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Established and Novel Methods of Interrogating Two-Dimensional Cell Migration

William J. Ashby[a] and **Andries Zijlstra**[a,b]

Andries Zijlstra: andries.zijlstra@vanderbilt.edu

[a]Department of Pathology, Microbiology and Immunology, Vanderbilt University Medical Center, Nashville, TN

[b]Department of Cancer Biology, Vanderbilt University, Nashville, Tennessee. Fax: 615-936-7040; Tel: 615-322-3295

Abstract

The regulation of cell motility is central to living systems. Consequently, cell migration assays are some of the most frequently used in vitro assays. This article provides a comprehensive, detailed review of in vitro cell migration assays both currently in use and possible with existing technology. Emphasis is given to two-dimensional migration assays using densely organized cells such as the scratch assay. Assays are compared and categorized in an outline format according to their primary biological readout and physical parameters. The individual benefits of the various methods and quantification strategies are also discussed. This review provides an in-depth, structured overview of in vitro cell migration assays as a means of enabling the reader to make informed decisions among the growing number of options available for their specific cell migration application.

Introduction

Cell migration is a dynamic and complex process guided by a vast array of chemical and physical signals. Controlled cell migration allows for normal development and function while misregulated motility potentates a multitude of pathologies, including inflammation and cancer metastasis. Not surprisingly, a variety of cell migration assays have been designed in order to investigate the critical components that control cell movement. These assays have unique strengths and weaknesses that define their utility. To judiciously select one assay from among the growing number of assays, knowledge of both the assay's capabilities and the surrounding context are needed. This review expounds on the capabilities and shortcomings of existing assays including a side-by-side comparison of current 2D cell migration assays. More importantly by including potential methods for new assays, this review provides the context needed to readily understand both existing and future assays.

The landscape of cell migration consists of numerous variables that fit into various categories. Four broad categories useful for capturing the various influences on migration are 1) cell autonomous properties, 2) soluble factors, 3) matrix properties, 4) cell-to-cell interactions (Fig. 1A).[1] To visualize these categories and further stratify the migratory

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landscape, we expanded Friedl's tuning model of migration to include variables within each of the four broad groups (Fig. 1B).[2] The various conditions possible for each variable are represented as possible vertical positions which together constitute the bandwidth of the tuning model. Specific conditions of variables across these four categories result in specific modes of migration. Unique modes of migration such as dense and single cell mesenchymal migration, collective cell migration, and single cell ameboid migration are illustrated as lines cutting across the bandwidth of the tuning model. This model helps convey the multifactorial nature of the various controls over cell migration.

For a review of cell migration itself this landscape and the various migratory modes would be sufficient; however, understanding the context of cell migration assays requires incorporation of the assays' physical means of operation into this migration-focused landscape. These means of operation are given in the two left-most columns of Table 1. In two-dimensional migration assays cells are either removed from the substrate or excluded. Cell removal methods utilize mechanical, electrical, chemical, and potentially thermal and optical means to remove or destroy cells and thus enable migration into the disrupted area. Exclusion methods rely on solids, gels, liquids, air interfaces, and potentially electromagnetic forces to prevent cells from adhering to the area into which they later migrate. These physical modes of operation each have limitations. Combining the cell migration landscape with the physical modes of operation creates a full context for understanding 2D cell migration assays.

With this complete context in mind the capabilities of specific assays can be readily compared and understood even without prior knowledge of the individual assays (Table 1). Just as a mode of migration can be displayed by a line across the bandwidth of the tuning model, each migration assay can be displayed as a custom bandwidth according to the assay's ability or lack of ability to provide experimental control of important variables such as geometry, cell-cell interaction, matrix composition, and soluble parameters. By comparing an assay's custom bandwidth to a mode-of-migration line, the ability of a given assay to study a specific mode of migration can be crudely estimated. For example single cell migration assays are, as expected, highly compatible with the single cell ameboid and single cell mesenchymal migration modes, and magnetically attachable stencil (MAts) assays are well-suited for studies of dense mesenchymal and epithelial migration. Visually depicting the capability and limitations of each assay facilitates rapid side-by-side comparison and thus selection of suitable migration assays (See Suppl. Fig. 2 for examples). This visual depiction can be readily expanded to accommodate new assays as they are developed.

In vivo, most migration occurs in three dimensions, and for this reason many three-dimensional in vitro migration assays are in development. Unfortunately, there are currently few consistent guidelines regarding the set up and analysis of 3D assays. Furthermore, 3D assays require sophisticated data collection and more advanced image analysis than 2D assays. Considering this, we have restricted our review to the more common two-dimensional in vitro migration assays. In this publication densely organized cell migration is used to refer to both collective epithelial cell migration and migration of densely organized mesenchymal cells.

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Migration of densely organized cells

1 Cell-Removing Methods

Cell-removing methods are frequently referred to as “wound healing” assays because of the damage caused by removing or destroying cells within a defined area of the culture surface. Migration of the cells into this denuded void can be recorded and analyzed under various experimental conditions. Cell removing (and cell excluding techniques) can be made with rectangular or circular voids or nests (see Fig. 2) and then quantified by various methods (see Fig. 3).

Pros: The main advantages of cell-removing assays are their simplicity and ease of use. Few, if any, modifications are made to the routine culture conditions under which cells are maintained and the experimenter can choose among a variety of removal options ranging from simple mechanical removal to enzymatic detachment. In some instances the damage caused by cell removal is advantageous, since it may simulate a migratory response representative of in vivo processes such as wound healing.[3]

Cons: Damage to the cells and the underlying matrix is a significant limitation of cell-removing assays. Generally physical cell removal damages and removes the matrix to which the migrating cells should adhere. Since the extent of damage to cells and matrix cannot be readily assessed its contribution to the migratory behavior is usually unknown. Another disadvantage is that as cells form a dense population they modify the underlying substrate. As these cells are removed from the monolayer the composition of the void into which they migrate will be influenced by 1) the culture surface material (glass or plastic), 2) the matrix protein coated onto this surface prior to cell plating, 3) the deposition, removal, and/or modification of matrix by cells during monolayer formation, and 4) the irregular disruption of the matrix during cell removal. For these reasons cell-removing methods though simple and easy to perform offer little control over underlying matrix conditions.

