Assays for Eukaryotic Cell Chemotaxis

Zac Pujic[1], Duncan Mortimer[1], Julia Feldner[1,3] and Geoffrey J. Goodhill[*,1,2,3]

[1]*Queensland Brain Institute,* [2]*School of Mathematics and Physics and* [3]*Institute for Molecular Bioscience, The University of Queensland, St Lucia 4072, Queensland, Australia*

Abstract: Chemotaxis is essential for many biological processes. Much of our understanding of the mechanisms underlying chemotaxis is based on a variety of *in vitro* assays. We review these assays, dividing them into groups depending on the process used to generate the gradient. We describe how each method works, its strengths and limitations, and provide some information about the kinds of cells that have been studied with each assay.

Keywords: Gradient, microfluidics, diffusion, axon guidance, cell migration, metastasis, Boyden, invasion assay.

INTRODUCTION

Many biological processes which occur during normal function, development, repair, regeneration or pathology require the movement of eukaryotic cells up or down chemical gradients. Some particularly well-studied examples include the movement of leukocytes towards sites of infection [1], slime molds during aggregation or towards cyclic AMP [2], spermatozoa towards factors released by follicles [3], metastatic cells towards sources of growth factors [4], and the orchestration of neuronal wiring during brain development by factors guiding neuronal growth cones [5].

Understanding how cells detect and respond to gradients is thus an important problem in many areas of biology. To develop such an understanding it is often useful to examine the response of cells in situations where gradient parameters can be known and controlled, and visualization of cell behaviour is easier than in *in vivo* settings.

This need has led to the development of many different types of *in vitro* chemotaxis assays, many of which can be applied to a wide variety of cell types. Improvements in technology over the last several decades have led to assays which can now provide high resolution imaging of live cells in controllable gradients. We review some of the most commonly used chemotaxis assays, including the cell types they have been used for, and the chemotaxis parameters they are suitable for measuring. A brief overview is shown in Table **1**.

ASSAYS EMPLOYING SOURCE AND SINK CHAMBERS

The simplest chemotaxis assays use fluid-filled chambers separated by a porous membrane or by small conduits through which a chemotactic factor, present at high concentration in one chamber, can diffuse into the second chamber (Box **1**). The resulting gradient of factor across the membrane or within the conduit influences cells growing in its vicinity. Chemotaxis can be quantitated either by directly observing cell motion, or by measuring the net movement of cells from one chamber to the other at the completion of the assay. Assays employing source and sink chambers are most suitable where high throughput is required or where the movement of large numbers of individual cells must be visualized.

Boyden Chamber

The Boyden chamber is one of the earliest and simplest assays of cell migration [6]. It uses an upper and a lower chamber separated by a porous membrane through which cells can migrate (Fig. **1A**, Table **1**). The gradients are formed by placing different concentrations of the chemotactic compound of interest in the upper and lower chambers. A linear gradient is quickly established across the membrane by diffusion (Box **1**). Cells respond to the gradient and move from one chamber to the other through pores in the membrane.

The Boyden chamber assay is an established method for the study of chemotaxis in leukocytes and fibroblasts, and is useful for screening large numbers of cells and potential chemotactic factors. However, the assay has some limitations for investigating the mechanisms of chemotaxis. The chamber provides an end-point assay in which the process of the migration is not directly viewable. As a result, it is difficult to discriminate between chemotaxis and other chemo-induced phenomena such as chemokinesis (though more complex protocols such as the checkerboard assay [7] can help to address this issue). Furthermore, the exact nature of the concentration gradient is unknown, and the cells may modify the gradient by obstructing the pores in the membrane. Nevertheless, the Boyden chamber remains a useful and sensitive method for identifying possible chemotactic responses in migrating cells [8, 9]. Boyden chambers are available from commercial sources, including a 1,536-well chamber version which allows for simultaneous high-throughput analysis of chemotaxis.

Zigmond Chamber

The Zigmond chamber [10] is similar to the Boyden chamber in that a gradient is formed by the diffusion of chemotactic factor between two chambers (Fig. **1B**, Table **1**); however in the Zigmond chamber, the permeable membrane of the Boyden chamber separating the two sources of chemotactic cue is replaced by a narrow space. The assay chamber

*Address correspondence to this author at the Queensland Brain Institute, The University of Queensland, St Lucia 4072, Queensland, Australia;
E-mail: g.goodhill@uq.edu.au

1386-2073/09 $55.00+.00 © 2009 Bentham Science Publishers Ltd.

Table 1. A Summary of the Main Chemotaxis Assays Reviewed and their Relative Merits

Assay	Direct Viewing	Time Scale	Rapid Bath Application	Precise Control	Dynamic Control
Boyden chamber	No	Hours	No	Intermediate	No
Zigmond chamber	Yes	Hours	No	Intermediate	No
Dunn chamber	Yes	Hours	No	Intermediate	No
Pipette	Yes	Hours	Yes	Good	Yes
Soon chamber	Yes	Days	Yes	Good	Yes
Gel blocks/Explants	Yes	Days	No	Poor	No
Printing	Yes	Days	No	Good	No
Microfluidics	Yes	Days	Yes	Good	Yes
Substrate bound	Yes	Days	Yes	Intermediate/Good	No

Direct viewing refers to the ability to observe cells (*via* microscopy) during the assay. Time scale indicates the approximate amount of time that a gradient is present. Rapid bath application indicates whether or not chemicals can be applied to the cells in the assay without disrupting the gradient. Precise control is an indicator of how much control is available over the gradient characteristics in the assay. Dynamic control is an indicator of whether the gradient characteristics can be controllably altered during the assay.

Box 1. Physical Principles of Source-Sink Assays

Many of the assays we describe rely on diffusion in order to develop gradients. The Boydon, Zigmond and Dunn chambers make use of diffusion from a source to a sink. In an idealised situation in which the source does not deplete and the sink is perfectly absorbing, this would create a stable gradient after an initial transient during which the gradient forms [74]. In practice, neither the source nor the sink will meet this criterion. As a result, the gradient gradually decays over time as the source becomes depleted and the concentration in the sink rises. Thus, three phases are involved: a stabilisation phase during which the long-term gradient is forming, a period of relative stability, followed by a period over which the gradient decays.

