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Determining whether observed eukaryotic cell migration indicates chemotactic responsiveness or random chemokinetic motion

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Abstract

Chemotaxis, the motion of cells directed by a gradient of chemoattractant molecules, guides cells in immune response, development, wound healing, and cancer. Unfortunately, this process is difficult to distinguish from chemokinesis, i.e., stimulated random cell motion. Chemotaxis is frequently inferred by determining how many cells cross a boundary in a chemotaxis assay, for example how many cells crawl into a chemoattractant-infused filter, or how many cells enter a defined region in an under-agarose assay or agarose spot assay. To mitigate possible ambiguity in whether motion observed in these assays is directed by the chemoattractant gradient or by chemokinesis, we developed a mathematical model to determine when such methods indeed indicate directed motion of cells. In contrast to previous analyses of chemotaxis assays, we report not just the gradients that arise in the assays but also resulting cell motion. We applied the model to data obtained from rigorous measurements and show, as examples, that MDA-MB-231 breast-cancer cells are at least 20 times less sensitive to gradients of EGF or CXCL12 than neutrophils are to formyl peptides; we then used this information to determine the extent to which gradient sensing increases the rate of boundary crossing relative to a random-motility control. Results show, for example, that in the filter assay, 2–4 times as many neutrophils pass through the filter when exposed to a gradient as when the gradient is absent. However, in the other combinations of cells and assays we considered, only 10–20% more cells are counted as having migrated in a directed, rather than random, motility condition. We also discuss the design of appropriate controls for these assays, which is difficult for the under-agarose and agarose spot assays. Moreover, although straightforward to perform with the filter assay, reliable controls are often not done. Consequently, we infer that chemotaxis is frequently over-reported, especially for cells like MDA-MB-231 cells, which move slowly and are relatively insensitive to gradients. Such results provide insights into the use of chemotaxis assays, particularly if one wants to acquire and analyze quantitative data.

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Author Contributions

ACS designed the study, developed the model, analyzed data, and wrote the manuscript. RN analyzed data and edited the manuscript.

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Graphical Abstract

Keywords

chemotaxis; migration; model

1. Introduction

Chemotaxis, the motion of cells directed by a gradient of chemoattractant molecules, is involved in development, immune response, and cancer metastasis (Ridley et al., 2003; Balkwill, 2004). The most rigorous measurements of chemotaxis use methods that subject cells to a controlled, constant chemoattractant gradient and analyze chemotaxis based on the migration paths taken by individual cells. Analysis of these migration paths can indicate the extent to which cell motion is directed (chemotactic) or random (chemokinetic). The Zigmond (1977) and Dunn (Zicha et al., 1991) chambers are classic means of subjecting cells to a stable, linear gradient; microfluidic devices can subject cells to stable gradients indefinitely (Wu et al., 2013) and can precisely distinguish directed and random motility (Boneschansker et al., 2014).

Although other assay types, including the filter migration assay (Boyden, 1962), the under-agarose assay (Nelson et al., 1975), and the agarose spot assay (Wiggins and Rappoport, 2010), generate gradients that vary over time, they are widely used due to a combination of their relative simplicity, lower cost, and greater amenability to multiplexing. Schematics of these chemotaxis assays are shown in Figure 1. In the filter migration assay (Fig. 1a), chemoattractant is pipetted into a lower well separated by a filter from another well into which cells are added. The cells settle on the filter. Diffusion of chemoattractant up through the filter forms a gradient. Cells crawl through the filter toward the chemoattractant source. (A single well was used initially (Boyden, 1962), but multi-well formats are also in use (Falk et al., 1980); we refer to both types as the filter assay.) In the under-agarose assay (Fig. 1B), a disk of soft agarose is formed in a cell culture dish. Two cylindrical wells are punched out of the gel. Chemoattractant is pipetted into one well, and cells are pipetted into the other. Chemoattractant diffuses from the source well, forming a gradient. In the agarose spot assay (Fig. 1C), a spot of agarose containing chemoattractant is pipetted onto a cell culture dish. Cells are then added to the dish; the gradient forms by diffusion of chemoattractant out of the spot (Wiggins and Rappoport, 2010).