1.1 Mechanical Removal

Scratch Assay—Expertise: low

Throughput: single to 384-well plates

Equipment: cultureware, camera, microscope (CCM)

The scratch assay is by far the most published method discussed in this review (see Table 2). It is well-established, versatile, and easy to perform. The basic scratch assay is implemented by creating a continuous monolayer of cells and then manually scratching away a portion of the cells with a plastic pipette tip or similar mechanical pin.[4] The cells adjacent to the scratch remain attached and migrate into the void or “wound” which is generally 300 to 900 μm wide. The primary advantage of the scratch assay is compatibility with most lab cultureware and cell culture microscopes. Positioning plates or dishes for imaging is often done manually by making fiducial marks on the bottom of the culture dish. The scratch assay is not restricted to specific culture dishes and can be performed on a variety of plastic

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and glassware of different sizes. Therefore, the assay is easily adaptable to standard cell culture protocols.

Beside damaging cells and matrix (a limitation of cell removing methods in general), scratch assays often create irregular voids with jagged edges and occasionally cause cells to pile-up densely alongside the void. Such piled-up cells rapidly expand back to normal density within a few hours. These variables decrease the accuracy of the scratch assay and confound analysis and interpretation of the results. These complications have motivated many modifications of the assays.

Variations of the Scratch Assay—Variations of the scratch assay generally involve alternative scratch mechanisms and alternative cell patterns. The scratching mechanism can significantly alter the amount of damage to both cells and substrate. Because metal objects readily damage plastic, they are rarely used to make scratches. Plastic pipette tips are the standard tool; however, silicone tips and Teflon® wedges have been used to reduce damage to cells and substrate. Within these scratch variations rectangular voids are still most common. Drill presses have been used to create circular geometries by gently pressing spinning silicone tips against the cells.[5,6] By placing the silicone tip off-center in the drill press, circular nests can be created.[7] Scratch-making devices may reduce human error and improve reproducibility but are most-often employed in order to achieve high throughput.[8,9] Although each of these variations offers some improvement over the standard scratch assay, the basic strategy is the same, and maintaining a defined substrate is not possible.

Stamp Wound Assay—Expertise: low

Throughput: single to 24-well plates

Equipment: CCM, PDMS stamps, and weights

Rather than scraping a silicone tip across a surface, stamp wounds are created by pressing polydimethylsiloxane (PDMS) stamps against a dense cell population using weights. After several minutes the weights and PDMS stamps are removed leaving behind an area of cell debris into which the surrounding cells migrate.[10] By stamping rather than scratching, the matrix generally remains intact as do many parts of the removed cells. Unfortunately stamping still requires the formation of a monolayer and therefore the underlying matrix may be modified by the occupying cells. This prevents analysis of a clean homogeneous matrix, In specific studies like those involving migration through dead or damaged tissue, the cell debris left after stamping may provide a more relevant environment.

1.2 Electrical Removal of Cells

Electric cell impedance sensing (ECIS)—Expertise: low-medium

Throughput: multiwell plates

Equipment: CCM, ECIS plates and control system

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Electric cell impedance sensing (ECIS) is an increasingly popular alternative to both traditional scratch and transwell migration assays. ECIS systems measure impedance which results from interactions between cells and an electrode-containing substrate. Changes in impedance occur as cells proliferate, migrate, spread, scatter, and alter cell-to-cell or cell-to-matrix adhesions.[11] This makes ECIS useful in a broad range of studies including epithelial barrier function.[12] ECIS platforms are available in a variety of formats; however, only the application of ECIS to migration is directly relevant to this publication. After establishing a dense cell population over the electrode-containing substrate, which can be coated with matrix proteins, pulses of high voltage are applied to the electrode resulting in electroporation and cell death after several seconds. The result is a circular void (though other geometries should be possible) over the electrode. As cells migrate and cover the electrode, the resulting impedance change reflects the rate of migration.

ECIS provides several advantages over scratch assays and introduces a few disadvantages. ECIS measurements can be made in real-time without removing cells from a controlled environment such as a cell culture incubator. The void for migration is automatically created with a pulsating high voltage electric field eliminating human error. Irregularities in cells and substrate may still occur from this process but are much less likely than for scratching or stamping. However, special plates designed for ECIS-readout of scratch assays are also available.

The major disadvantage of ECIS migration studies is the diversity of cell behaviors that change impedance. Changes in adhesion and cell density will alter the impedance. These changes are indistinguishable from changes due to migration. For this reason, the judicious use of controls and microscopic verification of migration are necessary for informative analysis.

1.3 Chemical Removal

Laminar Flow—Expertise: medium

Throughput: varies with microfluidic design

Equipment: CCM, microfluidic systems

Cells are routinely removed by chemical means such as trypsin. Using laminar flow within microfluidic devices can create rectangular voids and nests of cells when chemically removing cells with trypsin or other reagents.[13,14] Laminar flow is the flow of two different solutions side by side without mixing that depends on fluid viscosity and spatial dimensions. In the micrometer dimensions of microfluidic devices, fluids such as water and cell culture medium undergo laminar flow. After flowing cells into a microfluidic channel and allowing them to attach, two or more inputs into the large channel establish the laminar flow of trypsin (or another cell-removing agent) bounded by normal medium. As trypsin degrades cellular attachment proteins the cells detach from a portion of the substrate and are subsequently flushed away with fresh medium. The remaining cells migrate into the trypsinized area.

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In general microfluidics are especially useful for experiments requiring rare or costly reagents because they can utilize small volumes. An additional benefit of chemically removing cells via laminar flow is that the matrix left in the cell-free void is more uniform and predictable than the scratch assay's void.

The major disadvantage is that successful application of microfluidic devices requires expertise. Air bubbles, clumping of cells, and managing the fluidic systems needed for laminar flow are challenges common to microfluidic cell-based experiments. Fortunately, these challenges have been surmounted by several groups. For example, VanderMeer *et al*. implemented devices for migration analysis using pumps to exchange medium, while others (Nie *et al*.) implemented devices that use passive flow of medium which is driven by gravity, and evaporation.[13,14] The latter devices are attractive options because they are easier to use.

1.4 New, developing methods of Cell Removal

While we organized existing cell-removal methods, it became apparent that optical and thermal methods for initiating cell migration could be developed. A survey of the scientific literature revealed potential techniques that could be but are not yet applied to the migration of densely organized cells.

Chemical Removal: Alternatives to Laminar Flow—Although chemical removal is routinely achieved with microfluidic devices, it is also possible without them. Peterbauer *et al*. used a robotic clone selecting system (CellCelector, Aviso, Greiz-Gommla, Germany) to selectively remove small colonies of cells.[15] This approach should be adaptable to creating voids in densely organized cells. Alternatively, aqueous two-phase systems can pattern proteins or transfect cells with ~400 μm resolution.[16] Adaptation of this approach to selectively remove patterns of cells with trypsin or other chemical reagents should also be possible; however, a more promising approach would be to selectively pattern cells with aqueous two-phase solutions and thereby avoid cell-based substrate alterations (see “Immiscible Solutions”).