The timescale over which the gradient forms is determined by the diffusion coefficient, D, and the distance over which the gradient is set up, L:

$$T \sim \frac{L^2}{D}$$

In contrast, the period over which the gradient is stable is determined by the volumes of the resevoirs. During the period for which the gradient shape is reasonably constant, the rate at which material flows from one reservoir to the other, dn/dt, is determined by the difference in concentration between the reservoirs ΔC, the diffusion coefficient D, the length L and cross-sectional area A of the gradient:

$$\frac{dn}{dt} \approx \Delta C \frac{AD}{L}$$

The gradient retains its shape as long as the difference in concentration between the source and sink is sufficiently close to its initial value. This difference decays exponentially, with a rate inversely proportional to the volume of the reservoirs:

$$\Delta C(t) = \Delta C_0 \exp\left(-\frac{AD}{LV_{eff}}t\right)$$

where V_{eff} is the effective volume of the source and the sink (if both are equal in volume, then V_{eff} is just $V_{source} = V_{sink}$). Thus, the decay time is related to

$$\tau = \frac{V_{eff}L}{AD}$$

The time for which the gradient is stable can be increased by (*i*) increasing the volumes of source and sink, (*ii*) increasing the length or decreasing the cross-sectional area of the gradient, or (*iii*) using a more slowly diffusing cue. These expressions must be modified for gradient systems in which the cross-sectional area of the region in which the gradient is generated varies along the length of the gradient (for example, in the Dunn chamber, where the region between source and sink is annular); see Crank [74] or similar textbooks on diffusion for further information.

consists of two wells cut into a plexiglass slide. Both wells are 4 mm wide and 1 mm deep and hold ~100 μL of solution. Separating the wells is a bridge that is 1 mm wide and ~3-10 μm deep. A coverslip, on which cells are growing, is placed over the slide and constrained with a brass clip screwed into the plexiglass at each end. The coverslip thus seals the two wells and allows the transfer of fluid between the two wells only across the bridge.

The Zigmond chamber is relatively inexpensive and can be rapidly assembled. It is a direct-viewing chamber which permits the observation of cells moving across the bridge separating the two chambers (which is not possible in the Boyden chamber). Zigmond chambers are commercially available and have been used for studying a variety of cells including *Dictyostelium discoideum* [11], spermatozoa [12] and leukocytes [13, 14]. However this assay is poorly suited to high-throughput analysis of chemotaxis. Its main drawback is the instability of the gradient; due to the small volumes of the two chambers, the gradient is not stable and decays.

Dunn Chamber

The development of the Dunn chamber [15] was driven by a need for better optical characteristics, higher dimensional precision and increased long-term stability than the Zigmond chamber, on which it is based (Fig. **1C**, Table **1**). The chamber consists of a glass microscope slide with two concentric annular wells approximately half the depth of the slide. The annular bridge that separates the two wells is about 1 mm wide and lies about 20 μm below the level of the glass slide surface. Dissimilar solutions are introduced into the two annular wells, and cells are added to one well. A glass coverslip is then laid over the surface, allowing diffusion to generate a linear gradient across the bridge. Cells moving over the bridge can be directly visualized using microscopy.

As in the Boyden and Zigmond chambers, the formation of the gradient in the Dunn chamber is diffusion-driven and results in a radially directed gradient which is maintained for several hours. It suffers from similar drawbacks to its predecessors; the gradient is not stable and decays. The similarity

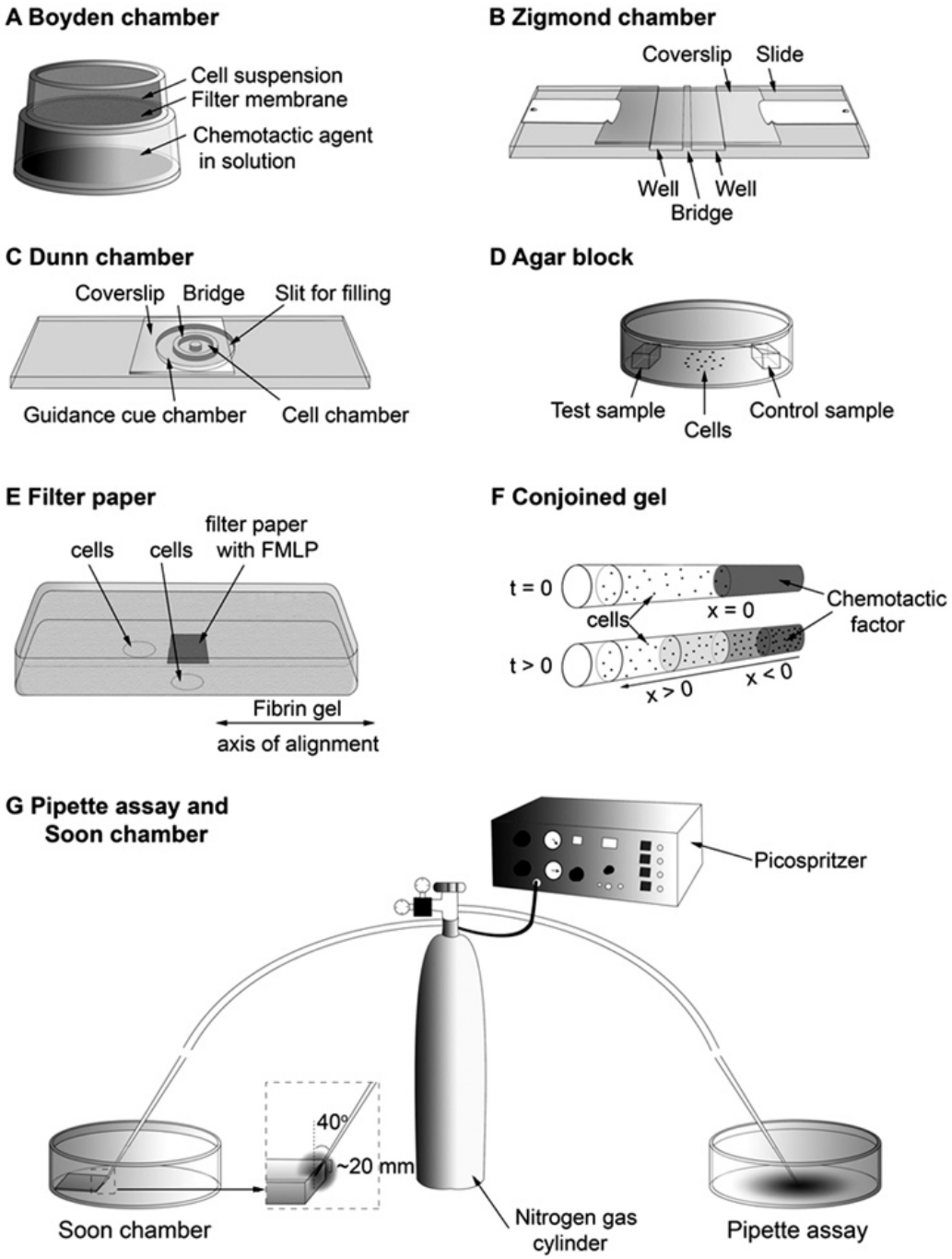

Fig. (1). Examples of chemotaxis assays which generate gradients by using chambers for the source and sink (**A-C**), gel substrates (**D-F**) and diffusion from a point-source (**G**). In **A-C**, a source of chemotactic agent in one chamber diffuses into a second to generate a gradient across which cells move. In **D-F**, cells are grown in the vicinity of sources of chemotactic agent (*e.g.*, gel block or filter paper impregnated with chemotactic agent which produces a gradient). In **F**, a chemotactic agent in a gel is poured next to a gel without chemotactic agent (but containing cells), at t = 0. For t > 0, the boundary (x = 0) between the two is lost due to diffusion of the chemotactic agent and a gradient is formed. Cells grown in the gels move in response to the gradient. In **G**, a chemotactic agent is released from a micropipette in the vicinity of cells growing in Petri dishes. In the Soon chamber, the cells are grown on coverslips which aids in the generation of a steeper gradient.

of the Dunn and Zigmond chambers makes them suitable for the study of similar types of cells [16, 17].

