediated protrusions form at the leading edge followed by attachment of the protrusions to the substrate by the formation of integrin-based focal adhesions.[7-9] Actomyosin contraction leads to tension along the length axis of the cells which in turn causes translocation of the cell body forward and retraction of the trailing end.[7-9] This basic process is relevant in most cell types and types of cell migration. However, each cell and migration type has its own specific variant (even depending on the cell environment) which can differ in terms of cell morphology, migration speed, cell-cell interactions and dynamics.[7,9]

Roughly speaking, cell migration can be divided into single cell migration and collective cell migration, which each consist of several different types of migration ("Figure 2.1 Types of cell migration."). Next to migration, cells can also display invasion. The difference between the two being that the surrounding environment is restructured by the cells in case of invasion. In the next sections, single cell migration ("2.2. Single cell migration".), collective cell migration ("2.3. Collective cell migration") and cell invasion ("2.4. Migration versus invasion") are discussed in more detail.

Figure 2.1 Types of cell migration.

Cell migration can be divided into single and collective cell migration.



2.2. Single cell migration

Single cell migration can roughly be divided into amoeboid and mesenchymal migration ("Figure 2.1 Types of cell migration."). The first form of ameboid migration is called ***blebby amoeboid migration*** and is characterized by movement of rounded or ellipsoid cells without mature focal adhesions and filopodia.[7,10] These blebby cells do not migrate by adhering or pulling on the substrate but rather use propulsive, pushing blebs.[11] This type of migration is used by e.g. leukocytes migrating through extracellular matrix (ECM).[4]

Pseudopodal ameboid migration is the second form of ameboid migration and is defined by displacement of more elongated cells having weak cell-substrate interactions and actin-rich filopodia at their leading edge.[10,12] Neutrophils and dentritic cells, for example, display this type of migration.[13]

Elongated, spindle-like, cells with strong focal adhesions and high cytoskeletal (actomyosin) contractility move using ***mesenchymal migration.***[12,14] This type of migration mostly resembles the general type of migration described in "2.1. Introduction". and is seen in cells such as fibroblasts[15] and sarcoma cells.[16]

2.3. Collective cell migration

Collective cell migration is characterized by the coordinated migration of a group of cells in which cells are influenced by interactions with each other. The exact definition of collective cell migration is still open for debate. Some argue that stable cell-cell junctions are required for collective cell migration.[17,18] Others, on the other hand, suggest that migration can be considered collective when cells moving as a group affect each other's movement by e.g. forming transient cell-cell contacts or secreting soluble factors that influence one another's migration.[9,19] Here, we comply with the latter definition and consider migration to be collective when some form of cell-cell interaction is displayed in a migrating group of cells. In that case, when looking at the extreme ends of the spectrum, collective cell migration can be split into collective migration of epithelial cells and of mesenchymal cells ("Figure 2.1 Types of cell migration.").[19] However, any intermediate between epithelial and mesenchymal collective cell migration can take place depending on cell type and state (e.g. level of Epithelial to Mesenchymal Transition (EMT)[20]).

Epithelial cells form cell-cell adhesions (adherens junctions, desmosomes, tight junctions and gap junctions) in order to fulfill their barrier function.[21] During collective migration, epithelial cells maintain stable cell-cell adhesions, thus still fulfilling their barrier function "Figure 2.1 Types of cell migration." and "Figure 2.2A"[19,22,23] Epithelial cells can undergo collective migration in several manners. Examples of ***collective epithelial cell migration*** are the formation of sprouts or branches as seen in neo-angiogenesis of blood vessels[24] and branching morphogenesis of mammary glands.[25] Epithelial cells can also migrate as separate groups of cells like the border cells in Drosophila egg chambers[26] or invasive groups of detached cancer cells[27]. Another form of epithelial cell migration is the migration of strands stretching out of a tissue such as those observed in invasive carcinomas[28]. However, the most studied form of collective epithelial migration is that of sheet migration, where cells migrate as a 2D interconnected sheet. ***Sheet migration*** is seen during wound healing, both in skin[22] and in other epithelial tissues such as the intestine[29] and cornea[30]. The common denominator between all these types of collective migration is that they rely on mechanical coupling of the cells via stable cell-cell adhesions.[19,22,23] These adhesions ensure coordinated cytoskeletal activity of all cells within the collective. In this manner, the group of cells can obtain a polarity and thus directionality at the collective level, similar to the polarity needed for migration at the single cell level.

In contrast to the tightly connected epithelial cells, mesenchymal cells only form transient connections with each other "Figure 2.1 Types of cell migration." and "Figure 2.2B". When two polarized mesenchymal cells collide, they form (N-cadherin based) cell-cell adhesions. This triggers the retraction of the cell protrusions (lamellipodia and/or filopodia), causing a loss in polarity. This subsequently halts the migration and the cells quickly repolarize in the opposite direction. The repolarization causes the cells to move away from each other. This process is called ***contact-inhibition of locomotion*** (CIL) and can occur between cells of the same type (homotypic CIL) or between two different cell types (heterotypic CIL).[31,32] Recent studies have shown that loss of

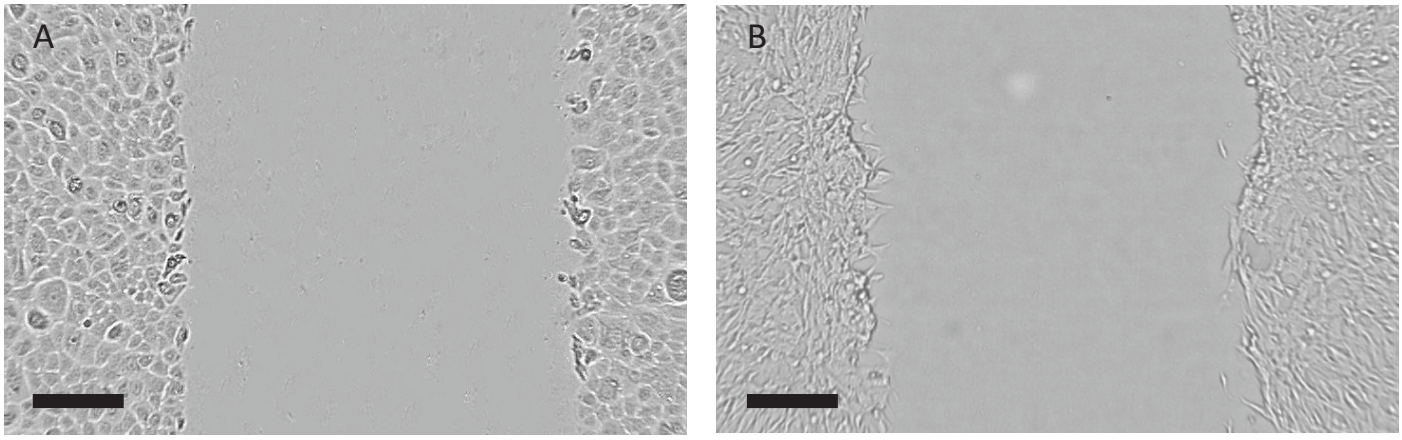
heterotypic CIL is involved in cancer metastasis and invasion.[33-35] Next to CIL, cells actively attract each other by secreting attractants ***(co-attraction)***.[19] In the case of neural crest cells, for example, C3a is secreted, which is a well-known attractant in the immune system.[36] Since each cell in the group produces the same attractant, the attractant concentration is high in regions with a high cell density. When a cell moves away from the group due to CIL, it can migrate back to the collective by following the local gradient of chemo-attractant (e.g. C3a gradient).[19] It is assumed that this continuous cycle of repulsion and attraction via respectively CIL and co-attraction maintains the ***collective migration of mesenchymal cells.*** Collective mesenchymal migration has mainly been studied in neural crest cells[36-38], but recent studies show that this type of migration is also involved in many other processes such as cancer metastasis[33,39] and the migration of the mesoderm during development.[40]

More in depth information about collective migration of epithelial and mesenchymal cells (including CIL) can be found in excellent reviews by Friedl and Gilmour[18], Friedl and Mayor[23], Rorth[9], Roycroft and Mayor[31], Scarpa and Mayor[1], Stramer and Mayor[32] and Theveneau and Mayor[19].

2.4. Migration versus invasion

Next to migrating inside the body, cells can also invade their surrounding environment. In biology, the terms migration and invasion are often used as interchangeable phrases. While the mechanisms are closely related, cell migration is defined as the directed translocation of cells on a 2D substrate or through a 3D matrix. ***Cell invasion***, on the other hand is defined as cell movement through a 3D matrix, which is accompanied by restructuring the 3D environment.[5,8] The process of cell invasion encompasses cell adherence to extracellular matrix (ECM) and subsequent remodeling of the ECM by means of degradation of existing ECM components and deposition of new ECM components before being able to migrate through the ECM.[5,8] Thus the term invasion describes a specific mode of 3D migration including ECM degradation whereas migration is used to describe non-destructive movement in both 2D and 3D environments.

Figure 2.2 Examples of collective migration.
(A) epithelial (B) mesenchymal migration in 2D wound healing assay



Example images of collective epithelial (A) and mesenchymal (B) migration in a 2D wound healing assay. A) The cobblestone morphology of the normal human epidermal keratinocytes indicates the presence of stable cell-cell adhesions during collective epithelial migration. B) 3T3 cells have transient cell-cell interactions due to their mesenchymal origin, indicated by their elongated morphology. Images made using the CytoSMART Lux2, scale bar represents 200 µm.

Chapter 3. Assays to invenstigate single-cell migration

3.1. Introduction

Cell migration is involved in a multitude of critical physiological and pathological processes. Cell motility can be divided into collective and single-cell migration. Single-cell migration is used by cells to move towards and between tissues, and it can be split into ***amoeboid and mesenchymal migration***,[42,43] as described in "2.2. Single cell migration". A variety of in vitro assays have been developed to study single-cell migration.

Several factors are essential in the design of single-cell migration assays. These factors include robust quantification, the use of a few step protocols, and a close resemblance to the cell microenvironment found in vivo. In addition to these factors, assays that use three-dimensional (3D) culture environments and can accommodate the co-culturing of multiple cell types can also be desirable.[44] This chapter will cover the most well-known methods for investigating two-dimensional (2D) single-cell migration. An overview of the advantages, disadvantages and commercially available products of these assays is available in "Table 3.1 Single cell migration assays and there advantages and disadvantages.".

3.2. Boyden chamber assay

The Boyden chamber assay, also referred to as the Transwell assay, is a standard method used for migration analysis.[45] The Boyden chamber assay is an ***end-point assay*** and indicates the number of cells that have migrated through a membrane.[44]

The setup consists of two stacked culture compartments separated by a ***porous membrane*** "Figure 3.1A". Cells are seeded in the upper compartment, and culture medium supplemented with a chemo-attractant is added to the lower compartment. The chamber is then incubated, allowing cells to adhere and form a cell monolayer. Cells can then migrate through the pores to the lower surface of the membrane. After incubation, the cells are fixed and stained. Cells that remain on the upper surface of the membrane are removed using a cotton swab. The stained migrated cells can then be counted using a microscope, or the stain can be resolubilized and quantified.[45,46] Chambers that exclude light can be used with fluorescent dyes, and this removes the need to count cells and reduces the amount of steps in the protocol.[47]

The benefit of this assay is that it allows the researcher to study 3D cell chemotaxis and it can be used for both migration (uncoated membrane) and invasion assays (coated membrane).[45] A design limitation of the method is that the end-point result is based upon a complete sample response; therefore, heterogenous populations cannot be distinguished.[44] Furthermore, migrating cells cannot be imaged in real-time.[48] An additional drawback is that the assay can be time-consuming as optimization is required to achieve significant differences between experimental groups[44]. Commercial Boyden chamber assays are available, including Boyden Chamber Assays from Cell Biolabs, Inc., and Transwell and FluoroBlok systems from Corning.