Thermal wounding—Thermal cell wounding is an undeveloped method, despite the existence of two technologies that could be readily adapted for creating voids in densely organized cells. First, electrical current flowing through a thermoresistive material embedded on a culture surface could be used to wound a portion of the cells. Existing ECIS systems already provide the needed electronics to controllably heat such thermoresistive strips. Although cells may not be removed by thermal damage, the subsequent migration of cells into the lifeless void may be very informative for studies of burn healing. The expertise and requirements for such a thermal cell “removing” migration assay would be very similar to those of ECIS. The second technology capable of thermal wounding cell populations is the heating of small volumes using infrared laser light.[17] By scanning such a laser across a cell-coated dish countless patterns could be created for cell migration. Both techniques could be utilized to heat and wound small areas and possibly induce heat shock without ablating cells or causing apoptosis. The ability to modify existing ECIS systems to study migration or

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heat response make such thermal wounding systems an attractive candidate for future development.

Optical Removal via Laser Ablation—Ultraviolet lasers are routinely used to perform microsurgery. These lasers successfully ablate cell monolayers *in vitro*[18] and *in vivo* in the drosophila embryo.[19] This laser ablation is significantly different from thermal wounding with infrared lasers because it relies on the brief formation of a plasma and cavitation bubbles to destroy individual cells.[20] Though already employed for cell migration and cell tension studies, the cost and maintenance of sophisticated lasers and optics currently prevent wide adoption. However, improvements in on-chip lasers and optics as found in micro-total analysis systems (μTAS) may enable wide adoption in the future.

2 Cell Excluding Methods

The alternative to removing cells from an area is to exclude them from settling into an area. The past two decades have introduced a variety of novel techniques for cell exclusion ranging from elastomeric solid barriers to laminar flow in microfluidic devices. Like cell removing methods, methods for excluding cells also employ rectangular or cylindrical nests and voids (see Fig. 2). The resulting images are also quantified with the same approaches used for cell removing methods (see Fig. 3).

Pros: Cell excluding methods have significant advantages over cell removing methods and will undoubtedly prove beneficial to our understanding of the role and effect of the environment of cells. The most significant advantage of excluding cells is that the matrix in the void is not altered directly by the cells because they are not allowed to cover the void until migration is initiated. Another important advantage is that certain methods can accommodate additional matrix complexity such as pliable surfaces, protein patterns and even textured surfaces.

Cons: The primary disadvantage of cell exclusion methods is the additional components required to exclude cells as they adhere and form dense populations. A disadvantage of certain cell excluding assays is that the barrier may leave residues on the matrix or in solution which may alter cell behavior.

2.1 Solid Barriers

The earliest documented method for excluding cells is the solid barrier. Originally, solid barriers were fabricated from nickel or stainless steel and could only exclude cells during adhesion.[21] Modern solid barriers are fabricated from elastomers and are able to prevent cell protrusion and migration until the barrier is removed.[22] These barriers are forcefully held against the bottom of a culture dish in order to successfully seal against the matrix, prevent cell protrusions, and protect the condition of the matrix. The original metal stencils relied on gravity to maintain contact and remain immobile on the culture surface. Current barrier strategies are held in place with forces generated from wedging, autoadhesion, or magnetism rather than relying upon gravity.

Wedging, Stoppers—Expertise: low

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Throughput: low-high

Equipment: CCM, specific cultureware and stoppers

Stoppers utilize friction and compression to wedge into a dish and press against the bottom of the dish. This wedging provides the force needed to seal against the substrate on the bottom of the dish. For this reason stoppers are large (even when the void created is small ∼1.5 mm). Because they must wedge against the walls of a culture dish, they can function only in the specific dishes for which they were designed. They can be solid or hollow in the center.[22,23]

The main advantage of stoppers is that they can be sealed against wet, protein-coated surfaces in order to study the effect of matrix conditions on cell migration.[22,24,25] However, excessive force while inserting the stopper can disrupt matrix proteins coated onto the culture surface. To avoid this problem, preinserted stoppers can be purchased in protein-coated plates. Alternatively, the risk of matrix disruption can be minimized to a small perimeter using stoppers with a hollow center.[22,24]

Regardless of being hollow or solid in the center, the main weakness of stoppers is the need to insert them into the well which must be done manually for custom-made substrates. Excessive force during insertion can disrupt matrix proteins and insufficient force will result in an incomplete seal allowing cells to enter into the void prior to the start of the migration assay.

Adhesion, Stencils—Expertise: low

Throughput: low-medium

Equipment: CCM, Stencils

Most modern stencils rely on autoadhesion rather than gravity. Autoadhesion provides a tight seal against the matrix but limits the application to hydrophobic materials and in a few cases to dry matrix proteins. Rudimentary stencils have been cut out of Parafilm.[26] High precision stencils are made using microfabrication techniques from PDMS[27,28] or parylene-c[29]. Though most stencils are thin membranes less than 200 μm in height, some are large such as Ibidi's Culture Inserts, 5 mm.[30] Stencils can also be made from a hybrid of rigid and conformal materials.[31,32]

Regardless of height or composition, a major advantage of stencils is their similarity to the scratch assay. The expertise and hardware for stencil assays is nearly identical to the scratch assay.

The main weakness of stencils is that autoadhesion requires a dry and generally clean. hydrophobic surface. Autoadhesion to wet protein-coated substrates is not possible and in many situations stencils placed on dry hydrophilic surfaces fail to successfully prevent cells from protruding into the void.

Magnetic Attraction, Magnetically Attachable Stencils (MAts)—Expertise: low

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Throughput: low-high

Equipment: CCM, MAts, and magnets

Magnetically Attachable Stencils (MAts) are fabricated from PDMS and magnetite. These stencils seal against a wide variety of substrates via magnetic attraction to magnets placed underneath the culture dish. Although they can be produced in various geometries, the most commonly used MAts are star-shaped with four arms that are 7mm in length and 5mm tall. Because MAts are attracted to magnets placed under the substrate, they seal successfully on wet, protein-coated surfaces, elastic polyacrylamide substrates, and polycaprolactone nanofibers (Nanofiber Solutions).[33]

The highly controlled magnetic force minimizes damage to the matrix and also improves reproducibility between experiments. By positioning MAts manually a few millimeters above the bottom of a dish containing several millimeters of solution and then releasing the MAts, the impact of the MAts on the matrix becomes dependent upon the magnetic force rather than the user. This eliminates the risk of damaging the substrate during MAts attachment. Another advantage of magnetic force is it can be customized to achieve similar compression on substrates of varying elasticity.