ASSAYS USING DIFFUSION FROM A POINT SOURCE

The formation of a gradient by the release of chemotactic factors from pipettes has been successfully used to measure chemotaxis in a wide variety of cells including *Dictyostelium discoideum* [18], cancer cells [19], and neuronal growth cones [20]. Although these assays are currently relatively low throughput, they offer some advantages over many other chemotaxis assays. They are amenable to the rapid application of other chemicals in the background (bath application), and cells may be observed directly during the assay using a

large range of microscopy techniques. In addition, the assays may be performed in a wide variety of dishes, unlike the Dunn, Zigmond or Boyden chambers which are purpose-built for the assay.

Pipette, or "Growth Cone Turning" Assay

Precursors of assays using material released from a micropipette to produce gradients of chemotactic factors date to the 1960's [21]. Early methods relied on gravity/diffusion to transfer the chemical from the pipette [22]. A modification of this approach is the use of compressed gas with a timer-driven gate to provide pulsatile ejection of the chemoattractant [20, 23], a method which can more precisely deliver small volumes (Fig. **1H**, Table **1**). Once the chemoattractant has exited, diffusion causes the material to form a gradient centred roughly on the pipette tip.

The method has significant benefits over chamber-based gradient generation. Cells can be directly and easily observed using a variety of microscope imaging methods, including DIC, phase, fluorescence and confocal microscopy. Since the Boyden, Zigmond and Dunn chambers require that no liquid currents occur for the formation of a stable gradient, it is difficult to apply chemicals after these assays have been started. For many applications, the simultaneous inclusion of other chemicals in the background is highly desirable. The pipette assay allows this by producing a gradient that is continuously generated; addition of test substances to the assay dish produces only transient disruption of the gradient, which is then quickly re-established [24].

Due to the flexibility of the assay, it has been used to provide information about the motility of a large range of cell types including neutrophils, *Dictyostelium discoideum* [18, 25-27] and neuronal growth cones [20, 24, 28, 29]. A limitation of pipette-based assays is the relatively small number of cells that can be studied simultaneously. Whereas chamber based assays can be used to provide information about chemotaxis for potentially many cells simultaneously, pipette-based assays provide information only about the cells in the vicinity of the pipette tip. It is also difficult to accurately determine the gradient parameters, including the steepness or concentration of the chemotactic factor [23]. Also, complete pipette assay systems are not readily available commercially, although separately the components are fairly standard.

Soon Chamber

The Soon chamber was established to allow the real-time imaging of chemotaxis by carcinoma cells towards chemoattractants correlated with metastasis (Fig. **1G**, Table **1**) [19]. It is similar to the pipette assay, but is modified to be able to provide steeper gradients. The Soon chamber assay consists of a micromanipulator which supports a micropipette filled with the chemoattractant of interest. Cells are grown on a coverslip which is placed in a glass-bottom dish, and the tip of the micropipette is placed approximately 20 μm below the coverslip top (near one of its edges) at an angle of 40 degrees from the vertical. The chemoattractant is continuously ejected from the micropipette. The coverslip acts as a dam and serves to increase the steepness of the gradient.

The gradient formed in the Soon chamber is characterized by rapid equilibration, high stability and high steepness. The chemoattractant gradient rapidly stabilizes by 20 seconds following the onset of flow from the micropipette and is then stable for at least 60 minutes. Since the cells are grown on glass coverslips, their movement can be documented using DIC or phase microscopy. Furthermore, the setup is inexpensive and rapid. The disadvantages of this assay are similar to those of the pipette assay described above.

GEL-BASED ASSAYS

In gel-based assays a gradient is obtained by diffusion of a chemotactic factor secreted from cells or tissue located on a filter or in a gel block, or by printing the chemotactic factor directly onto the gel (Table **1**). The responsive cells are grown in a tissue culture matrix made from extracellular matrix components such as collagen, laminin, fibrin, or Matrigel. Gel-based assays have been employed to study migration of fibroblasts [30], macrophages [31], vascular endothelial cells [32] and neutrophils [33] as well as for studying axon guidance [34, 35]. To analyse the direction of migration or extension of axons, cells are labelled with antibodies and imaged using fluorescence or light microscopy. Typically, migratory behaviour in these assays is analysed after migration has occurred, by measuring the final position of the "leading front" of invasion or the total number of cells invading a region of interest.

For several reasons, direct measurements of the rate of the decay of the gradient in gel-based assays have rarely been made. Firstly, if a biological response is seen in the assay then often this is considered sufficient evidence for the existence of a gradient, and its precise shape is of little concern. Secondly, fluorescent labeling of chemotactic factors can change their properties and the way they interact with the gel substrate. Thirdly, the concentrations of such factors needed for a detectable fluorescence signal to be observed can be much higher than the concentrations usually used to produce a biological response, and this can be prohibitively expensive. Some approaches that have been taken are radioactive labelling [36], and extrapolation from the measurement of fluorescently labeled compounds of similar molecular weight to the biological factor [37, 38]. Alternatively, inferences can be drawn from theoretical modeling of the expected shape of the gradient [39].

Most gel-based assays can also be assembled on glass coverslips or in glass bottom dishes and the behaviour of cells above, under, or within the gel (if the gel is transparent) can be observed continuously, using real time or time-lapse microscopy [40]. Tracks of individual cells can be traced continuously and parameters such as velocity or the straightness of the path can be obtained.

Filter Paper Assay

This assay is a quick and simple method that is commonly used to look at the response of migratory cells to a chemoattractant released from filter paper (Fig. **1E**) [40] used this assay to look at chemotactic attraction of human neutrophils to f-Met-Leu-Phe in a fibrin gel. A small square of filter paper is impregnated in a solution of chemotactic factor. It is then placed in the centre of a microscope slide or tissue culture dish, and a fibrin or collagen gel is poured over the filter paper. This is then overlaid with a suspension of cells. After

these have settled on the gel, a final layer of tissue culture medium is applied and the preparation is incubated or used for live cell imaging. A more recent study uses a gel-free variation of this method to show that nematodes are attracted by filter paper impregnated with slug mucus [41].

The filter paper assay is a quick and inexpensive method; however the low stability of the gradient makes this assay most suitable for fast moving cells such as neutrophils. Depending on the arrangement of filter paper and responsive cells the direction of migration can be vertical or horizontal, which impacts on the ease of visualization.