3.3. Colloidal particle assay

Another assay used to study single-cell migration is the colloidal particle assay, also known as the colloidal gold ***phagokinetic track assay*** — this a straightforward method for observing cell migration. The protocol involves monitoring tracks made by motile cells on specially coated surfaces.

Microplates are first coated using colloidal gold or quantum dots. After that, cells are added at a low cell density to the surface.[49-51] Using cells at low density prevents trail overlaps.[50] Motile cells will then phagocytize the coating as they move, creating a phagokinetic track free of colloidal gold or quantum dots "Figure 3.1B". This trail can be visualized under a light microscope, and the area of the path can be compared between different experimental groups.[50-53]

The advantages of this assay are that cells that are unable to pass through the Boyden chamber assay membranes can be tested, cells can be imaged in real-time, the assay has a higher sensitivity compared to the Boyden chamber assay, and the assay is automatable.[50,53] The limitations of the method are that the preparation of the coating requires optimization for best results and cells that backtrack on the path are not recorded. Furthermore, the method is not suitable for the measurement of bulk chemotactic effects, 3D migration, or the detailed investigation of cellular movement.[51] No commercial kits are available for this assay.

3.4. Microfluidic chips

A more sophisticated method for investigating cell migration makes use of custom microdevices and ***microfluidics***.[48] Microfluidic assays are flexible and can be used for either ***end-point or continuous analysis.***[44] These assays allow the researcher greater control of the experimental design and migration channel.[48] These microdevices usually consist of a silicon substrate fabricated of polydimethylsiloxane (PDMS) "Figure 3.1C".[44,48] These devices can also include gelatin or other extracellular matrix-based polymers to reflect the cell microenvironment better.[44] For visualization purposes, cells can also be stained with markers such as green fluorescent protein (GFP).[48]

The benefits of microfluidic assays are that they require minimal volumes and provide the researcher with precise microenvironment control.[48,54] Low volumes are particularly useful when examining small tumor biopsy samples. These devices compare well with Boyden chamber assays in terms of resolution, precision, and investigating chemotaxis.[44] A disadvantage of microfluidic devices is that they generally do not allow for distinguishing between individual cells, nor do they allow for the retrieval of cells after the migration assay.[48] An additional drawback of microfluidic chips is that they can be time- and cost-consuming because of microfluidic pumping systems and continuous recording under cell culture conditions. The time and cost can hinder its high-throughput capability and the use of the technique by researchers.[44] Commercial kits include the Millicell® µ-Migration Assay Kit from Merck and the µ-Slide Chemotaxis by ibidi.

3.5. Time-lapse cell tracking

This technique allows the researcher to ***track individual cells*** in real time.[55] In this protocol, cells are seeded on a surface and allowed to adhere. This surface is mounted onto an environmentally controlled microscope stage "Figure 3.1D" or on an in-incubator microscopy system such as the CytoSMART Lux2. After adhesion, images of the migrating cells are recorded at five to ten minute intervals.[46,55,56] These time-lapse movies are used to identify and track single cells that are not undergoing cell division. This technique can be combined with microfluidic devices.

The advantage of live-cell time-lapse video microscopy followed by cell tracking is that other cell migration methods often only report on end-point results and can be influenced by cell proliferation and cell-cell contacts.[46] Furthermore, not only the migratory path can be determined using this technique, but also the migration speed and persistence. Commercial software for single-cell monitoring exists such as MetaMorph by BioImaging Solutions Inc, and there are many free to download software that can be used, such as TrackMate by ImageJ. A comparative list of available software available to analyze single-cell migration data is summarized by Massuzo et al. (2017).[57]

Figure 3.1

Single cell migration assays.
A) Boyden chamber assays B) colloidal particle assay C) microfluidic chips D) Time lapsed cell tracking.

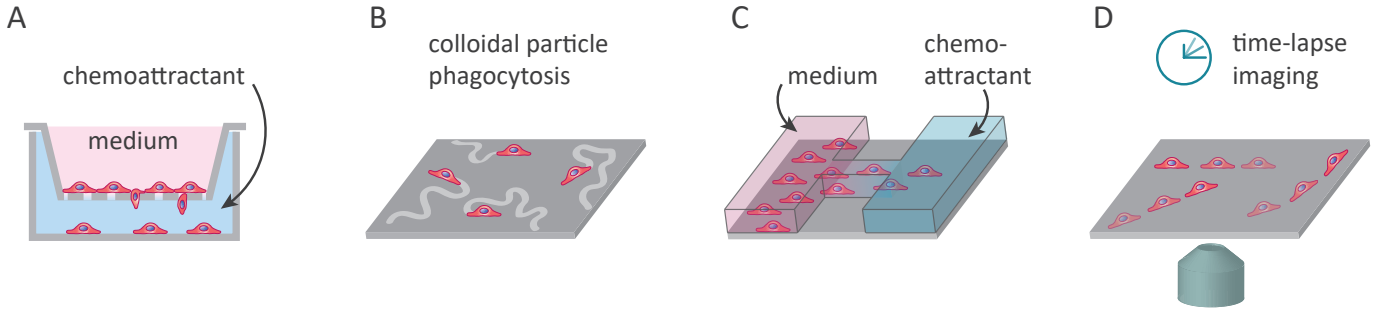


Table 3.1

Single cell migration assays and there advantages and disadvantages.

Method	Advantages	Disadvantages	Commercial Products	References
Boyden chamber assay	3D cell chemotaxis, migration /invasion assays	End-point analysis, no real-time imaging, time-consuming, not all cell type compatible	Boyden Chamber Assays (Cell Biolabs, Inc.), Transwell and FluoroBlok systems (Corning)	45
Colloidal particle assay	Real-time imaging, automatable	Coating requires optimization, cell backtracking not measured	-	51
Microfluidic chips	Minimal volumes, precise microenvironment control, end-point/ continuous analysis, customizable	Time-consuming, expensive, individual cells not usually analyzed.	Millicell® µ-Migration Assay Kit (Merck), µ-Slide Chemotaxis (ibidi)	44,48
Time-lapse cell tracking	Continuous analysis, no cell proliferation, no cell-cell contacts	Data-intensive	MetaMorph (BioImaging Solutions Inc), TrackMate (ImageJ)	55,57

3.6. Conclusion

In this chapter, commonly used assays investigating single-cell migration were discussed. The methods covered were the Boyden chamber assay, colloidal particle assay, microfluidic chips, and time-lapse cell tracking, however, many different (custom developed) methods for single cell tracking exist. These techniques include both end-point and continuous analysis, and can be used to quantify both the whole sample and individual cell responses. In addition to single-cell migration, cells can migrate collectively. Information relating to collective cell migration techniques, specifically cell removal, exclusion, and outgrowth assays, will be discussed in “Chapter 4. Comparision of 2D sheet migration assays”.

Chapter 4. Comparision of 2D sheet migration assays

4.1. Introduction

Collective 2D migration can be studied in several ways *in vitro*. The study method matters, because a barrier method could be sufficient to study migration of cells into a void, but a wound healing assay requires a degree of cell injury.[58] Moreover, wounding can result in debris accumulation in the wounded area or void, which can cause chemotactic effects and will thereby influence the cell culture environment.[59] Because the experimental method can have an effect on the outcome of the results, the assays are characterized into: 1) cell removal (wounding) assays in which cells are physically damaged upon creation of a void, 2) cell exclusion assays where a barrier is removed to create a void in which the cells can migrate and 3) cell outgrowth assays where cells can migrate out of a confined area after removal of a barrier.[7,17] Different variations of these three different types of 2D collective cell migration assays will be discussed in the next sections.

4.2. Cell removal assays

Cell removal assays are generally a low-tech solution to study ***migration and wound healing.*** The standard process entails damaging part of a confluent layer of cells, thus creating a cell-free zone in which cells can migrate. Thereafter, the cellular (and matrix) debris is removed by washing the sample with phosphate-buffered saline (PBS) or culture medium. As the last step, medium (supplemented with test compounds) is added to the samples, and the wound closure is measured. The wound size can be determined at the beginning and end of the assay, or at multiple time points throughout the assay. In the latter case, samples are either taken out of the incubator at each timepoint to take the images, or a live-cell imaging microscope is used with which the cells can be kept at constant temperature throughout the entire experiment.

The most cost-efficient and commonly used type of cell removal assay is the ***scratch assay,*** in which a pipet tip or other scratching tool is used to create a wound. However, many custom and commercially available variations of this assay have been developed in the last decade. These assays range from simply standardizing the mechanical scratch making process to chemical wounding of the cell monolayer “Figure 4.1 Cell removal assays.”. Each of these methods has its technical requirements, reproducibility, and type of damage. The most common methods and their advantages, disadvantages, as well as commercially available products, are described below and summarized in “Table 4.1 Methods of cell removal assays.”

4.2.1. Scratch assay

The scratch assay is the most commonly used wound healing assay in which a confluent layer of cells is mechanically damaged since it is straightforward and affordable.[41,60] Next to this, the scratch assay can be performed using readily available lab tools ranging from pipet tips to metal spatulas.[60,61] The downside to removing cells in this manner is that it often disrupts the underlying extracellular matrix (ECM) coating on culturing surfaces. In addition to this, the accumulation of cells on the edge of the scratch can affect results when studying wound closure.[62] ***Mechanical scratches*** “Figure 4.1A” are mainly made by dragging a scratch tool from one side to the other, thus creating a linear scratch with a specific length and width. Therefore, this assay is limited in the range of void shapes that can be created. Although easy to implement, ***manual scratching*** often

results in differences in the scratch shape and size between samples, which can severely affect the reproducibility between samples and experiments. To reduce variability, several ***commercial solutions*** have been developed. The most simple techniques comprise tools containing multiple identical pins that are moved simultaneously to create numerous comparable and reproducible scratches in one operation. Examples of these tools to form scratches of the same size are the Cell Comb™ Scratch Assay (Merck), IncuCyte® WoundMaker, HTSScratcher by Peira, and Wounding Pin Tools by V&P Scientific, Inc. More advanced tools, such as the BioTek AutoScratch™ make use of an automated system with multiple identical scratching pins to create comparable and reproducible voids. More examples of commercially available wound making tools can be found in "Table 4.1 Methods of cell removal assays."