The disadvantage of MAts, which is shared by stencils and stoppers, is their manual removal. Like stencils and stoppers, careless MAt removal can damage cells or substrates, or possibly both. However, with proper care MAts successfully pattern densely organized cells while protecting the underlying matrix. The matrix can have various conditions ranging from coated to uncoated, oriented to randomly oriented, and stiff to soft (see Table 1).

2.2 Gel Barriers

Degradable gel droplets—Expertise: low

Throughput: 24- to 384-well plates

Equipment: CCM, gel-containing plates

Rather than placing and removing a solid barrier on a substrate, gels can be used to prevent cell adhesion to a defined area. Gels are printed onto the center of multiwell plates and dried or polymerized prior to adding cells. After cells have adhered the gel is dissolved allowing migration into the void. These gels are currently proprietary technologies available as the Oris™Pro and Radius™ cell migration assays. Two strategies have been taken to dissolving gel barriers. One is to create gels which automatically dissolve in solution after a certain amount of time. This enables an assay to be setup and left in an automated analyzer; however, the disadvantage is that the edges of the gel which are thinner dissolve sooner resulting in irregularities along cell boundaries. Alternatively, a dissolving reagent can be used to initiate the dissolution of the gel. If done after cells are well adhered, this dissolution technique results in crisper cell boundaries at the initial time-point.[23]

The advantage gels over solid barriers is that the gel dissolves without any manual manipulation other than adding solutions. This eliminates the human error inherent in the removal of stencils, stoppers, and MAts.

The disadvantage of gels revolves around application of the gel before it polymerizes. This is currently done commercially with proprietary systems on 24, 96, and 384-well plates which are sold uncoated or collagen-coated. Other proteins and possibly custom-made matrices may be available upon request. In any case the matrix proteins have to be dried limiting the ability to investigate various cell-matrix interactions.

2.3 Liquid Barriers

Similar to gels, liquid barriers ensure that substrate conditions remain unchanged by physical damage from solid objects while excluding cells and preventing cell alteration of the substrate. Considering the advantages of liquid barriers, their limited use is surprising and may reflect lack of awareness of the technique or concerns of affecting migration with the additional reagents required to create two-phase solutions. We anticipate increased use of liquid barriers in migration assays as two-phase aqueous systems are adapted to cell patterning and as microfluidics become more commonplace.

Immiscible Solutions—Expertise: low-med

Throughput: low-high

Equipment: CCM, immiscible solutions

Liquids can function as barriers to cells. By placing cells in one part of an immiscible or two-phase solution, cells can be patterned as they adhere to a substrate. Immiscible solutions such as mineral oil and cell culture medium can create 2-3 mm diameter cell colonies.[34] However, better resolution is achievable. Tavana *et al*. recently used aqueous two-phase system consisting of polyethylene glycol and dextran solutions to pattern droplets ranging from 400 to 1400 μm diameter for substrate coating and cell transfection. With this system complex patterns can be created by dispensing a stream of dextran solution from a moving tip.[16] Adapting this approach to the patterning of cells should enable sub-millimeter features ranging from simple droplets to complex printed patterns on even softest of substrates and can likely be applied repeatedly to pattern multiple protein and cell containing solutions.

The major advantage of these techniques is that with proper care cells can be patterned on delicate matrices that would readily be altered by solid objects such as soft collagen gels.

The main disadvantage is the requirement of using solutions uncommon to cell culture which may affect cell behavior and migration or possibly alter matrix conditions. Further research is needed to verify or dismiss the possibility of such effects.

Laminar Flow in Microfluidics—Expertise: medium

Throughput: varies with microfluidic design

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Equipment: CCM, microfluidic systems

Because of the small dimensions of microfluidic devices, laminar flow can be achieved with various solutions such as cell culture medium. This has enabled chemical removal of cells as addressed earlier, patterning of protein gradients,[35] and patterning of cells for migration.[36] Utilizing liquids to pattern cells removes risks of substrate damage that is inherent with solid barriers.

However, microfluidic flow provides additional advantages. Medium conditions can be controlled dynamically to deliver treatments or used to maintain very stable conditions regardless of cell metabolism. Gradients in solution can be created and maintained, a feature that is important for many studies such as chemotaxis.

A disadvantage unique to excluding cells using laminar flow is that as cells adhere there is no surface tension or physical force to prevent cells from immediately migrating into the void after they adhere.

2.4 Air Interface as a Barrier

Droplets—Expertise: low

Throughput: low-med

Equipment: CCM

One of the simplest approaches to patterning cells in liquid is to add droplets of cells to a dry substrate. Essentially the air interface acts as a barrier because of the surface tension of the medium. After cells in droplets have begun to adhere (30-60 minutes), medium is added to re-immerse the dry surface surrounding the droplet preventing evaporation or exhaustion of the limited nutrients within the droplet, and subsequent cell death.

Key advantages of using droplets are that they do not require novel tools and cells can easily be patterned on delicate materials. Though cell patterning achieved in this way is highly variable, the approach has enabled analysis of cells migrating collectively on soft elastic polyacrylamide gels.[37] This represents one of the first investigations of collective cell migration on materials capable of recreating soft tissues such as breast.

The major disadvantages of this approach are that the cells used must be capable of adhering in a short period of time, the matrix outside the cell-occupied zone must be dried temporarily, and the cell patterns achieved are variable.

Microfluidics—Expertise: medium

Throughput: varies with microfluidic design

Equipment: CCM, microfluidic systems

Surface tension at the liquid air interface can also be used to create precise patterns of cells inside microfluidic devices. Generally, a large, main channel bordered by several small

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channels is filled with cells. Liquid does not enter the small channels because of surface tension. After the cells have adhered, migration is initiated by applying sufficient vacuum to the small channels to overcome the surface tension and fill them with culture medium.[38]

This approach has multiple advantages. The cell patterns are precise and reproducible. The substrate remains untouched until migration is initiated. Cells have ample time to adhere and form stable monolayers because culture medium can be replenished without initiating migration.

The disadvantages of the approach are that the matrix must be temporarily dried while cells adhere and unlike droplets microfluidic devices are not currently compatible with elastic surfaces such as polyacrylamide.

2.5 New, developing Methods for Excluding Cells

Electric Fields—ECIS migration assays rely on the removal of cells by brief, pulsed, intense electric fields that electroporate and disrupt cell monolayers. However, it may be possible to exclude cells from the void with a pulsating electric field, referred to as an "electric fence", during the adhesion and growth of cells. Turning off the fence initiates migration into the void which can be measured by changes in impedance.[39]

There are three advantages of the "electric fence" approach. First, it can be implemented using existing ECIS dishes and controllers. Second, the matrix does not have to be dried. Third, the matrix in the void is not modified by physical contact with solids, gels, or cells.