Under-Agarose Assay

This end-point assay was developed using agarose as a solid cell-impermeable gel in which a gradient is formed by diffusion of the chemotactic factor from a well cut into the gel [42]. The responsive cells are placed into a second well in the gel, and migrate towards the attractive gradient under the agarose along the surface of the tissue culture dish or microscope slide. After incubation for several hours and fixation of the whole set-up, the agarose gel is removed and the cells attached to the dish are stained for analysis. The migration patterns can then be analysed by measuring the distance the cells have moved towards the chemoattractant. In order to visualize the formation of the gradient, dyes such as methylene blue can be added to the well containing the chemoattractant. This assay is suitable for the analysis of chemotaxis of a large variety of cell types, including neuronal growth cones [43]. The main advantage of this assay is the 2-dimensional movement of cells, which simplifies imaging since there may be only a single focal plane. However, migration underneath a gradient rather than through it is less physiologically relevant.

Conjoined Gel Assay

In this assay, gradients of chemotactic factors are obtained by setting up two horizontally adjacent gels, one containing the chemoattractant and the other containing the responsive cells but not the attractant (Fig. **1F**) [44]. Initially, the two gels are separated by a barrier, which is then removed to initiate gradient formation by diffusion. After the gradient has formed, multiple cells can be tracked using time-lapse microscopy. It is possible to measure migration parameters such as the random motility and chemotaxis coefficients, and the absolute concentration and gradient steepness of the chemotactic factor can be modelled and determined experimentally by measuring the spatial variation in fluorescence of a FITC-dextran analog representing the chemotactic factor. A variation of this assay involves the presence of a physical barrier between the two gels in order to geometrically constrain the free diffusion of the chemotactic factors, thereby slowing the decay rate of the gradient [45]. The known geometrical shape of the two gels allows for high reproducibility and the characteristics of the gradient such as the steepness and absolute concentration can be measured or modelled. Since the gradients are relatively stable (up to 24 hrs), this assay is suitable for studying slow migrating cells such as fibroblasts [45].

Agarose Block Assay

In this assay, the chemotactic factor, or a suspension of transfected cells expressing the factor if purified factor is not available, is first mixed with the agarose that is then poured onto a microscope slide or into a culture dish to form a gel about 1 mm deep (Fig. **1D**). A small block of this agarose is cut out with a scalpel and transferred to a coverslip or tissue culture dish (alternatively a cube of agarose only can be prepared and soaked in chemotactic factor after it has set). A solution of collagen or fibrin gel is poured over the agarose block and allowed to set [33, 35, 46]. A gradient is then established by diffusion from the block. In this 3-dimensional assay, the responsive cells may be incorporated throughout the gel. Alternatively, explants derived from freshly prepared tissue may be embedded in the gel a short distance away from the agar block. The release of chemotactic factor by overexpression in cells is continuous over time which results in a slower decay of the gradient compared to that of guidance cue released from impregnated agar cubes. Using agar cubes rather than filter paper as a source of the chemotactic factor allows for greater quantities of the factor to be delivered. However due to variability in the size and shape of individual cubes, the absorbed amount of chemotactic factor, and the expression rate of cells in the cube, the gradient is not as reproducible as in filter paper or printing assays.

Co-Culture Assay

In co-culture assays freshly isolated tissue expressing the chemotactic factor is used instead of cell lines overexpressing the chemotactic factor. At the same time, another tissue that is responsive to this guidance cue is embedded in a 3-dimensional matrix a short distance from the source tissue [47]. The matrix is then overlaid with growth medium and cultured for up to several days. One variation of this assay is to include tumor cell aggregates embedded around a source of cell aggregates expressing a chemotactic factor in collagen gels [48].

Co-culture assays are commonly used for axon guidance studies [47, 49-51]. Because the nature of the gradient is not easily observed or manipulated, it is only practically possible to obtain qualitative information about the responsiveness of a tissue. However, the use of freshly isolated tissue resembles the *in vivo* situation more closely than the use of cells or isolated proteins.

Printing Assay

This method was introduced to provide precisely controllable gradient conditions in which the chemotaxis of neuronal growth cones can be studied, though many other cell types could in principle be used [37] (Fig. **2A**, Table **1**). The cells (or explants [37]) are first embedded in the middle of a layer of collagen approximately 1 mm thick in a standard 35 mm Petri dish. This embedding is most easily achieved by placing the cells on top of one 0.5 mm-thick layer of collagen (or other appropriate substrate) and then applying a second 0.5 mm-thick layer on top. A pattern of drops of spatially varying concentration of the chemotactic factor is then "printed" onto the surface using any suitable non-contact printing device which permits small drop volumes and fine spatial control of drop placement (we currently use a Nano-Plotter 2.0 (GeSiM, Germany), for this). The drops of chemotactic factor then diffuse into the collagen, producing a concentration profile in the gel that evolves in time in a predictable manner. A smooth gradient is rapidly established horizontally in the gel, and the shape of this gradient (at least