4.2.2. Stamp wounding

Stamping the confluent monolayer of cells is an ***alternative method*** to mechanically create a wound in cell cultures. Placing a weighted stamp on top of a layer of cells "Figure 4.1B", either manually or automatically, destroys the underlying cells but often keeps the underlying substrate intact.[59,63] Another advantage of using stamps to wound the cell culture is that many different wound shapes and sizes can be created, in contrast to the linear wounds that can be created by using the scratch method. The type of stamp material mainly determines to what extent cells and debris will be removed from the cell culture area. PDMS is commonly used for this reason, as cell debris easily attaches to it. However, caution should be taken as it is possible to disrupt the underlying structures severely, especially when applying too much pressure or when using specific stamp geometries that negatively affect the pressure distribution on the cells.[64]

Stamp wounding can be integrated into ***microfluidic assays***, as has recently been demonstrated by Sticker *et al.* (2017) and Kim *et al.* (2019). In both microdevices wounding was generated using pneumatically controlled circular stamps.[65,66] Pneumatic control allows accurate control of the pressure of the stamp. Furthermore, the advantages of stamping in a microfluidic environment include higher reproducibility and robustness, the removal of cell debris during perfusion and reduced assay duration. These microdevices also lend themselves to being automated further.[65,66]

Stamping can also be combined with ***heat***. Wounding via localized heating can be performed using a thermo-mechanical combination of a heated stamping method.[67] This type of wounding is useful to study the wound healing after skin injuries *in vitro*. All thermal wounding inherently suffers from heat spreading away from the site of damage, making it challenging to create reproducible wounds.[67]

4.2.3. PDMS barrier

A variation of the scratch assay is the removal of a barrier from a monolayer of cells. In this assay, a hydrophilic PDMS slab is used as a ***barrier in the cell culture area.***[68] When cells are seeded, they are allowed to grow and form a monolayer on the culture surface as well as on top of the PDMS barrier. Upon removal of the PDMS slab from the substrate, a void with wounded cells on the boundary is created for the cells to migrate into "Figure 4.1C". This method of wounding is much more reproducible compared to creating scratches due to the standardized shape and size of the PDMS slabs of each sample. It is also possible to cut PDMS into any geometry of interest. This method is particularly useful to study cell-substrate interactions, because no matrix proteins are deposited by cells that are removed when creating the void.[68]

A ***primary disadvantage*** is that only hydrophobic PDMS will auto-adhere to dry and uncoated surfaces. However, to culture cells, the PDMS should be made hydrophilic, which increases the chance of cells and proteins protruding underneath the PDMS slab before removal.[69,70] A minor disadvantage is that the PDMS slab needs to be attached and removed from the culture surface, which makes it difficult to automate this assay. No commercial products are available at the time of writing.

4.2.4. Laser-based wounding

Wounds can be generated using ***ablation*** by infrared (IR) or ultraviolet (UV) lasers "Figure 4.1D". Depending on the method, custom void geometries can be produced. The creation of a cell-free zone via laser ablation offers high reproducibility and enables high throughput under sterile conditions. Lasers can be of varying wavelengths but are most commonly UV-B (280nm-315nm) or UV-C (100nm-280nm).[71] The effects of thermal damage can also be investigated using IR-lasers. Localized heating of cell cultures simulates thermal-damage response leaving denatured ECM and cellular debris behind, creating a unique environment into which cells can migrate.[67] As already mentioned for thermo-mechanical stamping, a downside of using heat is the reduced reproducibility due to heat spreading. A commercial research laser system for this purpose is the Stiletto® by Hamilton Thorne.

4.2.5. Electrical wounding

Electrical wounding is a technique based on the electroporation of cells by applying a local current via gold-film electrodes embedded in the culture vessel "Figure 4.1E". By sending a high current through the ***electrode***, cells on the surface are electroporated leading to cell death.[72] These electrodes can also measure the impedance in the electron flow caused by the cells in the culture vessel. The impedance is used as a measure for cell migration; the more cells migrate into the wound, the higher the impedance.

The ***advantages*** of this method include the high reproducibility of results because of the use of impedance measurements instead of optical measurements. This automated, real-time measurement excludes the possibility of errors due to human intervention.[73] However, the impedance measurements are easily influenced by changes in temperature, pH, or medium.[72] Next to electroporating the cells, the electrodes could also cause local heat development that affects cell viability in the surrounding areas. Currently, two ***commercial systems*** are available, the Electric Cell-substrate Impedance Sensing system (ECIS™) by Applied Biophysics and the xCELLigence by Aligent. Both systems require the use of special (expensive) gold-coated well plates to be able to electrically wound the cells.

4.2.6. Chemical wounding

Chemical wounds can be created by ***chemical damage or removal*** of part of the cell monolayer "Figure 4.1F". As cell dissociation reagents (e.g. trypsin) are essential in any research involving cell culture, chemical wounding can be performed in any cell culture lab. Localized wound areas can be created by adding a small droplet of the dissociation reagent to the cell culture.

To control the dimensions of the cell free area, ***microfluidic devices*** are the norm for chemical wounding of cell cultures. A microfluidic device consists of two or more channels with inlets and outlets. Laminar flow prevents the solutions (e.g. culture medium with and without trypsin) from mixing to detach only one part of the cell monolayer.[74] After detachment, the trypsin is removed and cells can start to migrate into the void. This results in a fully integrated wound healing assay that can be precisely controlled.[75] The small volumes in microfluidics make these assays useful for studies with rare or costly compounds and cells.[74] One of the advantages of chemical wounding is the uniform matrix without substrate damage that is left in the cell-free area.[76] Other advantages of this system include the mechanical and chemical stimulation of cells by investigating the shear stress by fluid flow and by introducing chemical gradients in the channels.[76] However, microfluidic devices can be quite demanding as daily medium changes are necessary because of the small volume of the medium inside the device. Because of this, vigilant control of the humidity in the incubator is also essential. Next to this, the successful use of microfluidic devices requires expertise. Many challenges can arise with microfluidic devices, including cell clumping, air bubble formation (prevents cells from growing), and leakage.

4.2.7. Vacuum-based wounding

A recently described method by De Ieso and Pei (2018) uses ***vacuum suction*** to remove an area of cells "Figure 4.1G". In this way, a circular void is created using commonly available lab equipment (vacuum-pump and pipette tip).[77] The benefit of this method is that the circular wounds are smaller than the field of view (FOV) of the microscope. This makes the relocation of the sample at each analysis timepoint more reproducible compared to that of linear voids. Another benefit is that cellular and ECM debris is removed when creating the void. This manner of damaging offers higher reproducibility than the manual scratch assay. However, since it is still a manual technique, it is less reproducible in wound size and geometry as opposed to automated cell removal techniques.[77]

Figure 4.1 Cell removal assays.
A) scratch assay B) stamp wounding C) PDMS barrier D) Laser-based wounding E) electrical wounding F) chemical wounding G) vacuum-based wounding.

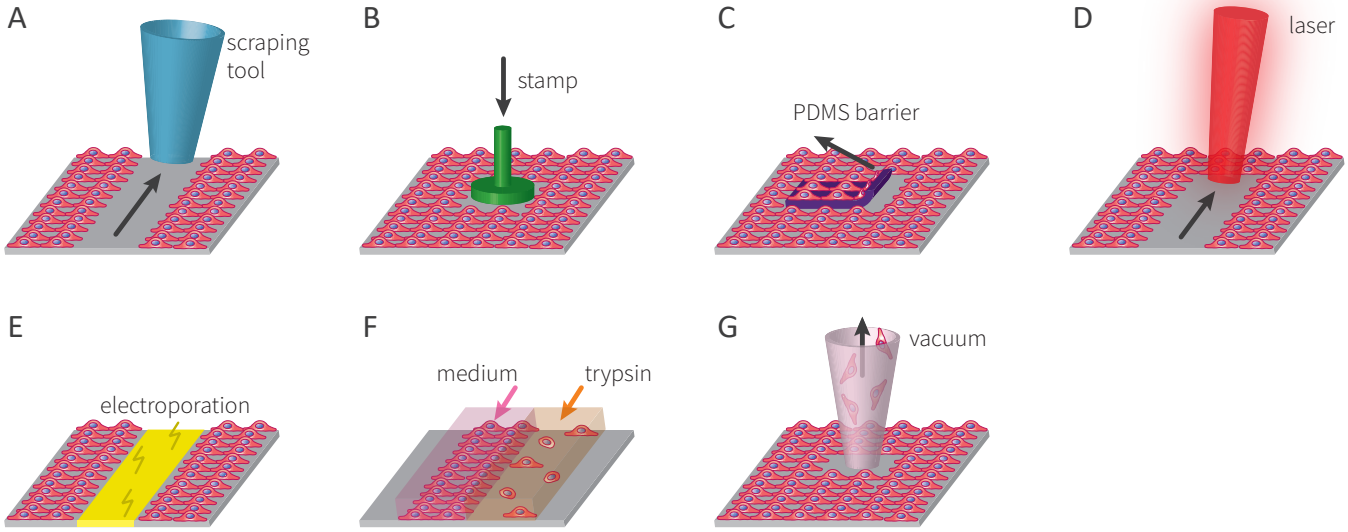


Table 4.1 Methods of cell removal assays.

Method	Advantages	Disadvantages	Commercial Products	References
Scratch assay (Mechanical)	Little to no requirements Easy to implement Cheap (manual) Fast	Disrupts extracellular matrix (coating) Low reproducibility (manual)	AutoScratch™ (BioTek) Cell Comb™ Scratch Assay (Merck) WoundMaker (IncuCyte®) HTSScratcher (Peira) Wounding Pin Tools (V&P Scientific, Inc.)	60, 61
Stamp Wounding ((Thermo-) mechanical)	Maintains substrate Cell debris removed with stamp High geometrical control	Manual stamping affects reproducibility Thermal stamping impairs reproducibility No commercial products available	-	59, 63
PDMS Barrier (Mechanical)	Reproducible way of cell wounding Reproducible void geometry No substrate/matrix in void	Difficult to automate No commercial products available	-	68
Laser-based wounding (Radiation or Thermal)	High reproducibility High throughput Custom shapes Sterile conditions	Thermal ablation affects reproducibility Expensive instruments	Stiletto® (Hamilton Thorne)	67, 71, 78
Electrical Wounding (Electroporation)	Submicroscopic resolution Real-time measurement Automated Quantitative and reproducible results	Special equipment required Expensive culture vessels Difficult detachment of cell monolayers Local heat development affects cell viability	ECIS (Applied Biophysics) xCELLigence (Agilent)	72, 79
Chemical Wounding (Chemical)	Chemicals widely available No matrix damage Useful for rare/expensive compounds/cells (microfluidics)	Droplet shape not reproducible Microfluidic devices required to control geometry Use of microfluidic devices requires expertise	-	74-76
Vacuum-based wounding (Mechanical)	Reproducible size Commonly available in cell lab Free from debris	Variability in wound sizes and shapes	-	77

4.2.8. Conclusion

Cell removal assays recreate wounds *in vitro* and can be used to study healing and cell migration. By far, the most common technique is the scratch assay that uses mechanical damage to remove cells from a monolayer, creating a void into which cells can migrate. This migration is then imaged using a microscope. Next to various types of mechanical wounding, cell monolayers can also be damaged using stamps, laser ablation, electricity, and chemicals. In addition to wounding, the cell monolayer can be limited to attach to a certain area by the use of physical barriers. The barriers can then be removed to create a void. Cell exclusion zone ("4.3. Cell exclusion zone assays") and outgrowth assays ("4.4. Cell outgrowth assays") both make use of a variety of physical obstacles and will be discussed below.