The main disadvantage is the unknown effect of oscillating electric fields on nearby cells, substrate, and possibly cell culture medium. However, protein coatings are expected to remain intact in the presence of the electric fence enabling densely organized cell migration onto custom, protein-coated substrates.[39]

Magnetic Particles—Various magnetic beads are routinely used to manipulate cells and perform magnetic based separations. Two technologies exist that could be implemented for cell migration assays. First, cells in solution can be patterned by exposure to cationic liposomes containing magnetite followed by application of static magnetic fields.[40-42] The second technology consists of, dynamic magnetic manipulators currently used in making force measurements on individual cells.[43,44] Both technologies could potentially enable magnetic particle-based cell patterning for cell migration assays.

Optical Traps—Since the introduction of optical traps for manipulating viruses and cells in 1987,[45] traps have found diverse applications ranging from subcellular and molecular manipulation to label-free discrimination of cancerous and non-cancerous cells.[46,47] State-of-the-art holographic optical tweezers enable dynamic control of the shape and position of large traps or numerous small tweezers simultaneously. Such capabilities can exclude and sort cells in real-time[48] and should allow cell exclusion from a defined region during adhesion. This would enable analysis of both single and densely organized cell migration on a variety of substrates including extremely soft materials. Another important application of optical traps will be to dynamically manipulate and probe cells while they migrate.

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3 Geometry

Densely organized cells can be arranged to migrate towards one another or away from one another by creating voids or nests of cells, respectively. Various shapes can also be created though generally only rectangles and circles are employed as shown in Figure 2. Understanding the differences between geometries is important to choosing and properly executing migration assays. The migration between void and nest geometries is nearly identical for rectangular geometries when experimental conditions and times are carefully selected. However, circular voids and nests are fundamentally different.

3.1 Voids

Voids are often created in large dense cell populations in order to measure migration. Generally culture surfaces are completely covered with cells except for the void. In this situation a large number of cells must be nourished by a limited volume of medium. As a result migration can be affected by changes in medium condition. Furthermore, time points must be selected carefully for each cell type in order to minimize variations in migration rate that occur as voids begin to close and cells undergo contact inhibition of migration.

Rectangular Voids—Traditional scratch assays create rectangular voids. Many other assays also use this geometry. The width of the void is generally less than 900 μm, and lengths may range from a few millimeters to a few centimeters. Acquisition of rectangular voids should include both sides of the void and employ fiducial marks or another positioning scheme. Alternatively time-lapse microscopy and automated microscope stages can be used to ensure proper positioning throughout all time points. This is necessary for precise quantitation of migration rates or percent closure. Fortunately, the rectangular geometry is forgiving of minor misalignment as long as both sides of the void remain completely visible in the image.

Circular Voids—Circular voids are popular in high-throughput formats where space is limited and often the entire culture surface is imaged. In some situations acquisition is possible for only partial images of the void and very precise alignment must be achieved using automated live-cell microscope systems or fiducial marks combined with image registration. Circular voids are quantified almost exclusively by percent closure because converting circular closure to a linear migration rate is mathematically complex and the migration rate itself is altered by the quadratic decrease in the area of the void as cells migrate inward. In some instances cells capable of rapidly closing a rectangular void may be seriously retarded when closing a circular void. Though this is most often considered a disadvantage, in some situations, this retardation may be beneficial by providing extra time to compare control and experimental groups.

3.2 Nests

Nest assays overcome the spatial hindrance that retards migration cells as they fill voids. Nests are dense populations of cells that migrate away from each other into a large open space. Nests use fewer cells for a given volume which can be beneficial when working with highly metabolic cell types but may result in dilution of factors that stimulate migration.

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Like voids, nest migration rates may also decrease in migration after a period of normal migration. The cause of such retardation is primarily because the cell density decreases rapidly as the nest empties into the surrounding area. By judiciously selecting the time frame and conditions for migration, these differences can be minimized.

Rectangular Nests—With proper care for medium conditions and time points, the migration of rectangular nests can be nearly identical to rectangular voids. In fact, if two or more rectangular nests are created side by side, one or more rectangular voids will be created between them. For many of the stencil assays such patterns are purposefully created and migration can be viewed as a rectangular void or nest. Like voids, images generally contain both sides of a nest in order to reduce the need for precise horizontal alignment.

Circular Nests—Unlike rectangular geometries, circular voids and circular nests will behave differently despite giving careful consideration to medium conditions and time points. This difference occurs because circular nests experience a quadratic increase in available surface area as they migrate outward whereas the area for circular voids decreases as cells migrate inward. Like circular voids, circular nests should be imaged in their entirety if possible. This eliminates the need for precise image alignment. An advantage of circular nests is their resemblance to the migration of cells away from a dense population such as a tumor.

4 Quantification

Equally important to the execution of cell migration experiments is the quantitative analysis of the resulting data. Generally, a couple of pictures or time series of pictures are taken and subsequently analyzed to determine widths between cells, migration rates, and/or percent closure. The two simplest analyses consist of determining the open area and the average width of the void. Though such analyses are suitable for answering many biological questions, much more information can be obtained by tracking cells or performing image-based calculations on a time series.

4.1 Area Analysis

The simplest and probably most common method of analyzing densely organized cell migration is to compare the void area of images from two time-points (Fig. 3A). This can be done by counting pixels uncovered by cells for both time-points and then calculating the percent closure (Fig. 3A′). For rectangular geometries this percent closure can be converted to average migration rate if the actual width of the image is known. Alternatively, average widths between cells can be obtained by measuring multiple horizontal lines (Fig. 3A″). From these widths the average migration rate or percent wound closure can be calculated which both represent the average productive movement into the void. The authors prefer average migration rate because percent closure varies depending on the initial size of the void.

For area analysis, TScratch stands out among the software platforms known by the authors (Fig. 3D). TScratch successfully distinguishes between cells and background artifacts and provides a graphical user interface to facilitate manual manipulation of void areas as needed.

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Alternatively, various standalone software as well as packages/algorithms for ImageJ[49], Cell Profiler[50], and Matlab (Mathworks) are readily available.