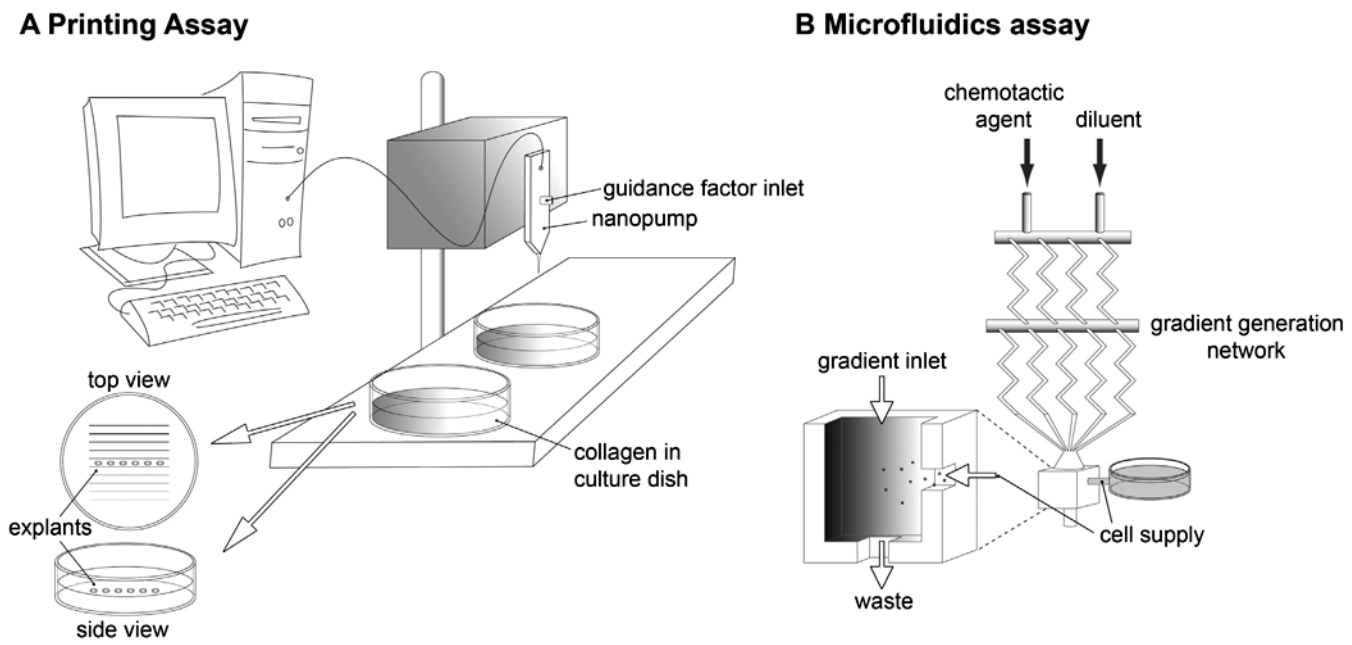

Fig. (2). Examples of chemotaxis assays which possess a high degree of reproducibility and control over gradient parameters; the printing assay (**A**) and microfluidics-based assays (**B**). In the printing assay, a computer-controlled piezoelectric pump is used to deliver lines of nanolitre-sized droplets of chemotactic cue to a gel containing cells (or explants). In a microfluidics assay (**B**), a source of chemotactic agent is introduced into mixing chambers and tubes with a diluent (*e.g.*, PBS). The mixing tubes converge onto a chemotaxis chamber in which are cells which have been introduced from a supply. The gradient is applied after the cells have entered the gradient chamber and their response to the chemotactic agent is observed using microscopy.

at certain positions in the gel) then remains fairly constant over periods of up to several days, despite the continued diffusion of the chemotactic factor [37, 38]. By varying the pattern of drops initially applied a wide variety of gradient shapes can be obtained. So far we have only examined fixed and antibody labelled axonal trajectories after 2 days, though timelapse imaging would also be possible.

Two disadvantages of this assay are that it requires potentially high concentrations of purified chemotactic factor, and that steep gradients are hard to produce for rapidly-diffusing factors. However, a significant advantage of this assay is that it allows fine control over both gradient steepness and absolute concentration. Furthermore, since gradients of multiple factors can be printed onto the same gel, unlike most other chemotaxis assays it allows the response of cells to simultaneous well-defined gradients with potentially arbitrary spatial relationships of multiple chemotactic factors to be examined.

ASSAYS USING SUBSTRATE-BOUND GRADIENTS

The majority of chemotaxis assays employ a gradient of diffusing chemoattractant formed in a liquid or gel medium (Table **1**). This is partly due to the ease with which systems such as the Boyden or Dunn chambers can be set up to provide these gradients. However *in vivo* gradients of chemoattractants may sometimes be anchored to the extracellular environment.

Developing substrate-bound gradients *in vitro* requires that a gradient be formed and attached to a substrate or scaffold. The earliest forms, the stripe assays [52], consisted of alternating stripes of chemotactic chemicals or different types of cell membranes attached to culture dish substrates. Although these do not constitute gradients per se, they do provide comparisons of, for instance, the mobility of cells between the different stripes. This approach has particularly been used to examine how the responses of different populations of retinal cell axons depend on the region of optic tectum on which they are growing [53]. A detailed protocol is available in [54].

The original stripe assay has since been modified to allow for the formation of a more continuous substrate-bound gradient of cell membranes [55, 56]. This was achieved by lifting one end of a coverslip to produce a "wedge" of a suspension of cell membranes, and then sucking the suspension down onto a substrate, thus producing a gradient of cell membrane density. In general this approach has fairly low reproducibility. More reproducible substrate-bound gradients can be formed by the delivery, *via* microfluidics (see below), of chemicals in a soluble form which adsorb onto the substrate [57, 58]. For instance, in [57] the response of axons of rat hippocampal neurons to gradients of laminin of variable steepness was examined.

A quite different approach was used in [59]. First a silicon master was etched using electron beam lithography to produce a pattern of spots. This was then used to produce a corresponding pattern of chemotactic factor by stamping onto a suitable substrate. The distance between spots can be as small as 1 μm or so, compared to cellular dimensions of roughly 10 μm. Thus, while each spot is of the same concentration, varying the size and spacing of the spots allows a quasi-continuous gradient to be formed when local concen-

tration is averaged over cellular length scales. Again this was used in [59] to examine the response of retinal cell axons.

In general substrate-bound gradients are more difficult to produce than diffusible gradients. However once formed, the gradients are more (in principle indefinitely) stable, and the characteristics of the gradient are potentially variable over a broader range than for chamber-or gel-block based assays. Since the gradients can be formed in small chambers and the growth is by definition in two dimensions, substrate-bound assays possess good optical characteristics, making them suitable for time-lapse imaging of chemotaxis.

MICROFLUIDICS-BASED ASSAYS

Advances in fabrication techniques have allowed the development of microfluidic devices: systems which allow the control and analysis of fluids at the micrometer (or nanoliter) scale (Fig. **2B**, Table **1**) [60]. At such scales, different physical forces become important, as compared to fluid dynamics on the macro-scale. Inertial forces exert negligible effects on fluid dynamics, being dominated by, for example, viscosity and surface tension. As a result, microfluidic flows are typically laminar, and fluid mixing occurs primarily through diffusion. Thus, stable gradients can be generated by directing fluid streams carrying different concentrations of target substances side-by-side into the same channel.

Fig. (**2B**) shows a typical design for such a constant flow system. There are two stages to the setup. Fluids carrying the substances of interest are injected into the device, and then directed through a mixing stage, in which the flows are repeatedly split and recombined in long, serpentine mixing channels. In the second stage of the process, the outputs from the mixing stage are brought back into contact, and fed side-by-side into the viewing channel, which contains the cells. In a typical experiment, cells are first injected into the device through the injection channel, and allowed to adhere. The gradient is then generated, and cell trajectories filmed. Such systems have been used to study numerous cell types, including neutrophils [61, 62], T cells [62], *Dictyostelium discoideum* [63] and cancer cells [64].

There are several advantages to this kind of system. 1-dimensional concentration profiles of almost arbitrary complexity can be set up with relative ease. Innovations to the basic design of these assays also allow rapid temporal control of gradient shape, enabling the quantitative study of both spatial and temporal components of chemotaxis [65-67]. Similarly, cells can easily be exposed to additional pharmacological reagents.

However, there are disadvantages to the continuous flow design. The size of the region within which the gradient retains the desired shape is determined by the flow rate, with faster flow giving a larger usable region. Flow rates in these devices are typically on the order of mm/s, which exerts considerable shear stress on the cells under study [68]. This limits their utility for studying shear-sensitive cells, such as primary neurons and stem cells. These stresses also exert a measurable influence on cell migration paths.

Several microfluidic devices have recently been developed which attempt to address this problem by generating gradients in stationary media, based on the same principles as the Dunn and Zigmond chambers [69, 70], or the pipette assay [71]. A further advantage of this kind of setup is that fluid growth media are not required, allowing the study of, for example, cell dynamics and chemotaxis in more physiologically relevant matrices.