Distinguishing between chemotactic and chemokinetic motion in these frequently-used assays is non-trivial. The typical readout for all of these assays is not based on the migration paths of individual cells, but on the total number of cells that cross a boundary by the end of an incubation period. In the filter assay, the boundary is the top or bottom of the filter itself, depending on whether the number of cells that crawl into or through the filter is counted. In the under-agarose assay, the boundary is the edge of the cell-containing well facing the chemoattractant well. In the agarose spot assay, the boundary is the interface between the spot and the medium. In general, simply counting the number of cells that cross a boundary cannot provide a definitive indication of directed cell migration without comparison to the number of cells that would cross the boundary when stimulated with chemoattractant at a corresponding uniform concentration.

Here, we calculate the gradients formed in these frequently-used chemotaxis assays, and use information from rigorous measurements of chemotaxis of neutrophils and MDA-MB-231 cells, as examples, to predict the increase in cell motion due to gradient sensing, relative to the level of chemokinetic cell motion. This analysis of cell migration distinguishes our work from previous mathematical investigations of these assays, which do not consider cell motion directly but only report the chemoattractant gradients (Lauffenburger and Zigmond, 1981; Szatmary et al., 2014). We show that MDA-MB-231 cells, although highly invasive, are more than 20 times less sensitive to gradients of EGF than neutrophils are to formyl peptides, indicating that epithelial-derived cancer cells require much steeper gradients than immune cells do to effect chemotaxis. Our analysis yields guidelines for suitable incubation durations and initial chemoattractant concentrations appropriate for observing chemotaxis of neutrophils and similar fast, sensitive cells. We conclude that the under-agarose and agarose spot assays are unsuitable for MDA-MB-231 and similar slow, insensitive cells, as these assays generate gradients that are relatively shallow; also, the gradients dissipate in less time than is needed for observation of directed motility of these cells. Moreover, the filter assay can be used to measure chemotaxis of slow, insensitive cells only if rigorous controls are performed to rule out random motility. Because it is difficult to distinguish directed and random motility, it is likely that chemotaxis is over-reported, especially for slow cells that are relatively insensitive to gradients, such as most well-studied epithelial-derived cancer cells.

2. Methods

2.1. Overview

To determine the extent to which direction sensing contributes to cell motion in chemotaxis assays, we developed a model that predicts cell response based on calculations of time-varying chemoattractant concentrations and gradients (see Fig. 2). Our model calculates the gradients that form in common chemotaxis assays (Sec. 2.2), which lead to biases in cell orientation (Sec. 2.3) and corresponding degrees of cell migration (Sec. 2.4). We calibrated the model using data obtained from direct measurements of chemotaxis in stable, well-defined gradients (Sec. 2.5), and then applied the model to the filter, under-agarose, and agarose spot assays. Throughout, we consider chemotactic motion of neutrophils due to formyl peptides (such as fMLP and fMMM), and of MDA-MB-231 breast cancer cells in

response to EGF (epidermal growth factor). The source code for this model is available as Electronic Supplementary Information.

2.2. Determination of chemoattractant gradients

The gradients developed in the filter, under-agarose, and agarose spot assays depend on the particular materials and geometry used. In all cases we assumed a diffusion coefficient for EGF of $1 \times 10^{-6} \text{cm}^2/\text{s}$ (Miyagi and Maruyama, 2010). We used a diffusion coefficient for fMLP of $4 \times 10^{-6} \text{cm}^2/\text{s}$, having estimated this value from the molecular weight of fMLP (Lauffenburger and Zigmond, 1981). For the filter assay, we modeled diffusion through a $10 \mu\text{m}$ -thick Transwell filter having 5% of its surface covered by $8 \mu\text{m}$ -diameter pores, which is a typical filter for studying the of invasion of large cells. We treated the filter as separating two 2-mm deep wells, approximating the height of the $30 \mu\text{l}$ wells in Neuroprobe plates. We modeled the under-agarose assay for 0.5% agarose with 2 mm diameter wells separated by 2 mm of gel. The agarose spot was treated as a $10 \mu\text{l}$ hemisphere of 0.5% agarose.