4.2 Individual Tracking

In order to obtain more information about the migratory behavior of individual cells at the periphery and within the population, time series images can be used to track individual cells (Fig. 3B). After creating x,y coordinates of the cell tracks, a variety of parameters such as turn angle, persistence, velocity, and displacement can be extrapolated. To facilitate the extraction of such parameters and to minimize the possibility of human error, an open-source, peer-reviewed software package called Cell_motility was created by Martens *et al*.[51] Comparing behavior of cells at various distances from the periphery will provide insight into how and when cells in a dense population are mobilized.

4.3 Image Calculations

To better understand how cells at the periphery and within a dense population migrate and change, various image-based calculations have been implemented as alternatives to individual cell tracking. By calculating pixel by pixel the difference between phase contrast images separated by 15 minute increments, Matsubayashi, Razzell, and Martin visualized and quantified the mobilization of periphery cells and cells within the population with a growing “white wave” (Fig. 3C).[52] Similarly, Poujade *et al*. applied particle image velocimetry to phase contrast time-lapse images of cell monolayers and created velocity fields showing complex motions among cells within the population and at the periphery.[28] Automated image calculations can provide quantification of additional parameters such as the shape of both individual cells and the migratory front, proliferation rates, and cell turning.[53,54] These calculations provide insight into the behavior of cells throughout the population not just at the periphery and enable visualization of otherwise unnoticeable phenomenon.

5 Assay Selection

Deciding on the migration assay that is most suitable for a specific experimental objective can be challenging. A decision diagram has been included (Fig. 4) to facilitate this process. The decisions are based on the primary research objective, cell density, the analytical requirements, cost and available expertise. Although the suitability of any assay must be confirmed empirically, Fig. 4 provides an overview of possible approaches suitable for specific research objectives. In all instances selection of the specific assay will involve balancing the complexity of the scientific question and the analytic requirements with the time, cost and resources available to the investigator.

Conclusions

Migration assays have been and will continue to be important tools in our investigation of the mechanisms that control both normal biology and pathology. The implementation and diversity of methods for analyzing cell migration have increased dramatically over the past two decades. *In vitro* 2D densely organized cell migration assays often require less equipment and are generally simpler to analyze and quantify than single cell migration

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assays. Recent technological advances enable unprecedented 2D migration studies of densely populated cells on a variety of substrates ranging from custom-coated tissue culture plastics to pliable hydrogels and microfabricated surfaces. Such assays are able to integrate multiple aspects of the four broad factors influencing migration: 1) cell autonomous properties, 2) soluble factors, 3) matrix properties, 4) cell-to-cell interactions (Figure 1). This level of control and integration enables more relevant *in vitro* investigation of development, disease, and other biological processes that depend on cell migration.

Of the currently available densely organized cell migration assays, several demonstrate unique strengths. ECIS is unsurpassed in ability to perform nearly real-time acquisition. To achieve similar time-lapse results with other assays would require live-cell microscopy systems capable of observing multiple wells in parallel. The scratch assay remains unrivaled in cost since it can be performed with standard equipment already available in labs performing cell culture. However, as cell excluding methods, such as stencils, stoppers, MAts, and gels, become more common, their costs will decrease making them more and more competitive. Of the cell excluding methods, the simplest and most versatile is the magnetically attachable stencil, MAts. Any dish under which a magnet can be placed can be used with MAts and magnetic force can be customized to accommodate soft and stiff substrates.

Analyzing the fundamental mode of operation of existing assays reveals undeveloped methods with promise. Though thermal wounding has been performed on animals to better understand healing of burns, the technique has not been applied *in vitro* to the migration of cells. Such studies could provide insights into burn healing and also to other heat-related conditions. Another promising method for studying cell migration is the application of two-phase aqueous solutions to form sub-millimeter diameter droplets or patterns of cells and proteins. Because contact between a solid material and substrate is completely avoided, two-phase aqueous solutions are an attractive approach to patterning cells on very delicate substrates. In terms of control and ability to manipulate cells and even molecules during migration, the capabilities of holographic optical tweezers are unrivaled. Implementation of the above techniques for cell migration studies promise to provide significant, unique insights into the behavior of individual and densely organized cells. Furthermore, such studies are expected to reveal ways to improve the relevance of 2D migration assays to in vivo cell migration.

By analyzing existing assays and areas for future assay development, this review illuminates the unique qualities of individual assays and provides the necessary context for readily understanding the strengths and weaknesses of individual assays. Though the specific assays included in this review will evolve over time, the organization of their fundamental modes of operation provides a context that will remain important to understanding 2D cell migration assays far into the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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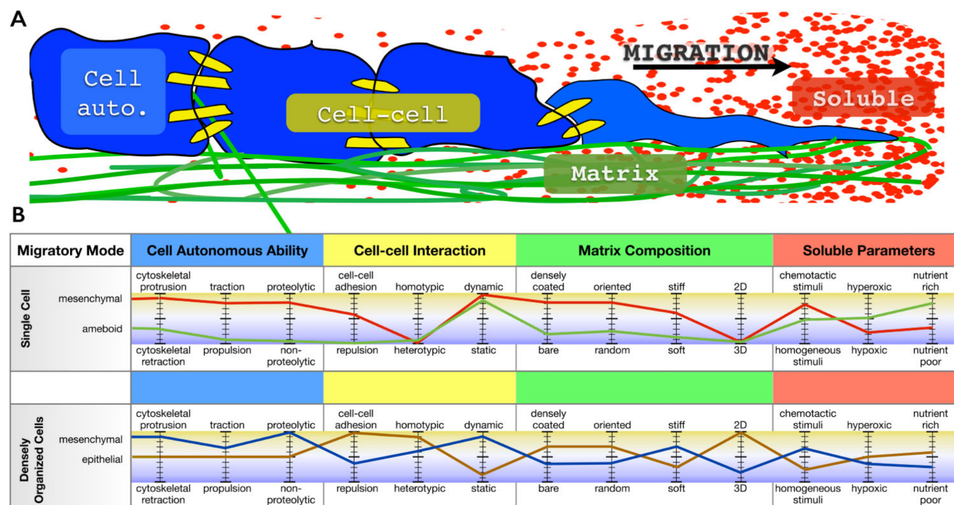

Figure 1. Parameters of cell migration

A multi-scale model of cell migration presents multiple interdependent parameters classified under four distinct categories (cell autonomous ability, cell-cell interaction, matrix composition and soluble parameters. (A) Cells are constantly integrating elements that contribute to their ability to migrate including those from within (cell autonomous ability), those created by interacting with neighboring cells (cell-cell interaction) or with the surrounding matrix composition and by those received as soluble stimuli (soluble parameters). The integration of these parameters determines the mode and capacity of migration. (B) The range of many migratory parameters can be displayed in a tuning model similar to those commonly used in audio equalizers (supplement). The magnitude of any parameter influences its impact on the mode and means of migration as well as the influence of related parameters. Each migration mode is represented by a colored line, and the position at which this line crosses each tuner represents the magnitude of that parameter for this migration mode. This enables a visual display of the general conditions for various types of cell migration including single isolated mesenchymal cells (red line) and ameboid cells (green line) as well as densely organized mesenchymal (blue line) and epithelial cells (brown line). For additional definition of terms and interpretation please see Supplemental Figure 1 and Table I.