There are a number of technologies available for the manufacture of microfluidic devices [72]. One of the most popular is soft lithography, in which an embossed master mold is generated through photolithography, which is then used to imprint a polymer, polydimethylsiloxane (PDMS). PDMS is optically transparent to wavelengths as short as ~300 nm light, which allows for visible light microscopy, and is also gas permeable allowing for cell respiration. For a thorough review of applications of this fabrication technique in biology, see [73].

CONCLUSION

A wide array of assay strategies has emerged for the analysis of chemotaxis. There are many variations in how the gradient is generated, how the chemotaxis is detected, the potential for high throughput analysis, and the type of information which may be obtained by each assay. A brief summary is shown in Table **1**.

The simplest assays, such as those based on the Boyden chamber, provide only an estimate of whether a chemical can elicit a chemotactic or chemokinetic response, and do so without any direct imaging of cell movement. The principle advantage of more sophisticated chamber assays (such as the Zigmond or Dunn chambers) is that they allow for the direct imaging of cell movement with microscopy. It is also not possible to rapidly include chemicals in the background in any of the chamber assays. In gel-based assays, chemotactic cues are released from sources into which they have been impregnated, or are secreted directly by cells or tissue expressing the factor of interest. These assays possess many of the disadvantages of chamber assays: temporal instability of the gradient, poor knowledge and control of the gradient parameters, and poor reproducibility. As a result, they remain poorly suited for studies which require quantitative data. However, gel-based assays can be set up both cheaply and rapidly, and can offer a more *in vivo* like environment than chamber based assays.

A different approach to the generation of the gradient is used in chemotaxis assays which employ diffusion from a point source such as a pipette tip. The continuous delivery of a chemotactic cue in the vicinity of growing cells helps ensure a high degree of temporal stability of the gradient, and may allow some control over the gradient parameters. The ability to introduce chemicals into the background during the assay, and the ability to move the position of the pipette tip gives these assays significant advantages compared to chamber- or gel-based assays. Setup time and cost however are generally greater, and their ability to be modified for high throughput or for the generation of quantitative data is poor.

Assays which provide the most potential for the generation of quantitative chemotaxis data include microfluidics-based assays and the printing assay. Neither are currently available commercially in complete form, and setup time and cost are relatively large compared to other assays. However, both offer a high degree of reproducibility and may be modified for high throughput analysis. They also offer a high degree of control of gradient steepness, duration of assay, abil-

ity to directly observe the cells under study and ability to introduce chemicals into the background.

ACKNOWLEDGMENTS

We thank Charlotta Lindwall for help with preparation of the figures. We gratefully acknowledge financial support from an Australian Postgraduate Award (DM), grants from the Australian Research Council (Discovery Grant DP0666126) and Australian National Health and Medical Research Council (Project Grant 456003), and from the Queensland Brain Institute, School of Mathematics and Physics, and Institute for Molecular Bioscience at The University of Queensland.

REFERENCES

[1] Thelen, M. Dancing to the tune of chemokines. *Nat. Immunol.*, **2001**, *2(2)*, 129-34.

[2] Manahan, C.L.; Iglesias, P.A.; Long, Y.; Devreotes, P.N. Chemoattractant signaling in dictyostelium discoideum. *Annu. Rev. Cell Dev. Biol.*, **2004**, *20*, 223-53.

[3] Eisenbach, M. Sperm chemotaxis. *Rev. Reprod.*, **1999**, *4(1)*, 56-66.

[4] Condeelis, J.; Singer, R.H.; Segall, J.E. The great escape: when cancer cells hijack the genes for chemotaxis and motility. *Annu. Rev. Cell Dev. Biol.*, **2005**, *21*, 695-718.

[5] Dickson, B.J. Molecular mechanisms of axon guidance. *Science*, **2002**, *298*(5600), 1959-64.

[6] Boyden, S. The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes. *J. Exp. Med.*, **1962**, *115*, 453-66.

[7] Zigmond, S.H.; Hirsch, J.G. Leukocyte locomotion and chemotaxis. New methods for evaluation, and demonstration of a cell-derived chemotactic factor. *J. Exp. Med.*, **1973**, *137*(2), 387-410.

[8] Albini, A.; Benelli, R. The chemoinvasion assay: a method to assess tumor and endothelial cell invasion and its modulation. *Nat. Protoc.*, **2007**, *2*(3), 504-11.

[9] Takayama, N.; Ueda, H. Morphine-induced chemotaxis and brain-derived neurotrophic factor expression in microglia. *Peptides*, **2005**, *25*(2), 430-5.

[10] Zigmond, S.H. Ability of polymorphonuclear leukocytes to orient in gradients of chemotactic factors. *J. Cell Biol.*, **1977**, *75*(2 Pt 1), 606-16.

[11] Rubin, H.; Ravid, S. Polarization of myosin II heavy chain-protein kinase C in chemotaxing dictyostelium cells. *J. Biol. Chem.*, **2002**, *277*(39), 36005-8.

[12] Fabro, G.; Rovasio, R.A.; Civalero, S.; Frenkel, A.; Caplan, S.R.; Eisenbach, M.Giojalas, L.C. Chemotaxis of capacitated rabbit spermatozoa to follicular fluid revealed by a novel directionality-based assay. *Biol. Reprod.*, **2002**, *67*(5), 1565-71.

[13] Hannigan, M.; Zhan, L.; Li, Z.; Ai, Y.; Wu, D.; Huang, C.K. Neutrophils lacking phosphoinositide 3-kinase gamma show loss of directionality during N-formyl-Met-Leu-Phe-induced chemotaxis. *Proc. Natl. Acad. Sci. U. S. A.*, **2002**, *99*(6), 3603-8.

[14] Veltman, D.M.; Van Haastert, P.J. Guanylyl cyclase protein and cGMP product independently control front and back of chemotaxing Dictyostelium cells. *Mol. Biol. Cell*, **2006**, *17*(9), 3921-9.

[15] Zicha, D.; Dunn, G.A.; Brown, A.F. A new direct-viewing chemotaxis chamber. *J. Cell Sci.*, **1991**, 99 (Pt 4), 769-75.

[16] Takeda, K.; Sasaki, A.T.; Ha, H.; Seung, H.A.; Firtel, R.A. Role of phosphatidylinositol 3-kinases in chemotaxis in Dictyostelium. *J. Biol. Chem.*, **2007**, *282*(16), 11874-84.

[17] Chen, X.; Mcclurg, A.; Zhou, G.Q.; Mccaigue, M.; Armstrong, M.A.; Li, G. Chondrogenic differentiation alters the immunosuppressive property of bone marrow-derived mesenchymal stem cells, and the effect is partially due to the upregulated expression of B7 molecules. *Stem Cells*, **2007**, *25*(2), 364-70.

[18] Parent, C.A.; Blacklock, B.J.; Froehlich, W.M.; Murphy, D.B.; Devreotes, P.N. G protein signaling events are activated at the leading edge of chemotactic cells. *Cell*, **1998**, *95*(1), 81-91.