4.3. Cell exclusion zone assays

Cell exclusion zone assays differ fundamentally from wounding assays; instead of removing part of the culture, they provide an ***artificial barrier*** to the culturing area. This assay is currently the only method that can investigate the effects of extracellular matrix proteins on cell motility.[80] The cell exclusion zone assay also provides researchers with several benefits over traditional wounding assays.

The main advantage is that the assay does not destroy the ***cell monolayer.*** Damage to the monolayer during wounding assays has several important implications. Firstly, cells that are damaged release intracellular contents into the medium and generate reactive oxygen species.[69,81,82] Cells at the border of the mechanical injury also often transiently retract, and this not observed when exclusion zone assays are used.[81] Mechanical wounding can also trigger other processes such as anoikis (detachment induced cell death), cell membrane repair, phagocytosis, and cytokine production. These additional processes can increase experimental noise that can affect data analysis.[82,83]

The second advantage of using cell exclusion zone assays focuses on the ***cell-free surface*** that is made when the barrier is removed. The exact geometry of the surface can be easily defined and reproduced based on the design of the barrier. Multiple barriers even allow for parallel testing in the same cell monolayer.[81,83] When the barrier is removed, the cell-free surface that is created has delineated borders in comparison to those produced in wounding assays and these defined boundaries improve overall assay reproducibility.[81] The chemistry of the cell-free surface can also be configured in cell exclusion assays.[80,81,84] In contrast, the surface chemistry generated in wounding assays is dependent on the extracellular matrix that is deposited by the cell monolayer.[81,83]

Cell exclusion zone assays are also cost-effective when compared to more repeatable and commercial scratch assays that create wounds, and fewer handlings are required.[83] Several ways exist to create cell-free zones, as will be discussed below and are summarized in "Table 4.2 Cell-exclusion zone assays methods"

4.3.1. Solid barriers

Zones of cell exclusion can be created with the use of solid barriers such as ***stoppers and stencils.*** These barriers are placed on the microplate surface before plating the cells. Cells are then seeded into the microplate and reach confluency in the presence of the barrier. Once a monolayer has formed, the barrier can then be removed to produce a cell-free zone into which migration can occur "Figure 4.2A".[81–85] Solid barriers can be designed specifically to meet the requirements of the researcher.

Barriers require two essential characteristics, namely that they have a high degree of adhesiveness to the surface of the dish and that they can be easily removed after attachment.[80] To meet these requirements, the material used in the design of the barrier can vary from PDMS, silicon, silicone rubber and Teflon to agarose.[69,80–83,85-87] These materials are then generally formed into circular or rectangular shaped barriers

capable of being inserted in microplates depending on the experiment.[88] The use of solid barriers is cost-effective as the barriers can be made to a predefined size negating the need to collect premigration images. In the case of silicon stoppers, these can be sterilized and reused.[82,85] Assays that make use of solid barriers are compatible with high-content imaging (HCI) techniques, and commercial kits are available such as the Oris™ Cell Migration Assay manufactured by Platypus Technologies and Culture-Inserts by ibidi.[83,89]

The disadvantage of using mechanical barriers is that there are underlying physiological differences in cell migration based on mechanical wounding or the sudden availability of space when a barrier is removed.[69,82] These physiological differences must be taken into consideration when designing an experiment. The type of material used for the solid barrier may, under high confluency, cause the cells to attach to the barrier itself.[90] When the barrier is removed sheets of cells are then pulled from the monolayer affecting reproducibility. The manual removal of the stopper also limits this assay for high throughput screening (HTS) operations.[80,83] Barrier removal can circumvented altogether using microfluidics and pneumatic control of barriers.[65]

4.3.2. Degradable gels

The manual removal of solid stoppers can also be made redundant with the use of ***degradable gel stoppers.*** As with cell exclusion zone assays that make use of solid barriers, the gel barrier is placed onto the surface of the microplate before cell seeding. The gel barrier either degrades when cell media is added, or a gel removal solution is used to remove the barrier once a cell monolayer has formed "Figure 4.2B". Complete degradation of the gel stopper can take 30-60 minutes. Cells then migrate into the newly exposed cell-free surface.[91,92]

These water-soluble gels can be made using poly(N-acryloylmorpholine), amongst other polymers.[93] Drawbacks with this system can be that cells need to adhere rapidly, cells can settle on the degrading barrier, or cells can detach when the gel is being removed. Cell exclusion zone assays that use degradable gel barriers are well suited for HTS operations.[91,94] There are commercial kits available for this assay, including the Radius™ Cell Migration Assay by Cell Biolabs, Oris™ Pro Cell Migration Assays by Platypus Technologies, and Cell migration BioGel assay by Enzo Life Sciences.[91,95-97]

4.3.3. Liquid barrier

Aqueous two-phase systems (ATPS), which create a liquid barrier between the cells and a void, can be used as a cell exclusion zone assay. In this technique, an ATPS can be produced when solutions of two incompatible polymers are mixed at threshold concentrations. The most well understood ATPS is the ***polyethylene glycol (PEG)/dextran (DEX) system.*** PEG and DEX phase separation occurs at low polymer concentrations and under non-denaturing conditions making it viable for mammalian cells.[98] In addition to ATPS, the use of other immiscible liquids has been applied to cell exclusion zone assays though this has not been widely used.[99] To set up a cell exclusion zone assay using ATPS, droplets of DEX can be printed onto a substrate and covered with a solution of PEG containing mammalian cells "Figure 4.2C". The cells included in the PEG phase are excluded from entering the DEX phase due to PEG/DEX interfacial tension. A cell-free surface is maintained in the DEX droplet that can then be used for the assay.[98,100]

The advantages of ATPS are that these systems are inexpensive to establish and do not require sophisticated equipment. The assay is rapid, compatible with a variety of cell types, and can be automated for ***high throughput.***[98,100,101] The geometry of the cell-free surface can also be controlled in more sophisticated setups.[102] The drawbacks of this approach are that the viscosity of the DEX phase can result in increased variability of pipetted volumes, and the DEX droplets can be disrupted when the PEG solution is added.[98]

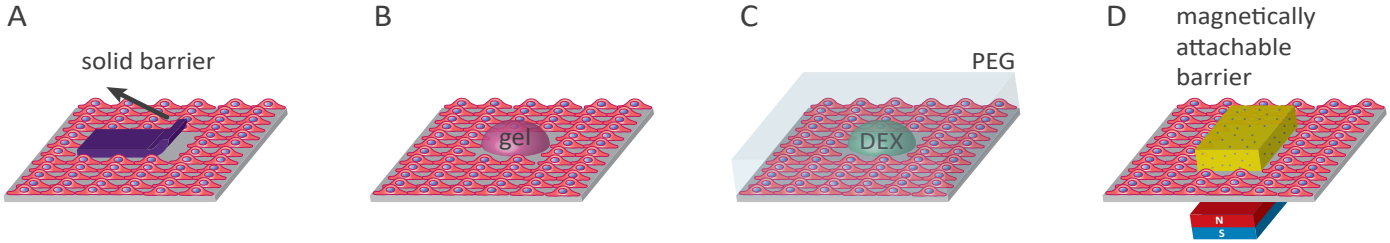
4.3.4. Magnetically attachable barriers

Cell migration is very much dependent on the underlying matrix onto which cells move. Given the importance of the matrix, *in vitro* assays are needed to evaluate the effects of surface composition, organization, and presentation on cell migration. Magnetically attachable barriers can be used to ***preserve the underlying substrate*** of the cell-free surface "Figure 4.2D". To date, these barriers have been produced using PEG and magnetized with iron ferrous microparticles.[103,104]

Magnetically attachable barriers allow researchers to evaluate unique surfaces such as micropatterned proteins, nano-textured surfaces, and pliable hydrogels.[103,105] The barriers also do not affect cell viability.[104] A drawback to this method is that these barriers are time-consuming to produce.[88]

Figure 4.2 Cell exclusion zone assays.

A) Solid barriers B) Degradable gels C) Liquid barrier using polyethylene glycol (PEG) and dextran (DEX) D) Magnetically attachable barriers.



4.3.5. Conclusion

Cell exclusion zone assays use physical barriers to prevent cell migration. The underlying surface is then exposed by removing the barriers, creating a void that the surrounding cells then close. This technique makes use of a variety of barriers to exclude cells. These include solid and degradable barriers, ATPS systems and magnetically attachable stencils. The cell exclusion zone assay provides the scientist with a technique of investigating cell migration without wounding the cells and for investigating the effect of the cell-free surface chemistry on collective migration. Other cell migration assays using barriers include outgrowth assays used to study collective migration, as will be discussed in "4.4. Cell outgrowth assays".

Table 4.2		Cell-exclusion zone assays methods		
Method	**Advantages**	**Disadvantages**	**Commercial Products**	**References**
Solid barriers	Low expertise required	Limited throughput	Oris™ Cell Migration Assay (Platypus Technologies) Culture-Inserts (ibidi)	80
Degradable gels	High throughput No barrier removal step	Cells must adhere when gel degrades Proprietary technology limits availability of surface coatings	Radius™ Cell Migration (Cell Biolabs, Inc) Oris™ Pro Cell Migration Assays (Platypus Technologies) Cell migration BioGel assay (Enzo Life Sciences)	91
Liquid barrier	Broad cell range can be tested High throughput Delicate surfaces can be used	Phase viscosity can affect variability	-	98
Magnetically attachable barriers	Unique cell-free surfaces can be investigated	Time-consuming barrier production	-	103

4.4. Cell outgrowth assays

Cell outgrowth assays, also referred to as ***nest assays or radial migration assays,*** can be used to investigate collective cell migration in fields such as oncology and drug discovery.[106-110] The outgrowth assay is essentially the opposite of the cell exclusion zone assay and wound healing assay in that cells expand outward from a nest as opposed to inward to close a void.[90,111] A further difference between the outgrowth assay and the wound healing assay is that it reflects the normal cellular microenvironment better.[112] Both cell outgrowth assays and cell exclusion zone assays resemble the normal cell microenvironment; therefore, these two assays share many of the same advantages. The two main benefits of the outgrowth assay are that the cell monolayer is not wounded and the cell-free surface can be defined before migration.[80,113]

There are a few disadvantages to the outgrowth assay that should be taken into consideration when designing an experiment. Firstly, the initial amount of cells in the nest cell number may have an effect on the rate of migration. Therefore it is important to repeat assays with different initial cell densities so that these effects can be observed and quantified beforehand.[114] In addition, the geometry of the initial cell area affects cell migration and, therefore, the outcome of the assay.[113] For most experiments, however, the geometries used in outgrowth assays are either square or circular.[111] Well designed experiments can mitigate these disadvantages. The following sections will elaborate on the various outgrowth assays available. The advantages and disadvantages are summarized in "Table 4.3 Methods for cell outgrowth assays."