To determine the gradients, we solved the diffusion equation using numerical methods. We previously reported chemoattractant gradients that develop in the agarose spot assay (Szatmary et al., 2014), which were obtained by solving the diffusion equation in spherical coordinates. Here, gradients in the under-agarose and filter assays were similarly determined by solving the diffusion equation, but in cylindrical and linear coordinates, respectively. Chemoattractant concentration profiles were calculated by solving the diffusion equation with the finite difference method using Adams predictor-corrector methods for timestepping; this was implemented with the `odeint` function from SciPy (Oliphant, 2007), which is an interface for the LSODE solver from ODEPACK (Hindmarsh, 1983). We validated these results by comparing them with the findings of Lauffenburger and Zigmond (Lauffenburger and Zigmond, 1981), who mathematically determined the chemoattractant gradients in the under-agarose and filter assays. The mathematically-determined gradient in the under-agarose assay was validated experimentally by Uden et al. (Uden et al., 1986).

In their analysis of the filter assay, Lauffenburger and Zigmond neglected hindrance of chemoattractant diffusion by the filter. This may be an appropriate assumption for assays employing nitrocellulose fiber filters, which were commonly used at the time, but typical filters used now, such as Transwell and Nuclepore, are thinner and have a low porosity. This low porosity hinders diffusion by limiting the cross-sectional area through which chemoattractant molecules can diffuse; to model diffusion through a Transwell filter with 5% of its surface permeated by holes, we treated the filter as a $10 \mu\text{m}$ -thick zone in which the cross-sectional area through which diffusive transport occurs is 5% of that outside the filter.

In Sec. 2.5, we describe how we calibrated our model with data from previous studies that used stable chemoattractant gradients (Zigmond, 1977; Lauffenburger and Zigmond, 1981; Wang et al., 2004; Kim et al., 2013). In those studies, the gradients were characterized using calculations and were validated by imaging distributions of fluorescent dyes.

2.3. Prediction of cell orientation distributions from gradient conditions

Previous mathematical analyses of these assays simply report the chemoattractant concentration and gradient slopes (Lauffenburger and Zigmond, 1981; Szatmary et al., 2014). However, predicting the extent of directed cell migration that is induced in these assays requires considering how chemoattractant concentrations and gradients affect cell motion. Direction sensing affects the rate of cell migration across a boundary by biasing the motion of cells toward the direction of increasing concentration. Based on current evidence, the change in receptor occupancy across the length of the cell is the best predictor of cell orientation bias (see below). The fractional receptor occupancy, FRO, at a point on the cell surface is,

$$\text{FRO} = \frac{c}{c + K_d}, \quad (1)$$

where c is the chemoattractant concentration on the surface and K_d is the dissociation coefficient for the chemoattractant-receptor interaction, that is, K_d is the concentration at which half of the receptors would be bound. The difference in fractional receptor occupancy, DFRO, across the length of the cell, is obtained by taking the derivative of FRO with respect to x (the direction in which concentration varies), and scaling by the length, ℓ of the cell,

$$\text{DFRO} = \frac{\ell}{K_d} \frac{dc}{dx} \frac{1}{(c/K_d + 1)^2}. \quad (2)$$

This is approximately equal to the difference in fractional receptor occupancy between the points on the cell located farthest up and down the gradient. A schematic illustrating the meaning of DFRO is shown in Figure 3. DFRO has been shown to be roughly proportional to the chemotactic index or mean cell velocity in the direction of the gradient (Herzmark et al., 2007; Haessler et al., 2011; Kim et al., 2013). DFRO is similar to the “chemotaxis coefficient” used by Tranquillo et al. to interpret and model neutrophil motion (Tranquillo et al., 1988). The difference in number of bound receptors on a cell has been used to predict T-cell and dendritic cell chemotaxis (Wang and Irvine, 2013). Although DFRO was originally posed as a difference in receptor occupancy between extreme points on a cell (Wang et al., 2004), this is an oversimplified interpretation, as cells transduce signals from receptors all over their surfaces, not just extremes. Moreover, the orientation of a cell and the distribution of its receptors affect how reliably the cell perceives the gradient (Nguyen et al., 2014). For these reasons, rather than conceptually representing cell response in terms of FRO at extreme points on a cell, we use DFRO because it is an effective way to account for chemoattractant concentration and gradient slopes in interpreting experimental data.