[19] Soon, L.; Mouneimne, G.; Segall, J.; Wyckoff, J.; Condeelis, J. Description and characterization of a chamber for viewing and quantifying cancer cell chemotaxis. *Cell Motil. Cytoskeleton*, **2005**, *62*(1), 27-34.

[20] Lohof, A.M.; Quillan, M.; Dan, Y.; Poo, M.M. Asymmetric modulation of cytosolic cAMP activity induces growth cone turning. *J.. Neurosci.*, **1992**, *12*(4), 1253-61.

[21] Adler, J. Chemotaxis in bacteria. *Science*, **1966**, *153*(737), 708-16.

[22] Gundersen, R.W.; Barrett, J.N. Neuronal chemotaxis: chick dorsal-root axons turn toward high concentrations of nerve growth factor. *Science*, **1979**, *206*(4422), 1079-80.

[23] Pujic, Z.; Giacomantonio, C.E.; Unni, D.; Rosoff, W.J.; Goodhill, G.J. Analysis of the growth cone turning assay for studying axon guidance. *J. Neurosci. Methods*, **2008**, *170*(2), 220-8.

[24] Song, H.; Ming, G.; He, Z.; Lehmann, M.; Mckerracher, L.; Tessier-Lavigne, M.; Poo, M. Conversion of neuronal growth cone responses from repulsion to attraction by cyclic nucleotides. *Science*, **1998**, *281*(5382), 1515-8.

[25] Langridge, P.D.; Kay, R.R. Mutants in the Dictyostelium Arp2/3 complex and chemoattractant-induced actin polymerization. *Exp. Cell Res.*, **2007**, *313*(12), 2563-74.

[26] Van Haastert, P.J.; Keizer-Gunnink, I.; Kortholt, A. Essential role of PI3-kinase and phospholipase A2 in Dictyostelium discoideum chemotaxis. *J. Cell Biol.*, **2007**, *177*(5), 809-16.

[27] Gomez-Mouton, C.; Lacalle, R.A.; Mira, E.; Jimenez-Baranda, S.; Barber, D.F.; Carrera, A.C.; Martinez, A.C.; Manes, S. Dynamic redistribution of raft domains as an organizing platform for signaling during cell chemotaxis. *J. Cell Biol.*, **2004**, *164*(5), 759-68.

[28] Song, H.J.; Ming, G.L.; Poo, M.M. cAMP-induced switching in turning direction of nerve growth cones. *Nature*, **1997**, *388*(6639), 275-9.

[29] Xu, X.; Fu, A.K.; Ip, F.C.; Wu, C.P.; Duan, S.; Poo, M.M.; Yuan, X.B.; Ip, N.Y. Agrin regulates growth cone turning of Xenopus spinal motoneurons. *Development*, **2005**, *132*(19), 4309-16.

[30] Brown, L.F.; Lanir, N.; Mcdonagh, J.; Tognazzi, K.; Dvorak, A.M.; Dvorak, H.F. Fibroblast migration in fibrin gel matrices. *Am. J. Pathol.*, **1993**, *142*(1), 273-83.

[31] Ciano, P.S.; Colvin, R.B.; Dvorak, A.M.; Mcdonagh, J.; Dvorak, H.F. Macrophage migration in fibrin gel matrices. *Lab. Invest.*, **1986**, *54*(1), 62-70.

[32] Dvorak, H.F.; Harvey, V.S.; Estrella, P.; Brown, L.F.; Mcdonagh, J.; Dvorak, A.M. Fibrin containing gels induce angiogenesis. Implications for tumor stroma generation and wound healing. *Lab. Invest.*, **1987**, *57*(6), 673-86.

[33] Islam, L.N.; Mckay, I.C.; Wilkinson, P.C. The use of collagen or fibrin gels for the assay of human neutrophil chemotaxis. *J. Immunol. Methods*, **1985**, *85*(1), 137-51.

[34] Tessier-Lavigne, M.; Placzek, M.; Lumsden, A.G.; Dodd, J.; Jessell, T.M. Chemotropic guidance of developing axons in the mammalian central nervous system. *Nature*, **1988**, *336*(6201), 775-8.

[35] Richards, L.J.; Koester, S.E.; Tuttle, R.; O'leary, D.D. Directed growth of early cortical axons is influenced by a chemoattractant released from an intermediate target. *J. Neurosci.*, **1997**, *17*(7), 2445-58.

[36] Letourneau, P.C. Chemotactic response of nerve fiber elongation to nerve growth factor. *Dev. Biol.*, **1978**, *66*(1), 183-96.

[37] Rosoff, W.J.; Urbach, J.S.; Esrick, M.A.; Mcallister, R.G.; Richards, L.J.; Goodhill, G.J. A new chemotaxis assay shows the extreme sensitivity of axons to molecular gradients. *Nat. Neurosci.*, **2004**, *7*(6), 678-82.

[38] Rosoff, W.J.; Mcallister, R.; Esrick, M.A.; Goodhill, G.J.; Urbach, J.S. Generating controlled molecular gradients in 3D gels. *Biotechnol. Bioeng.*, **2005**, *91*(6), 754-9.

[39] Goodhill, G.J. Diffusion in axon guidance. *Eur. J. Neurosci.*, **1997**, *9*(7), 1414-21.

[40] Wilkinson, P.C.; Lackie, J.M. The influence of contact guidance on chemotaxis of human neutrophil leukocytes. *Exp. Cell Res.*, **1983**, *145*(2), 255-64.

[41] Hapca, S.; Crawford, J.W.; Macmillan, K.; Wilson, M.J.; Young, I.M. Modelling nematode movement using time-fractional dynamics. *J. Theor. Biol.*, **2007**, *248*(1), 212-24.

[42] Nelson, R.D.; Quie, P.G.; Simmons, R.L. Chemotaxis under agarose: a new and simple method for measuring chemotaxis and spontaneous migration of human polymorphonuclear leukocytes and monocytes. *J. Immunol.*, **1975**, *115*(6), 1650-6.

[43] Cao, X.; Shoichet, M.S. Investigating the synergistic effect of combined neurotrophic factor concentration gradients to guide axonal growth. *Neuroscience*, **2003**, *122*(2), 381-9.

[44] Moghe, P.V.; Nelson, R.D.; Tranquillo, R.T. Cytokine-stimulated chemotaxis of human neutrophils in a 3-D conjoined fibrin gel assay. *J. Immunol. Methods*, **1995**, *180*(2), 193-211.

[45] Knapp, D.M.; Helou, E.F.; Tranquillo, R.T. A fibrin or collagen gel assay for tissue cell chemotaxis: assessment of fibroblast chemotaxis to GRGDSP. *Exp. Cell Res.*, **1999**, *247*(2), 543-53.

[46] Puschel, A.W.; Adams, R.H.; Betz, H. Murine semaphorin D/collapsin is a member of a diverse gene family and creates domains inhibitory for axonal extension. *Neuron*, **1995**, *14*(5), 941-8.

[47] Lumsden, A.G.; Davies, A.M. Earliest sensory nerve fibres are guided to peripheral targets by attractants other than nerve growth factor. *Nature*, **1983**, *306*(5945), 786-8.