4.4.1. Explant outgrowth assays

One assay that lies between *in vivo* studies and *in vitro* experiments is the explant outgrowth assay. The method involves the collection of tissue samples from an organism, ***culturing tissue specimens,*** and monitoring the outward migration of cells from these explants "Figure 4.3A".[115] This assay has been used to monitor the outgrowth of keratinocytes from skin explants, study glomerular diseases using outgrowth from kidney explants, investigate tenocyte migration from tendon explants, and examine lymphangiogenesis using lymphatic duct explants.[115-118] The advantage of using explants in cell outgrowth assays is that the unique features present within diseased tissues are preserved.[115]

4.4.2. Solid barrier

The most common outgrowth assay features a solid barrier to initially ***confine cultured cells*** before migration. This migration assay is most commonly known as the ***fence assay.***[119] Cells are limited to an area of the microplate with the use of a solid barrier "Figure 4.3B". The barrier is usually cylindrical, with the cells added inside the cylinder and allowed to adhere to the surface of the microplate. The cylinder can be made from glass or metal-silicone.[112,119-122] Alternatively, stencils made from PDMS can be used to create uniform cell islands on the surface.[123,124] Before the experiment, the barrier is removed, and any unattached cells are rinsed off. After that, the remaining attached cells migrate onto the surrounding cell-free substrate.[112,119–122]

The advantages of solid barriers include that they are reusable and that they can be adapted for high throughput automated imaging systems.[108,123] An example of a high throughput system is the injection-molded gaskets developed by Oliver et al. (2020), which enable the performance of 24 radial migration assays simultaneously.[108] However, drawbacks of this system are the non-uniformity of clamping pressure of the gaskets, the formation of bubbles, and thorough cleaning that is required after each experiment.[108] To date, only commercial cylindrical barriers are available such as the metal-silicone barriers from Aix Scientifics and Pyrex® Cloning Cylinders.[112,114,121] Culture-Inserts from ibidi can also be used for cell outgrowth assays.

4.4.3. Liquid barrier

The use of liquid barriers, created by ***aqueous two-phase systems (ATPS)*** can be applied to both outgrowth and cell exclusion zone assays. As mentioned in "4.3.3. Liquid barrier", an ATPS can be produced when solutions of two incompatible polymers are mixed at threshold concentrations. Polyethylene glycol (PEG) and dextran (DEX) are most commonly used in ATPS because phase separation occurs at low polymer concentrations and under non-denaturing conditions, making it viable for mammalian cells.[98] Other immiscible liquids have been applied to cell exclusion zone assays, however they are not widely used.[99]

To set up an outgrowth assay using ATPS, droplets of DEX containing mammalian cells can be printed onto a substrate and covered with a solution of PEG. The cells included in the DEX phase are excluded from entering the PEG phase due to PEG/DEX interfacial tension "Figure 4.3C". A cell-free surface is maintained outside the DEX droplets that can then be used for the assay.[98,100] The advantages of ATPS are that these systems are inexpensive to establish and do not require sophisticated equipment. The assay is rapid, compatible with a variety of cell types, and can be automated for high throughput.[98,100,101] The geometry of the cell-free surface can also be controlled in more sophisticated setups.[102] The drawbacks of this approach are that the viscosity of the DEX phase can result in increased variability of pipetted volumes, and the DEX droplets can be disrupted when the PEG solution is added.[98]

4.4.4. Air barrier

The ***liquid-gas interphase*** can also be used to generate cell-free surfaces for cell outgrowth assays. In this method, droplets containing cells are added to a dry surface, and are allowed to adhere (between 30-60 minutes). Following adherence, the surface is then entirely covered in cell medium, enabling the cells to migrate out from original droplets "Figure 4.3D".[88]

This technique is simple to set up and only requires standard cell culture materials. Limitations of this system include the requirement that cells must adhere rapidly, the cell-free surface is initially dry, and the cell patterning can vary due to the short adherence time to maintain cell viability in the droplets.[88]

4.4.5. Spheroids outgrowth

Another regularly used assay is the flat surface spheroid migration assay or simply the ***spheroid migration assay.***[125-128] This assay is a combination of three-dimensional (3D) ***cell spheroids*** and the two-dimensional outgrowth assay. In this assay, spheroids, produced from cells in suspension culture, are attached to the microplate surface and measured for outgrowth "Figure 4.3E".[107,109,110,129] The surface of the plate can be coated to alter attachment affinity. Laminin, which is a strong adhesion protein for epithelial cells, can be used to increase attachment, whereas, agarose can be used to prevent attachment to the surface.[107,130]

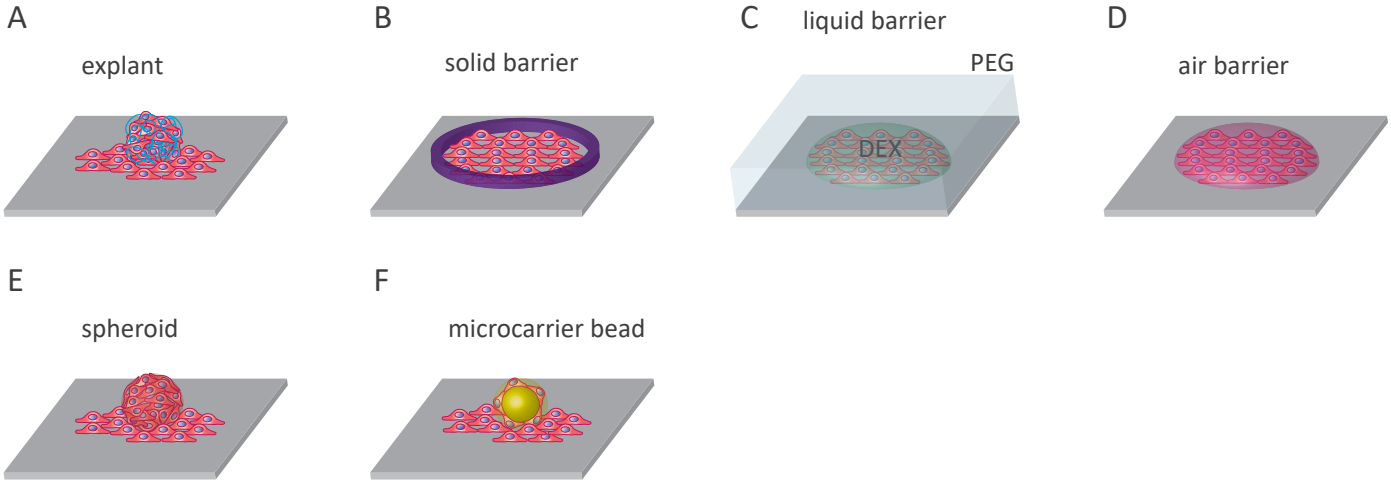
A significant advantage of using spheroids is that, because of their 3D structure, they more closely represent the tissue physiology being studied, such as small cancer clusters.[111] Another advantage of starting with spheroids is that they can be produced with a consistent diameter. This uniformity makes the assay accurate and easily miniaturized and automated for high content imaging.[107] A limiting factor to this assay is that not all cells can form spheroids, and experience with spheroid formation is required.[111] Commercial plates such as the 15-well µ-Slide Angiogenesis plates by ibidi have been used to prepare gels for spheroid attachment.[109]

4.4.6. Micro-carrier beads

The use of micro-carrier beads is a wholly different approach to tackle reproducibility. Pre-seeded micro-carrier beads provide a highly reproducible basis from which cells can migrate outwards "Figure 4.3F". This assay can be used to study cell migration via analysis of spreading from the point of contact between the bead and culturing surface, or invasion when the bead is embedded in a layer of gel such as fibrin gels.[131] Beads can be made from ***hydrated collagen-coated dextran beads.***[132] Commercial beads are available such as Dextran-coated Cytodex 3 microcarrier beads from Amersham Biosciences.[133] This method yields highly reproducible results; however, there are a few drawbacks. The disadvantages include the cost of the beads, and beads that are insufficiently coated in cells must be excluded from the assay.[111]

Figure 4.3 Cell outgrowth assays.

A) Explants B) Solid barrier C) Liquid barrier D) Air barrier E) Spheroids outgrowth F) Microcarrier beads.



4.4.7 Conclusion

A multitude of outgrowth assays are available to investigate collective cell migration. These can range in sophistication from spheroid outgrowth assays and ATPS systems to air and solid barriers to create cell nests for expansion. All these assays, except explants, spare the cell monolayer from wounding. The scientist can also specify the substrate onto which cells migrate. The outgrowth can be used in parallel with other cell migration assays.

Table 4.3 Methods for cell outgrowth assays.

Method	Advantages	Disadvantages	Commercial Products	References
Explants	Preserves features of diseased tissue	Requires ethics approval, wounding	-	115
Solid barrier	Reusable, autom-atable	Incomplete contact with surface	Metal-silicone barriers (Aix Scientifics) Pyrex® Cloning Cylinders (Fisher Scientific and Sigma)	119
Liquid barrier	Basic setup, automatable	DEX viscosity increase variability	-	98
Air barrier	Basic setup	Fast adhering cells required	-	88
Spheroids outgrowth	Consistent spheroid size, closer to *in vivo* physiology, automatable	Limited to cell that form spheroids, experience required	µ-Slide Angiogenesis plates (ibidi)	107
Microcarrier beads	Reproducible	Expensive	-	133

4.5. Conclusion

In this chapter, commonly used assays for investigating collective cell migration were discussed. The methods covered were the cell removal, cell exclusion and cell outgrowth assays. The cell monolayer can be damaged using a mechanical wounding, laser ablation, electricity, and chemicals. Several different types of physical boundaries can be used to refrain cells from entering the void or in case of cell outgrowth assays, the boundaries can create cell nests for expansion. Some assays damage the cells and others spare the underlying ECM. Depending on the research goal, a suitable assay can be selected. Guidelines relating to selecting a suitable collective cell migration assay, will be discussed in "Chapter 5. Guidelines to select the most suitable collective cell migration assay".

Chapter 5. Guidelines to select the most suitable collective cell migration assay

5.1. Introduction

In order to set-up a successful cell migration assay, several factors have to be considered that can influence the outcome of the experiment. When studying collective cell migration, research goals could involve angiogenesis[2], wound healing[3], tumor invasion[5], inflammation[6], drug discovery[107,110] and investigating the effect of the ECM on cell motility[80]. In case of single cell migration, it could involve immune surveillance[4], chemotaxis studies[44,45], researching early stages of cell invasion in metastatic processes[46], and studying individual cell responses[51,55,56]. In all cases, selecting the suitable assay will be a balance between answering the scientific question and the analytic requirements that are available to the researcher in terms of time, cost and resources for increasing reproducibility and throughput.[134]

Based on the research interest, first a specific cell type is selected. For single cell migration, leukocytes[4], neutrophils[13], dendritic cells[13], fibroblasts[15] and sarcomacells[16] are mostly studied. As discussed in "Chapter 3. Assays to invenstigate single-cell migration", this could be investigated by using a Boyden chamber assay[44,45], colloidal particle assay[51], microfluidic chips[48] or time-lapse cell tracking[55]. More information on single cell tracking and analysis can be found in an extensive review by Massuzo et al. (2017).[57] For collective cell migration, epithelial cells, mesenchymal cells and cells at any intermediate state are used.[20]

This chapter will focus on guidelines to select a suitable collective cell migration assay. Depending on features such as cell damage, cell free zone geometry and boundary control, the most suitable type of collective cell migration assay (wounding assay, cell exclusion zone assay or cell outgrowth assay) can be chosen. The assay selection criteria ("5.3. Assay selection criteria") and assay properties ("5.4 Assay properties") will be discussed below. Before selecting the correct assay, one should not only select the correct cell type, but also take the effect of cell proliferation into account ("5.2 Limiting cell proliferation").