We assume that the cell direction is based only on the current chemoattractant concentration and gradient slope, that the cells move at a constant speed, and that the cells do not affect each other’s motion. Although making these assumptions neglects some of the complexity

of the problem, because little is known about each of these features, attempting to account for them would not increase the accuracy of the model but would make it more difficult to interpret results.

To model the effect of gradient sensing on cell motion we treat the cells as having orientations that fall on a von Mises–Fisher distribution, a kind of bell curve; stronger gradient signals (i.e., higher DFRO) cause the cell orientations to be more biased. The 2D von Mises–Fisher distribution on a circle is given by,

$$f(\theta; \kappa) = \frac{\exp(\kappa \cos(\theta))}{2\pi I_0(\kappa)}, \quad (3)$$

where θ is the angle of the cell with respect to the chemoattractant gradient, such that $\theta = 0$ if the cell is oriented up the gradient and $\theta = \pi$ if the cell is oriented down the gradient. The function $I_0(\cdot)$ is the modified Bessel function of order 0; κ represents the “bias” in the cell orientation distribution. A more biased distribution has a greater number of cells oriented close to the direction of the gradient. Figure 4 shows angle distributions for different levels of bias. We use $f(\theta; \kappa)$ to represent 2D cell migration. The von Mises distribution previously has been used to characterize cell orientation and motion: it can represent a system in which cell orientation has a random “diffusive” component and a “drift” component due to gradient sensing. It has been used to account for the directions of paths taken by *Dictyostelium* cells (Fisher et al., 1989) and to model *Dictyostelium* pseudopod extension (van Haastert, 2010a,b). Neutrophil orientations also appear to fall on bell curves (Zigmond, 1977).

We shall assume that bias is proportional to the difference in fractional receptor occupancy, that is,

$$\kappa = S \cdot \text{DFRO}, \quad (4)$$

where S is the “sensitivity”. This S parameter depends on the cell type and identity of the chemoattractant.

2.4. Cell orientation distributions describe cell behavior

In this subsection, we develop functions that relate the bias in cell orientations, κ , to experimental observables, namely the normalized flux (the rate at which cells cross a boundary), the chemotactic index, and the fraction of cells that are oriented. We use a function for normalized cell flux to predict cell behavior in chemotaxis assays, and we use the fraction of cells oriented and the chemotactic index to calibrate our model. Figure 5 shows how each of these factors varies with κ , which is affected by cell type, gradient slope, and concentration.

A major readout for the filter, under-agarose, and agarose spot assays is the number of cells that cross a boundary, crawling into or through the filter, or under the gel in the under-agarose and agarose spot assays. The flux of cells, i.e., the number of cells that cross the boundary per unit time, depends on the angle distribution: with a greater fraction of cells oriented up the gradient, more cells would cross the boundary in a given interval of time. Moreover, cells are more likely to cross the boundary if they are pointed directly perpendicular to the boundary rather than at some angle. With cell orientations on an angular distribution, $f(\theta; \kappa)$, the flux of cells in 2D across a boundary defined by a line perpendicular to the gradient direction would be,

$$\Phi(\kappa) = A(c) \int_{-\pi/2}^{\pi/2} f(\theta; \kappa) \cos(\theta) d\theta, \quad (5)$$

where $A(c)$ accounts for factors such as the concentration-dependent speed of the cells and the plating density. This formula is applicable for assays in which cells cross a boundary irreversibly, which is generally the case for the under-agarose and agarose spot assays. The $A(c)$ term cannot be easily determined, but can be accounted for by comparing measurements of directed with random flux: for random motility, the probability distribution is uniform: $f(\theta; 0) = 1/(2\pi)$. The flux of a set of randomly oriented cells is,

$$\Phi_r(\kappa) = A(c) \int_{-\pi/2}^{\pi/2} \frac{1}{2\pi} \cos(\theta) d\theta = \frac{A(c)}{\pi}. \quad (6)$$

We express the effect of gradient sensing on the number of cells that cross a boundary over time as the “normalized flux”,

$$\Phi'(\kappa) = \frac{\Phi(\kappa)}{\Phi_r(\kappa)} = \pi \int_{-\pi/2}^{\pi/2} f(\theta; \kappa) \cos(\theta) d\theta, \quad (7)$$

where Φ and Φ_r follow from Eqs. 5 and 6.