[48] Nasarre, P.; Kusy, S.; Constantin, B.; Castellani, V.; Drabkin, H.A.; Bagnard, D.; Roche, J. Semaphorin SEMA3F has a repulsing activity on breast cancer cells and inhibits E-cadherin-mediated cell adhesion. *Neoplasia*, **2005**, *7*(2), 180-9.

[49] Messersmith, E.K.; Leonardo, E.D.; Shatz, C.J.; Tessier-Lavigne, M.; Goodman, C.S.; Kolodkin, A.L. Semaphorin III can function as a selective chemorepellent to pattern sensory projections in the spinal cord. *Neuron*, **1995**, *14*(5), 949-59.

[50] Kuang, R.Z.; Merline, M.; Kalil, K. Topographic specificity of corticospinal connections formed in explant coculture. *Development*, **1994**, *120*(7), 1937-47.

[51] Moore, S.W.; Kennedy, T.E. Protein kinase A regulates the sensitivity of spinal commissural axon turning to netrin-1 but does not switch between chemoattraction and chemorepulsion. *J. Neurosci.*, **2006**, *26*(9), 2419-23.

[52] Walter, J.; Kern-Veits, B.; Huf, J.; Stolze, B.; Bonhoeffer, F. Recognition of position-specific properties of tectal cell membranes by retinal axons in vitro. *Development*, **1987**, *101*(4), 685-96.

[53] Mclaughlin, T.; O'leary, D.D. Molecular gradients and development of retinotopic maps. *Annu. Rev. Neurosci.*, **2005**, *28*, 327-55.

[54] Knoll, B.; Weinl, C.; Nordheim, A.; Bonhoeffer, F. Stripe assay to examine axonal guidance and cell migration. *Nat. Protoc.*, **2007**, *2*(5), 1216-24.

[55] Baier, H.; Bonhoeffer, F. Axon guidance by gradients of a target-derived component. *Science*, **1992**, *255*(5043), 472-5.

[56] Rosentreter, S.M.; Davenport, R.W.; Loschinger, J.; Huf, J.; Jung, J.; Bonhoeffer, F. Response of retinal ganglion cell axons to striped linear gradients of repellent guidance molecules. *J. Neurobiol.*, **1998**, *37*(4), 541-62.

[57] Dertinger, S.K.; Jiang, X.; Li, Z.; Murthy, V.N.; Whitesides, G.M. Gradients of substrate-bound laminin orient axonal specification of neurons. *Proc. Natl. Acad. Sci. U. S. A.*, **2002**, *99*(20), 12542-7.

[58] Lang, S.; Von Philipsborn, A.C.; Bernard, A.; Bonhoeffer, F.; Bastmeyer, M. Growth cone response to ephrin gradients produced by microfluidic networks. *Anal. Bioanal. Chem.*, **2007**, *390*(3), 809-16.

[59] Von Philipsborn, A.C.; Lang, S.; Loeschinger, J.; Bernard, A.; David, C.; Lehnert, D.; Bonhoeffer, F.; Bastmeyer, M. Growth cone navigation in substrate-bound ephrin gradients. *Development*, **2006**, *133*(13), 2487-95.

[60] Whitesides, G.M. The origins and the future of microfluidics. *Nature*, **2006**, *442*(7101), 368-73.

[61] Li Jeon, N.; Baskaran, H.; Dertinger, S.K.; Whitesides, G.M.; Van De Water, L.; Toner, M. Neutrophil chemotaxis in linear and complex gradients of interleukin-8 formed in a microfabricated device. *Nat. Biotechnol.*, **2002**, *20*(8), 826-30.

[62] Lin, F.; Nguyen, C.M.; Wang, S.J.; Saadi, W.; Gross, S.P.; Jeon, N.L. Neutrophil migration in opposing chemoattractant gradients using microfluidic chemotaxis devices. *Ann. Biomed. Eng.*, **2005**, *33*(4), 475-82.

[63] Song, L.; Nadkarni, S.M.; Bodeker, H.U.; Beta, C.; Bae, A.; Franck, C.; Rappel, W.J.; Loomis, W.F.; Bodenschatz, E. Dictyostelium discoideum chemotaxis: threshold for directed motion. *Eur. J. Cell Biol.*, **2006**, *85*(9-10), 981-9.

[64] Saadi, W.; Wang, S.J.; Lin, F.; Jeon, N.L. A parallel-gradient microfluidic chamber for quantitative analysis of breast cancer cell chemotaxis. *Biomed. Microdevices*, **2006**, *8*(2), 109-18.

[65] Lin, F.; Saadi, W.; Rhee, S.W.; Wang, S.J.; Mittal, S.; Jeon, N.L. Generation of dynamic temporal and spatial concentration gradients using microfluidic devices. *Lab Chip*, **2004**, *4*(3), 164-7.

[66] Irimia, D.; Liu, S.Y.; Tharp, W.G.; Samadani, A.; Toner, M.; Poznansky, M.C. Microfluidic system for measuring neutrophil migratory responses to fast switches of chemical gradients. *Lab Chip*, **2006**, *6*(2), 191-8.

[67] Hsu, C.-H.; Folch, A. Spatio-temporally-complex concentration profiles using a tunable chaotic micromixer. *Appl. Phys. Lett.*, **2006**, *89*, 144102.

[68] Walker, G.M.; Sai, J.; Richmond, A.; Stremler, M.; Chung, C.Y.; Wikswo, J.P. Effects of flow and diffusion on chemotaxis studies in a microfabricated gradient generator. *Lab Chip*, **2005**, *5*(6), 611-8.

[69] Abhyankar, V.V.; Lokuta, M.A.; Huttenlocher, A.; Beebe, D.J. Characterization of a membrane-based gradient generator for use in cell-signaling studies. *Lab Chip*, **2006**, *6*(3), 389-93.

[70] Saadi, W.; Rhee, S.W.; Lin, F.; Vahidi, B.; Chung, B.G.; Jeon, N.L. Generation of stable concentration gradients in 2D and 3D environments using a microfluidic ladder chamber. *Biomed. Microdevices*, **2007**, *9*(5), 627-35.

[71] Chung, B.G.; Lin, F.; Jeon, N.L. A microfluidic multi-injector for gradient generation. *Lab Chip*, **2006**, *6*(6), 764-8.

[72] Beebe, D.J.; Mensing, G.A.; Walker, G.M. Physics and applications of microfluidics in biology. *Annu. Rev. Biomed. Eng.*, **2002**, *4*, 261-86.

[73] Whitesides, G.M.; Ostuni, E.; Takayama, S.; Jiang, X.; Ingber, D.E. Soft lithography in biology and biochemistry. *Annu. Rev. Biomed. Eng.*, **2001**, *3*, 335-73.

[74] Crank, J. *The Mathematics of Diffusion*, Oxford University Press: Oxford, **1975**.

Received: October 7, 2007 **Revised: December 20, 2007** **Accepted: January 7, 2008**