5.2 Limiting cell proliferation

Cell proliferation is one of the confounding factors when studying cell migration. When a confluent layer of cells is reached, cell migration is suppressed by contact inhibition. Upon introducing a cell free zone, cells on the edge of the cell free zone do not experience contact inhibition anymore, which induces cell proliferation. In order to differentiate between migration and cell proliferation, the timepoints and duration of the experiment should be chosen carefully. The duration of a scratch assay is generally limited to 24 hours to prevent the contribution of cell proliferation to filling the gap. Timepoints should be chosen carefully to minimize variations in migration rate that can occur when cells undergo contact inhibition.[134] Specific characteristics depend on the cell type that is studied, and the timepoints should be adjusted accordingly.[41]

One possible solution to limit cell proliferation is using a low dose of mitomycin C, an antitumor antibiotic that inhibits DNA synthesis.[114] Care should be taken when using mitomycin C as this antibiotic could also lower the migration rate of the cells and could influence changes in cell size because cell division into two daughter cells is inhibited. Another commonly used solution is the process of serum starvation to suppress cell proliferation, by using low serum concentrations in culture medium for up to 24 hours before performing the migration assay.[41] The concentrations of mitomycin C or serum should be carefully evaluated for each cell type, to prevent toxic and other complex cell-type-dependent effects.

Once the appropriate cells and, if necessary, cell proliferation inhibition method are chosen, the best fitting collective cell migration assay needs to be selected. Several criteria that can help to select the most suitable assay are discussed below.

5.3. Assay selection criteria

When setting up a migration assay, the researcher should take several criteria into account that will lead to a suitable assay selection. Although suitability can always be confirmed empirically, depending on the primary objective of the study, the researcher should consider features such as: cell damage, boundary control of cells, surface control and geometry of the initial cell-free zone/cell nest before selecting the migration assay.

5.3.1 Cell damage

Cell damage will influence the cell culture environment and thus cell migration, hence it is necessary to make a decision to include or exclude the damaging of cells in the study method. In a wounding assay, the wound edges contain surface damage, cell damage and cell debris. Subsequently, damaged cells and cell debris could release chemotactic reagents locally, thereby influencing the cell migration process.[59] Depending on the nature of the experiment, cell injury may or may not be studied. For example, when studying collective migration in tumor invasion, inflammation and angiogenesis, an assay should be chosen in which limited cell damage occurs.[136]

The cell exclusion zone assay uses a physical cell barrier where cells cannot grow, thereby creating a reproducible boundary with minimal cell damage.[80] One should take into account that a solid barrier might induce some mechanical wounding, as opposed to the other cell exclusion assays that allow the researcher to investigate cell migration without wounding the cells. Cells are also not damaged in outgrowth assays.[80] However when using an explant for the outgrowth assay, cells in the explant itself are wounded upon obtaining the tissue sample.

5.3.2 Surface matrix control

The surface matrix is important to resemble the *in vivo* microenvironment.[112] The cell culture surface can be modified using an ECM coating, however caution should be taken for wounding assays to prevent damage of the underlying ECM when scratching the monolayer.[69,81,82] This could be circumvented by using a physical barrier in a cell exclusion assay that allows for preservation of the surface chemistry configuration in the cell-free region.[82] It should be noted that using a solid barrier in the cell exclusion assay might induce some physiological differences to the ECM structure in the void.[69,82,90] In addition, in a cell outgrowth assay it is generally possible to control the surface matrix properties.[113]

5.3.3 Geometry

Upon creating a cell-free zone in a confluent monolayer of cells, cells at the edges will migrate in a defined direction into the void.[137] Cell migration in a cell outgrowth assay is measured from a dense population of cells (nest) that migrate away from each other into a large open space.[106–110] It is important to realize that cells migrating out of a nest can behave differently compared to cells migrating into a void.[134] Next to the effect of cells migrating out of a nest or into a void, the geometry of the nest or void itself can affect the cell migration.[90,111] Currently, techniques such as soft lithography allow the design of many different shapes of voids or nest, however, rectangles and circles are still the most used shapes in collective migration assay. The migration in rectangular voids and nest is nearly identical, while that is completely different for circular voids and nests. Therefore, understanding the differences between void and nest geometries can aid in choosing the proper cell migration assay.

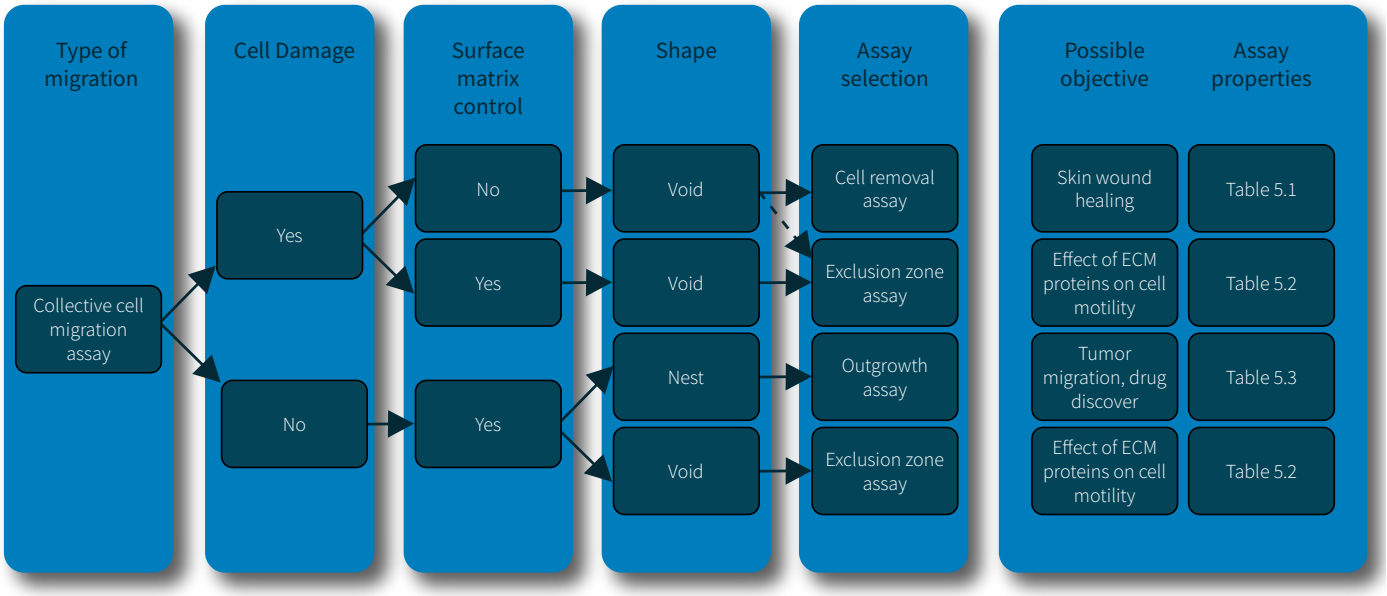
In traditional scratch assays ***rectangular voids*** are created with a width up to 900 µm and length from millimeters to centimeters. It is of importance to include both edges of the void into the images of the void and to take images of the same position at each timepoint. This is necessary for proper calculation of ***average migration rates*** or ***percent closure***. An advantage of rectangular shapes is that this geometry has the least influence on the migratory behavior. ***Rectangular nests*** are used in cell outgrowth assays where cells migrate outward. Cell migration from rectangular nests is comparable to migration into rectangular voids but nests use fewer cells per volume. This could be advantageous when the researcher can only use a sparse number of cells.[114]

Circular voids affect the cell migration rate as there is an quadratic decrease in void area as cells migrate inward. Since this quadratic decrease in area affects the migration rate as cells migrate inward, circular voids are mainly used to quantify ***percent closure*** of the area.[113] Cells in ***circular nests***, on the other hand, behave differently because of the quadratic increase in surface area when cells migrate outward.[138] It should be taken into account that migration rates will decrease over time because the cell density decreases when the nest area increase. Therefore, ***percent increase*** of the nest area is generally used as a measure for migration. An advantage of circular nests is that they mimic the outward migration of cells out of a tumor *in vivo*.[138]

5.3.4 Assay selection guidelines

When selecting the proper assay for your collective cell migration assay, we recommend to first consider whether cell damage is of importance for your experiment. Secondly, the importance of a surface matrix coating and its integrity should be taken into account. Lastly, we recommend to determine whether the cells need to migrate into a void or out from a nest. This approach is summarized in the flowchart in "Figure 5.1 Guideline for assay selection of collective cell migration assays."and leads to the choice of a cell removal assay, exclusion zone assay or outgrowth assay. Each of these three main types of collective migration assays has a variety of subtypes of assays, each with their own specific properties such as reproducibility and cost effectiveness. The next section provides an overview of the most important assay properties and tools to help select the most appropriate cell migration assay.

Figure 5.1 Guideline for assay selection of collective cell migration assays.



5.4 Assay properties

After selecting the most suitable type of cell migration assay, the exact method needs to be determined. Several assay properties can influence the choice for a specific assay, such as the expertise and costs required to execute the assay, but also the desired reproducibility and throughput. The tables below summarize the necessary skills needed for and properties of the cell removal ("Table 5.1 Properties for cell removal assays."), cell exclusion ("Table 5.2 Properties for cell exclusion assays.") and cell outgrowth assays ("Table 5.3 Properties for cell outgrowth assays.").

5.4.1 Time/Expertise

The expertise required to perform the experiment varies per assay and hence influences the time the assay will take to execute. Simple and quick assays include the scratch assay and the vacuum based assay, as well as the different (solid) barriers that are used in cell exclusion and cell outgrowth assays. Microfluidic chips, on the other hand, require more expertise and time, both in creating the chips and in performing the assays themselves.