Cells in the filter assay are essentially undergoing 3D migration, with an extra degree of freedom for the cell orientation. As this extra degree of freedom only affects motion parallel to the filter, we do not account for it explicitly. However, this extra degree of freedom affects the probability distribution of θ , the angle with respect to the gradient. Therefore, we represent cell orientations in the filter assay using the von Mises–Fisher distribution on a sphere,

$$f_{3D}(\theta; \kappa) = \frac{\kappa \exp(\kappa \cos(\theta))}{2\pi (\exp(\kappa) - \exp(-\kappa))}. \quad (8)$$

Because $f_{3D}(\theta, 0) = 1/(4\pi)$, the flux due to random motility in 3D is $\Phi_{r3D}(0) = A(c)/4$, and thus the normalized flux is given by,

$$\Phi'_{3D}(\kappa) = 4 \int_0^{\pi/2} f(\theta; \kappa) \cos(\theta) \cdot 2\pi \sin(\theta) d\theta. \quad (9)$$

We use the spherical von Mises distribution only to analyze flux in the filter; the remainder of this subsection uses the 2D von Mises distribution (Eq. 3).

The rest of this subsection concerns development of mathematical formulas for calibrating the model. In 2D, the fraction of cells pointing up the gradient rather than down is given as,

$$\Omega(\kappa) = \int_{-\pi/2}^{\pi/2} f(\theta; \kappa) d\theta, \quad (10)$$

which can be used to infer κ from experimental results, as reported by Zigmond (1977); $\theta = 0$ is the direction in which concentration increases, thus, cells with angles between $-\pi/2$ and $\pi/2$ are pointed at least somewhat in the right direction. The chemotactic index (CI) is defined as the total distance a cell travels up a gradient, normalized by the total length of the path taken by the cell, or, equivalently, the mean velocity of a cell up the gradient normalized by the mean speed of the cell. Typically, the mean CI for all cells in a sample is reported. For a cell moving at a constant speed, CI is equal to the mean of the time-averaged cosine of the angle of the cell relative to the gradient. (If the cell is moving at a time-varying speed, the chemotactic index is equal to a weighted average of the direction cosine.) With cell orientations falling on a von Mises distribution,

$$CI(\kappa) = \int_{-\pi}^{\pi} f(\theta; \kappa) \cos(\theta) d\theta. \quad (11)$$

2.5. Model calibration

We calibrated our model to analyze neutrophil and MDA-MB-231 breast cancer cell response to gradients. Zigmond (1977) subjected neutrophils to defined gradients of the bacterial formyl peptide fMMM, at varying concentrations, and measured the fraction of neutrophils that were oriented up the gradient; the resulting data is shown in Fig. 6. Although fMLP, another bacterial tripeptide, is currently more commonly used than fMMM, we use Zigmond's data because it is unmatched in terms of showing neutrophil response to a range of concentrations and gradient slopes. We assume that the sensitivity of neutrophils to fMMM is the same as for fMLP, and that the differences in response to these two species arise solely due to their distinct dissociation coefficients with FPR1, the main receptor for these molecules.

To determine the chemotactic sensitivity of neutrophils, we calculated DFRO for each concentration and gradient used, treating the neutrophils as having a length of $10\mu\text{m}$ and

assuming that the binding constant for fMMM to its receptor was 10^{-5}M (corresponding to the peak in the fraction of oriented neutrophils). The percent of oriented cells can be related to DFRO by,

$$\% \text{Oriented} = \Omega (S \cdot \text{DFRO}) \times 100\%, \quad (12)$$

where Ω is the fraction of cells oriented with the gradient (Eq. 10) and $S \cdot \text{DFRO}$ is substituted for κ . Different methods based on Eq. 12 yielded somewhat different values for sensitivity. Details of each method follow. For comparison with sensitivities inferred for MDA-MB-231 cells, we conservatively used the lowest value obtained from these calculations, that is, $S = 230$.