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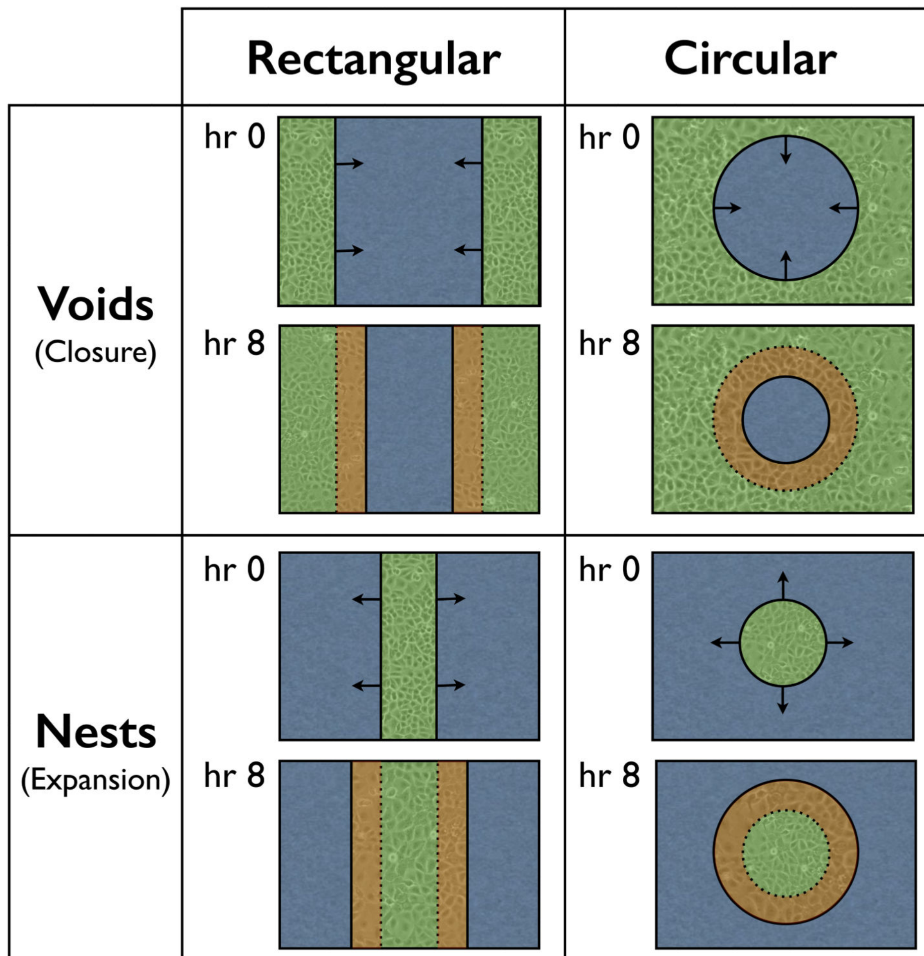

Figure 2. Common geometries

The geometries employed for analyzing *in vitro* two-dimensional migration of densely organized cells are classified by direction of migration or by shape. Generally cell migration is measured as an inward closure of a void or outward expansion of a nest of cells. Both voids and nests can be created with rectangular and circular shapes. However, average migration rates are generally only calculated from rectangles.

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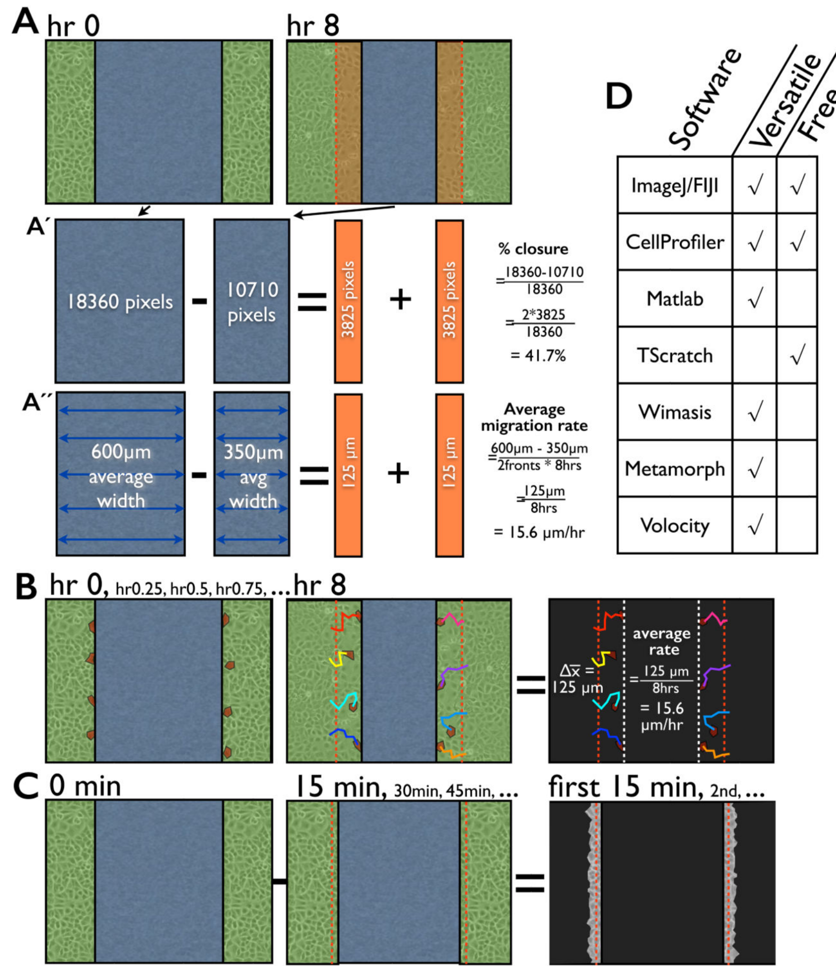

Figure 3. Quantification

Various methods are used to quantify migration data. (A) Cell covered areas are determined from corresponding images of initial and final timepoints. A′ The open area for each is calculated precisely or A″ the open average width is estimated by measuring lengths of 5 lines. The percent closure or average migration rate are calculated from the values. (B) Individual cells are tracked through all timepoints. The distance traveled is averaged and used to calculate average migration rate. (C) By subtracting the current image and following image a movie is created showing movement of cells both migrating into the void and mobilizing behind the initial cell boundary (red line). (D) Commonly used commercial and free software for migration analysis.