5.4.2 Reproducibility

To ensure reproducibility of the experiment, it is important to use cell monolayers with the same degree of confluence and reproducing the same size, shape and spacing of the area that is being studied. It is common use to perform experiments in triplicate to increase accuracy of the results.[41] Of the cell removal assays, the manual scratch and stamp assays have the lowest reproducibility due to the inconsistencies caused by manual void creating. In case of a scratch assay, automated (robotic) wound-making devices have been developed to increase reproducibility and standardization.[139] The most reproducible cell exclusion assay are the magnetically attachable barriers because they create voids of consistent size and also preserve the underlying substrate.[103,104] Concerning outgrowth assays, the most reproducible assays use spheroids or microcarrier beads since spheroids and microcarrier beads can be produced with a consistent size.[107,131]

5.4.3 Throughput

Throughput of experiments can be increased by running multiple experiments in parallel and using robotic systems for automation. High throughput screening (HTS) is useful for rapid screening of samples treated with many different conditions and could therefore accelerate the drug discovery process.[140] This includes a combination of robotic processing such as automatic liquid handling and high density, low volume assay formats. Multi-well plates that contain 96 or more wells are used to reduce the amounts of test compounds and reagents. In addition, high content screening (HCS) entails the use of automatic live-cell imaging and image analysis to achieve the accurate detection and measurement of (single) cells.[141]

For wounding assays, automated scratching systems, laser ablation and electrical wounding assays can achieve the highest throughput.[139] Cell exclusion assays can achieve high throughput with degradable gels.[91,94] Aqueous two-phase systems could be used for either cell exclusion and cell outgrowth assays, and can achieve a high throughput because they are automatable.[98,100,101] High throughput for solid barriers can be achieved with pneumatic control of the barrier.[108] Spheroids outgrowth can be interesting for high content imaging because of the control over the uniformity of the spheroids.[107]

5.4.4 Cost effectiveness

In general, the assay that requires the lowest amount of time and expertise is also the most cost effective one. However, high throughput screening can minimize testing costs by reducing the amounts of test compounds and reagents used.[140] In addition, high content imaging systems deliver vast amounts of information with reduced additional costs for extra assay points.[140]

The different techniques for wounding assays differ greatly in costs, where a mechanical scratch assay using only a pipette tip is the most cost effective.[142] Vacuum based wounding is also highly cost effective because all the necessary supplies are generally available in a lab. Of the cell exclusion assays, solid barriers are cost-effective as they can be sterilized and re-used.[82,85] Next to this, aqueous two-phase systems (cell exclusion and cell outgrowth assays) are inexpensive to establish and can be automated for high throughput, as stated before. This makes it an ideal candidate for cost-effective studies. The air barrier assay (cell outgrowth) is simple to set-up and only requires standard cell culture materials.

Table 5.1 Properties for removal assays.

Green indicates the most convenient choice, yellow requires specific effort and red indicates the least advantageous properties of the assay.

Cell removal assays	§	Time/ Expertise	Reproducibility	Throughput	Cost Effectiveness
Scratch wounding (manual)	4.2.1	🙂	☹	☹	🙂
Scratch wounding (automated)	4.2.1	🙂	🙂	🙂	☹
Stamp wounding	4.2.2	😐	😐	😐	😐
PDMS barrier	4.2.3	😐	🙂	☹	😐
Laser- based wounding	4.2.4	😐	🙂	🙂	☹
Electrical wounding	4.2.5	😐	😐	🙂	☹
Chemical wounding	4.2.6	☹	🙂	😐	😐
Vacuum- based wounding	4.2.7	🙂	🙂	😐	🙂

5.4.5 Property based assay selection

For each subtype of the collective migration assays, the properties and necessary skills are listed in the tables below. “Table 5.1 Properties for cell removal assays.” contains an overview of all cell removal assays, “Table 5.2 Properties for cell exclusion assays.” of the cell exclusion assays and “Table 5.3 Properties for cell outgrowth assays.” of the cell outgrowth assays. Based on the most important properties for your specific situation, the tables below can help you select the most suitable assay. After following the selection process in “Figure 5.1 Guideline for assay selection of collective cell migration assays.”, you will be guided to one of the tables below to continue the selection process.

Table 5.2 Properties for cell exclusion assays.

Green indicates the most convenient choice, yellow requires specific effort and red indicates the least advantageous properties of the assay.

Cell exclusion assays	§	Time/ Expertise	Reproducibility	Throughput	Cost Effectiveness
Solid barrier	4.3.1				
Degradable gels	4.3.2				
Liquid barrier	4.3.3				
Magnetically attachable barriers	4.3.4				

Table 5.3		Properties for cell outgrowth assays. *Green indicates the most convenient choice, yellow requires specific effort and red indicates the least advantageous properties of the assay.* ** pneumatic control of the barrier increases throughput*			
Cell outgrowth assays	**§**	**Time/ Expertise**	**Reproducibility**	**Throughput**	**Cost Effectiveness**
Explant outgrowth assay	4.4.1				
Solid barrier	4.4.2				
Liquid barrier	4.4.3				
Air barrier	4.4.4				
Spheroids outgrowth	4.4.5				
Microcarrier beads	4.4.6				

5.5 Conclusion

In this chapter, guidelines for selecting a suitable collective cell migration assay were discussed. This included selecting a specific cell type and limiting cell proliferation. Thereafter, the assay selection criteria cell damage, surface matrix control and geometry were explained. Those criteria lead to a choice between cell removal, cell exclusion and cell outgrowth assays. Next, an overview of specific assay properties was given, including the expertise required and cost effectiveness to execute the assays, as well as the reproducibility and throughput that can be achieved. This can aid in selecting a collective migration assay that meets the research objective best. In chapter 6, data acquisition and analysis methods for these assays will be discussed.

Chapter 6. Data acquisition & analysis

6.1 Introduction

After choosing the correct method to analyze collective cell migration, the experiment not only needs to be performed but also analyzed. The first step in analysis is to obtain images of the void or nest at several timepoints. Subsequently, the images need to be analyzed using image analysis tools. In this chapters tools for image acquisition ("6.2 Image acquisition") and image analysis ("6.3 Image analysis") will be discussed.

6.2 Image acquisition

For collective cell migration assays, transmitted-light microscopy (e.g. brightfield and phase-contrast microscopy) is often preferred over fluorescence microscopy since long-term imaging with fluorescence can lead to both photobleaching and phototoxicity.[143] Unless fluorescence imaging is required for visualizing specific cellular processes within the collective of cells, transmitted-light imaging is the method of choice for monitoring void closure/nest expansion over time. Therefore, we will focus on transmitted-light microscopy in this section.

To visualize collective cell migration, many different microscopes can be used, ranging from basic, bench-top, cell-culture microscopes to fully automated, in-incubator, microscopy systems. The easiest, but also least accurate, method to analyze gap closure/nest expansion is to take the sample out of the incubator to take images at regular time intervals (every 3 hours for example). However, manually capturing the images can be very time consuming and labor intensive, especially when experiments take 24 hours or longer. Not only because the samples need to be taken out of the incubator every timepoint, but also because it is difficult to take images of the same field-of-view of each sample at each timepoint. Therefore, some degree of automation is desirable for monitoring collective cell migration assays.

One form of automation consists of bench-top microscopes equipped with image acquisition software, an incubation chamber, digital camera and a motorized stage for image acquisition at multiple positions. The image acquisition software can be set-up in such a way that images of the migrating cells of each sample are collected at regular time intervals. The incubation chamber acts as a replacement of a cell culture incubator and thus allows the cells to remain on the microscope rather than being taken out of the incubator at each timepoint. This not only eliminates the manual labor but also the temperature shock the cells experience when transferred from the incubator to the microscope. These incubation chambers come in different complexity and thus also price levels, ranging from very simple temperature control boxes to complex systems regulating temperature, CO_2-level and humidity.[144,145] Most of the well known microscope manufacturers also provide incubation chambers for their automated microscopes. However, numerous labs have also built their own incubation setups.[144]

A recurring issue of microscopes equipped with an incubation chamber is the maintenance of the CO_2- and humidity level, generally caused by gas leaking from the incubation chamber. Maintaining the correct humidity level prevents medium evaporation and subsequently changes in osmolarity of the medium.[143] The correct CO_2-level is of importance for maintaining the proper pH-value of the medium.[143] Because of the difficulties in maintaining the correct osmolarity and pH-level, microscopy setups with incubation chambers cannot be used for long-term collective migration assays. A solution for this are automated live-cell imaging systems that can be placed inside regular cell-culture incubators. These in-incubator systems not only have the advantage of optimum environmental control throughout the entire migration assay, but the migration assay can also be performed under specialized

environmental circumstances such as those inside hypoxia incubators. In-incubator live-cell imaging systems can be as small as a few well plates stacked on top of each other or can fill up an entire incubator. Depending on your needs, a small, automated, single position microscope such as the CytoSMART Lux2 can be sufficient or a larger, automated, full well plate scanner such as the CytoSMART Omni is required. These kind of live-cell imaging systems are highly suitable of long term monitoring of cells under physiological conditions and can therefore provide valuable information about collective migration that cannot be obtained using bench-top microscopes (with or without an incubation chamber). "Table 6.1 Overview of microscopy systems for image acquisition." provides an overview of the advantages and disadvantages of the three types of imaging systems discussed in this section.

Table 6.1 Overview of microscopy systems for image acquistion.

System	Advantages	Disadvantages
Bench-top microscope	Already available in most labs Cost effective Easy to operate	Laborious Difficult to find the same field-of-view each timepoint Cells experience temperature shocks
Bench-top microscope with incubation chamber	Automated image acquisition Constant temperature throughout experiment	Can be expensive depending on the type of incubation chamber Difficult to maintain the correct humidity and CO_2-level
In-incubator microscope	Automated image acquisition Constant temperature, humidity and CO_2-level Can be used under specialized culture conditions (e.g. hypoxia)	Can be expensive depending on the type of microscope

6.3 Image analysis

Once images of the collective migration are taken using your microscopy system of choice, they need to be analyzed for e.g.: void/nest dimensions (e.g. area, width or diameter), cell migration rate and percentage of closure/expansion. As mentioned in "5.3.3 Geometry", rectangular and circular voids and nest are most commonly used to analyze collective cell migration, therefore, the analysis of these two geometries will be discussed below. Similar methods can be used to analyze voids and nest with different geometries.

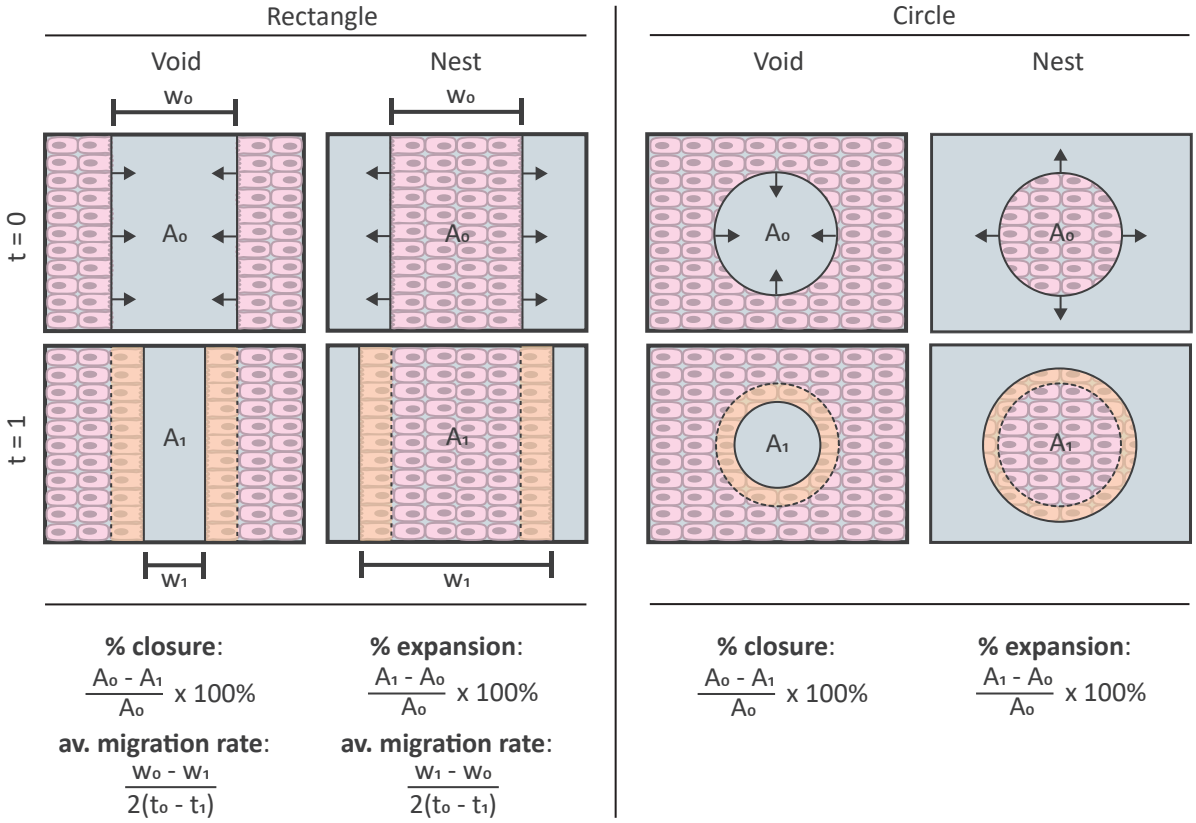
The most simple and common method to analyze rectangular voids is to compare the void area between two time-points and subsequently calculate the percent closure ("Figure 6.1").[134] By using the decrease in width of the rectangular void, the average migration rate of the cells can also be calculated. Similar methods are used to analyze rectangular nests, where instead of the decrease in void size/width the increase in nest size/width is used to calculate percent expansion and cell migration rate ("Figure 6.1").