The simplest way to determine the sensitivity, S , is to solve Eq. 12 for each DFRO and corresponding fraction of oriented cells. This yielded S values ranging from 230–36,000, but we note that numbers at the high end of the range may be unreliable because they come from data taken at very low concentrations (see Fig. 6). The second method we used was to fit the data to Eq. 12 using the curve fit function in SciPy (Oliphant, 2007). This fit yielded $S = 475$, near the low end of the range of S values obtained from the simple fit.

Zigmond observed neutrophils orienting in response to the gradient over an orders-of-magnitude concentration range, to a greater extent than would be predicted by Eq. 10. Neutrophils can sense formyl peptides via multiple isoforms of the formyl peptide receptor; by binding chemoattractant with different affinities, these receptors could enable gradient sensing over large concentration ranges. Another possible explanation for the difference between the data and the model is that systematic error affected the scoring of cell orientations. To account for this, we fit the data to the equation,

$$\% \text{Oriented} - b = \Omega (S \cdot \text{DFRO}), \quad (13)$$

where b is a correction factor for possible systematic error. This yielded $S = 235$ and $b = 0.11$, which would correspond to the cell orientations given by Zigmond (1977) being over-reported by 11%. In summary, individual data points led to values of S falling within an orders-of-magnitude range with $S = 230$ at the low end; a simple fit yielded $S = 475$, and a more complex fit to account for possible systematic error in the data gave $S = 235$. For the rest of the paper, we use the conservative value of $S = 230$ for neutrophils.

The ideal way to measure cell sensitivity to gradients is to subject cells to stable, well-defined gradients, measuring cell orientation or chemotactic index, and repeating these measurements at a variety of chemoattractant concentrations and gradient slopes. Unfortunately, measurements with this level of rigor, such as Zigmond's (1977), are rare. To analyze epithelial-derived cancer cell migration in assays, we focus on MDA-MB-231 breast cancer cells, which are among the cell lines for which chemotaxis is most-studied. Kim et al. measured MDA-MB-231 migration in gradients of SDF-1 α (Kim et al., 2013). In that study,

the greatest directed response was observed under conditions corresponding to a DFRO of 0.05 leading to cells having a CI of 0.02; by Eq. 11 this gives $\kappa = 0.04$ so, by Eq. 4, the cells had a sensitivity $S = \kappa/\text{DFRO} = 0.8$. (To be precise, the cells had a velocity up the gradient, normalized by the speed of a random motility control, of 0.021 and a speed of 1.05 normalized by the same random motility control. We find the CI by dividing the normalized velocity by the normalized speed.)

MDA-MB-231 cells also have been measured in an EGF gradient resulting in a DFRO greater than 0.1 (Wang et al., 2004); the cell motion exhibited a chemotactic index of almost 0.4 (for which $\kappa = 0.87$, from Eq. 11). The corresponding sensitivity would be $S = 8.7$. For simplicity, we use $S = 10$ for MDA-MB-231 response to chemoattractant gradients. It is possible that these cells are more sensitive to another chemoattractant, but we have not found evidence of this. Even using conservative estimates of neutrophil sensitivity and generous estimates of MDA-MB-231 sensitivity, we estimate neutrophils to have a sensitivity to formyl peptide gradients at least 20 times greater than the sensitivity of MDA-MB-231 cells to gradients of EGF.

3. Results

3.1. Overview

Using numerical methods, we determined the gradient that develops in the filter assay (Sec. 3.2). We compared this with the gradients developed in the under-agarose and agarose spot assays (Sec. 3.3), which were determined previously. In Sec. 3.4 we report modeling results that show how, in the filter assay, our ability to detect directed cell migration depends on the cell type. We do the same for the under-agarose and agarose spot assays (Sec. 3.5).

3.2. Filter assay generates steep gradients inside the filter

The chemoattractant distributions developed in the filter assay are shown in Figure 7; assay and chemoattractant properties are as described in Sec. 2.2. We found that, for a filter with 5% porosity, the gradient inside the filter was consistently 20 times greater than that above the filter. Unlike the smooth gradients typically generated in the under-agarose assay, agarose spot assay, Zigmond chamber, and most microfluidic chambers, this gradient changes abruptly at the top and bottom of the filter.