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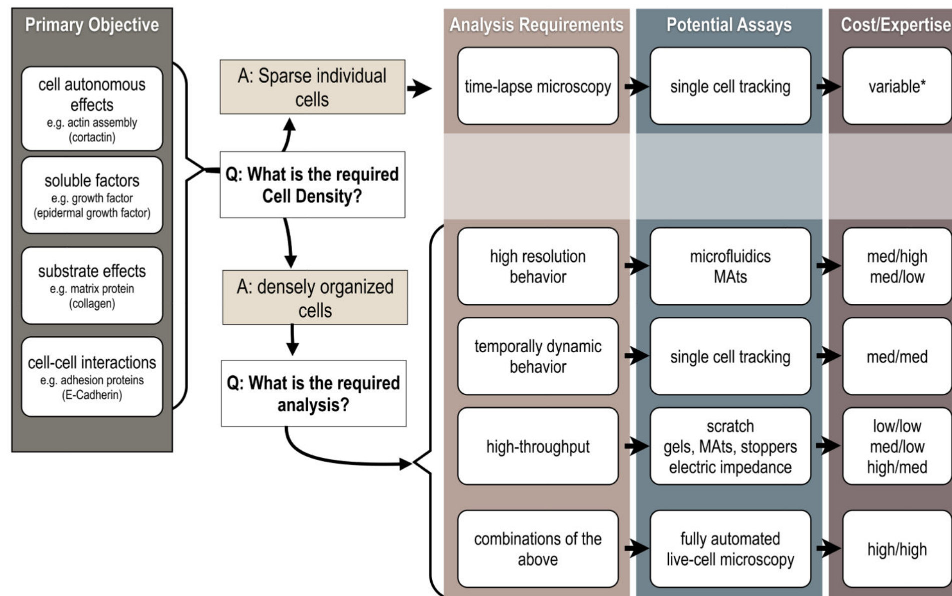

Figure 4. Assay selection

The first step in assay selection is to consider the study's research objective ("Primary Objective"). Once the research objective is defined, the cell density at which migration is analyzed must be determined. Selecting the analytical parameters ("Analysis Requirements") guide the final selection of an assay ("Potential Assays") most appropriate for the research objective. Frequently there are several options that are appropriate and the final choice is determined by the available resources and expertise ("Cost/Expertise").

* Expertise and expense of single cell tracking is highly dependent on the strategy taken and equipment used. Those elements are beyond the scope of this review. For further perspective on this topic consider references 55-57.

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Table 1

The bandwidth of 2D migration assays.

Assay Bandwidth — Upper Range … Full Range … Lower Range. ? = unknown NA = not applicable

			Geometry		Cell-cell Interaction		Matrix Composition				Soluble Parameters		
			rectangular / circular	voids / nests	homotypic / heterotypic	dynamic / static	densely coated / bare	oriented / random	stiff / soft	2D / 2.5D / 3D	chemotactic stimuli / homogeneous stimuli	hyperoxic / normoxic / hypoxic	migration inducing factors / inhibiting factors
Isolated, Individual Cells													
		Single Cell Tracking	NA	NA	NA	NA							
Densely Organized Cells													
Cell Removal (Wounding)													
	Mechanical	Scratch Assay											
		Scratch Variations	or	or									
	Electrical	ECIS											
Undeveloped	Chemical	Laminar Flow of Trypsin											
Undeveloped	Thermal	Thermal Damage	?	?									
Undeveloped	Optical	Laser Ablation	?	?									
Cell Exclusion (Void Filling)													
	Solids	Stencils											
		Stoppers											
		MAtS											
	Gels	Barricade Gels											
	Liquids	Immiscible Solutions											
		Laminar Flow											
	Air	Droplets											
		Microfluidic Surface Tension											
	Electromagnetic	Magnetic Particles	?	?									
Undeveloped		Electric Cell Repulsion	?										
Undeveloped		Optical Trapping											

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Table 2

Comparison and Publications of Cell Migration Assays

Migration Assay	Defining characteristic	Examples and (references)	Custom substrates		Compatible with variety of culture dishes		Google Scholar search term/s	Publications (2011)
Cell Removing								
Scratch Assay	scraping	pipette tip, silicone wedge, (4-9)	**no**	scratch disrupts the substrate	**yes**		"scratch assay"	**488**
Electric Impedance	electric readout of cell adhesion and migration	electric cell-substrate impedance sensing (ECIS), (11-12)	**no**	confluent cells alter the substrate	**no**	requires special plates with electrodes	migration "cell-substrate impedance sensing"	**96**
Miscellaneous								
Microfluidics	small fluidic channels and chambers	cell trypsinization, cell patterning via capillary force, (13-14, 35-36)	**?**	varies with each device	**no**	requires microfluidic device and equipment	microfluidic "wound healing" "cell migration"	**214**
Colony Migration	growth of small cell population into collective colony for migration analysis	oil drop assay, collective migration on elastic substrate, (16,34)	**yes**	requires either exposing substrate to atmosphere or to oil drops while cells attach	**yes**		colony expansion "collective migration"	**17**
Cell Blocking								
Stencils	contact between stencil and substrate relies on auto-adhesion	PDMS membrane, Culture-Inserts by Ibidi, (26-32)	**no**	substrate must be completely dry prior to attaching inserts	**yes**	requires uncoated or dry surface	"collective cell migration" OR "collective migration" PDMS membrane OR stencil/Ibidi culture inserts migration	**121**
Stoppers	wedging into specific size wells	Cytoselect™ Wound Healing Inserts, Oris™ Cell migration assay, teflon stoppers, (22-25)	**?**	physical insertion of stoppers may disrupt the substrate	no	requires specific dimensions	Oris cell migration/ Cytoselect cell migration	**211**
Barricade Gels	blocking cell adhesion with a dissolvable gel	Radius™ Gel, Oris™ Pro (NA)	**no**	manufacturer determines substrate which must be dryable	**no**	sold in limited variety of plates	"Radius gel" OR "Oris Pro" cell migration	**6**
MAts	magnetic force maintains contact between stencil and substrate	accepted and pending publication, (55)	**yes**	magnetic force attaches MAts without disrupting the substrate	**yes**		n/a developed in 2010	**n/a**