Analyzing circular voids can be done by measuring change in void radius. This method is highly sensitive to the circularity of the void, making it unsuitable for analysis of voids with irregular geometries.[146] An alternative, more commonly used, method is the analysis of the percent closure of the circular void area since it is less sensitive of wound circularity (Fig. 6.1). Because of the mathematical complexity of translating the

circular closure to linear migration rates, the migration rate of circular voids is rarely calculated. Unlike cells in rectangular voids and nests, cells in circular voids and nest behave completely different. Cells in circular voids experience a quadratic decrease in available migration area while cells in circular nests experience a quadratic increase in available migration area. However, similar to circular voids, the precent nest expansion can be calculated for circular nests by comparing the nest area between different experimental timepoints ("Figure 6.1").

Void width, migration rate and percentage of closure/expansion are generally suitable for answering the biological question of interest. However, sometimes more information is needed such as how individual cells migrate within the collective. This is can be analyzed by tracking the *x,y* coordinates of individual cells throughout all timepoints in a time-lapse series of images.[134] However, to enable individual cell tracking within the population, generally a higher imaging frequency and more sophisticated image analysis tools are required.

Figure 6.1 How to calculate void closure and nest expansion in case of rectangular and circular voids/nests.



6.3.1 Image analysis software

Software for migration analysis should be selected based on the ability of the software to distinguish artifacts from cells and the ability to detect the void or nest properly. As reviewed by Ashby *et al.* (2012) and Stamm *et al.* (2016), several (free) software packages exist for automated image analysis, including algorithms for ImageJ and the image processing toolbox by Matlab.[58,134] Most software is based on the principle of recognizing the edges in a wound healing assay by edge detection and segmentation.[58] Next to ImageJ and Matlab, more software is available such as CellProfiler, Metamorph, Image-Pro, TScratch and WimScratch.[58,134] An overview of the advantages and disadvantages of the above mentioned image analysis tools can be found in "Table 6.2 Overview of image analysis software for would healing assays."

Table 6.2

Overview of image analysis software for would healing assays.

Software	Free?	Advantages	Disadvantages
Cell Profiler	Yes	Easy to use	Low robustness to challenging imaging conditions
MATLAB	No	Many MATLAB scripts available Scripts customizable to your needs	Low robustness to challenging imaging conditions
Metamorph	No	Software can be used for both image acquisition and analysis	Complex software
ImageJ (FIJI)	Yes	Easy to use Many macros and plugins available	Might need switch macros/ adjust the macro dependent on cell type and imaging conditions.
ImagePro	No	Easy to use	No macros/scripts available
TScratch	Yes	Easy to use Fast	Low robustness to different cell types Smaller wound areas are difficult to detect
WimScratch	No	Online tool Fast	Low robustness to challenging imaging conditions

Moreover, automated live-cell imaging systems, such as those available for in-incubator microscopy, are generally equipped with integrated image analysis software, making the image acquisition and analysis even less labor intensive. Generally, you place the sample on/in the live-cell imaging systems situated inside the incubator, select the time-interval, duration and type of experiment. Thereafter, image acquisition starts and the image analysis is performed after all images of the entire experiment are captured. However, currently there are cloud-based systems available (e.g. CytoSMART Lux2 and Omni) that upload and analyze the images immediately after they have been taken. This not only provides the researcher information about collective cell migration already during the experiment, but also ensures data safety due to the automated storage and backups of the images and data in the cloud.

Chapter 7. Summary

Cell migration is involved in many developmental, regenerative and pathological processes. Cell migration occurs in many different forms *in vivo*; from blebby amoeboid single cell migration to mesenchymal single cell migration and from epithelial collective migration with tight cell-cell interactions to mesenchymal collective migration with transient cell-cell interactions ("Chapter 2. Modes of cell migration").

As numerous as the types of cell migration are, as numerous are the assays to analyze them *in vitro*. Single cell migration can be analyzed using assays ranging from the well-known Boyden chamber assay to the more sophisticated and complex time-lapse cell tracking method ("Chapter 3. Assays to invenstigate single-cell migration"). In case of collective cell migration assays, the number of different assays is even more extensive. The collective cell migration assays can be subdivided into three categories: cell removal assays, cell exclusion assays and cell outgrowth assays. Cell removal assays are most representative of situations in which cell damage occurs, such as in healing wounds. Cell removal assays can be as simple as a traditional scratch assay performed with a pipette tip or as complex as electrical wounding assays analyzed using impedance measurements ("4.2. Cell removal assays"). In cell exclusion assays, cells are not damaged but hindered to migration into a certain area until a barrier is removed. Solid inserts can be used to create such barriers, however, gels and liquids are also used ("4.3. Cell exclusion zone assays"). Cell outgrowth assays are most representative of conditions in which cells migrate out of a (dense) population of cells, such as cells migrating out of a tumor. Dense cell populations (nests) can be created *in vitro* by using explants, solid barriers, spheroids and many other methods ("4.4. Cell outgrowth assays").

In this eBook, we have provided tools to help you select the most suitable assay for your research ("Chapter 5. Guidelines to select the most suitable collective cell migration assay"). First, we have given guidelines to select one of the three categories of collective cell migration assays. Thereafter, different assay properties such as throughput, cost effectiveness and expertise were evaluated per assay to help you select the most relevant assay based on your needs.

Since collective cell migration assays not only need to be performed, but also analyzed, the eBook ends with an overview of methods for image acquisition and analysis ("Chapter 6. Data acquisition & analysis"). Different types of microscopes for image acquisition were discussed, ranging from simple bench-top microscopes to fully automated in-incubator systems. Lastly, different types of image analysis methods were covered and an overview of (free and paid) image analysis software programs was given.

In conclusion, since many factors can play a role in collective cell migration, many different assays have been developed over the years. Selecting the most suitable assay for your research of interest is therefore not an easy task. With this eBook we aim to provide guidelines to make this task a bit easier.

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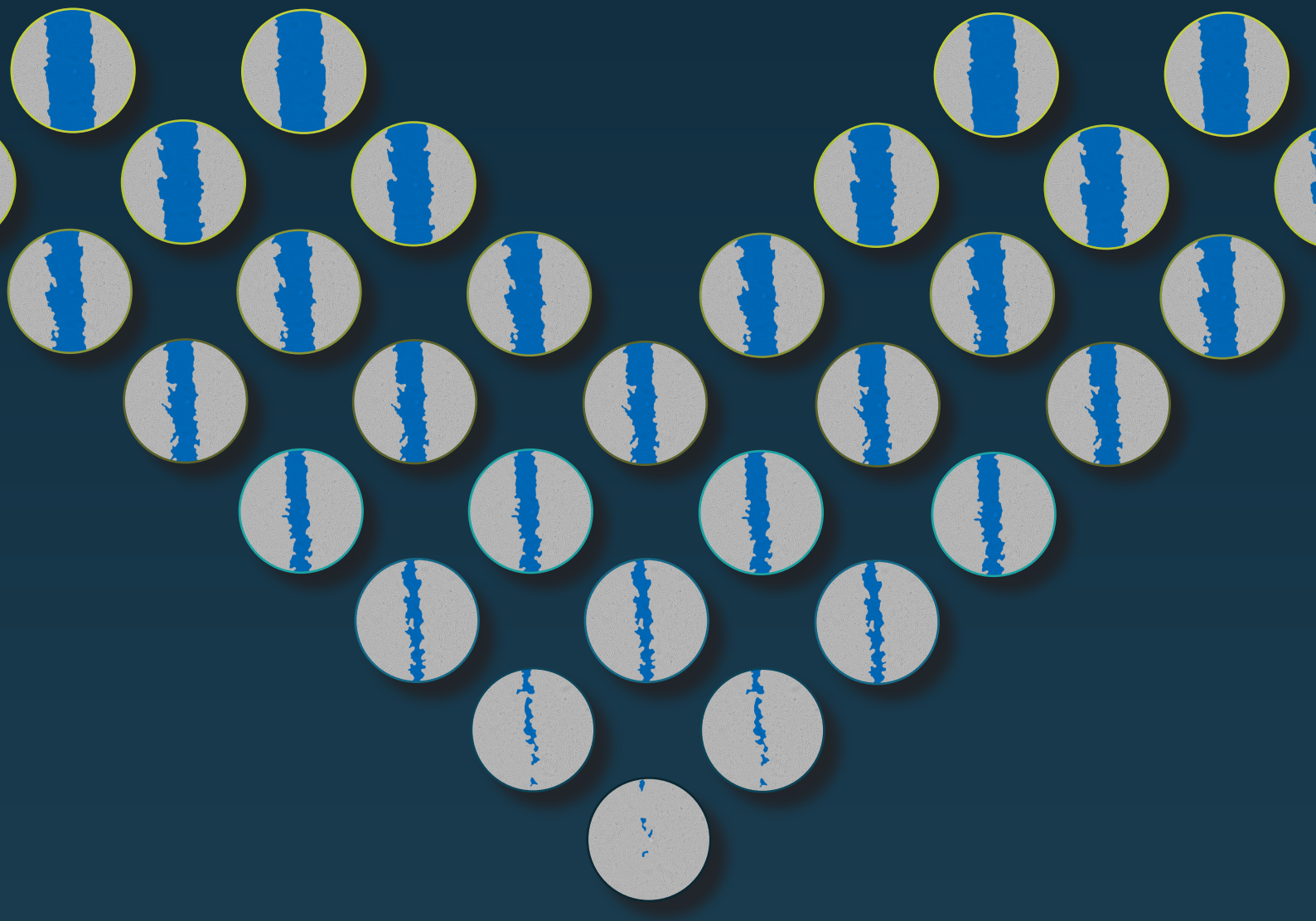

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