3.3. Comparison of gradients generated by several methods

Figure 8 shows comparisons of the concentration, gradient, and difference in fractional receptor occupancy (DFRO) for each of the assays considered; these were determined at the relevant boundary, i.e., the point immediately below the top of the filter in the filter assay, the edge of the cell well facing the chemoattractant well in the under-agarose assay, or the interface between the spot and the surrounding medium in the agarose spot assay (See Fig. 1). The methods used to determine gradient evolution, along with the specific assay and chemoattractant properties used, are described in Sec. 2.2. Here, we focus on the temporal response of MDA-MB-231 cells to EGF, but results are qualitatively similar for other chemoattractants and cell types. Concentration rapidly rises to a steady value in the filter assay (Fig. 8a). It rises over time in the under-agarose assay but it falls in the agarose spot

assay. Correspondingly (Fig. 8b), the magnitude of the chemoattractant gradient at the filter surface falls in the filter assay, rises and then gradually falls in the under-agarose assay, and falls steadily in the agarose spot assay. In the filter assay, DFRO decays slowly (Fig. 8c). In the under-agarose assay, the rising gradient leads to a rise in DFRO, but then falls due to rising chemoattractant concentration, which leads to receptor saturation. DFRO falls rapidly and monotonically in the agarose spot assay.

3.4. Gradient sensing in the filter assay

Let us now consider the filter assay. In the absence of a chemoattractant gradient some number of cells would enter and move through the filter randomly. Chemotaxis increases the rate at which cells move up the gradient, relative to a random motility control. If the gradient is too shallow, counting the number of cells that cross a boundary while moving up the gradient is a poor measure of chemotaxis, as this number would not differ much from that resulting merely from random motility. Figure 9 shows the calculated flux, normalized by a random motility control, for MDA-MB-231 breast cancer cells, neutrophils, and cells with intermediate sensitivities to gradients moving into the filter.

The initial chemoattractant concentration below the filter was set to $2K_d$, which led to the concentration in the filter being approximately K_d within a few minutes (see Fig. 7a). (For a given relative gradient, i.e., fractional change in concentration across the length of a single cell, the cell can sense the gradient most reliably at concentrations near K_d (Zigmond, 1977) so an initial concentration of $2K_d$ maximizes directed cell migration.) The slope of the chemoattractant gradient in the filter decreases over time, causing the rate of directed cell migration to likewise decrease. As shown in Figure 7C, the gradient in the filter is much steeper than the gradient immediately above, causing cells that happen to enter pores to robustly sense the gradient. However, after 6–8 h, the gradient becomes so shallow that gradient sensing by MDA-MB-231 cells would not be reliably measurable because, at that point, gradient sensing would only increase cell flux by 10%, which is comparable to the experimental variability of this assay. In general, the best observation duration for measuring chemotaxis is long enough that many cells migrate, but short enough that the gradient is still sufficiently sharp to have a large effect on directed cell motion. Longer incubation durations can be helpful for cells that are more sensitive to gradients than MDA-MB-231 cells are. Selecting an optimal observation duration is less crucial for more sensitive cells such as neutrophils, as direction sensing leads to a robust two- to three-fold increase in the number of cells that migrate through the filter compared to a random motility control if an incubation period of 2 h or less is used.

In the filter assay, by simply varying the concentrations of chemoattractant above and below the filter, one can set up forward, reverse, or no-gradient conditions. (By reverse gradient we mean that the gradient points up through the filter, i.e., that the concentration above the filter is higher than below.) Results from these measurements can be compared, as the concentrations in the filters rapidly reach the equilibrium concentrations.

Robust measurements of chemotaxis often quantify cell motion in terms of a chemotactic index, in short, the ratio of directed to total motion (see discussion around Eq. 11). For the sake of comparing with results obtained using such methods, measurements of the number

of migrated cells in the filter assay under forward and reverse gradient conditions can be used to estimate an equivalent chemotactic index (CI),

$$CI = \frac{3}{4} \frac{n_f - n_r}{n_f + n_r}, \quad (14)$$

where n_f and n_r are the number of migrated cells in forward and reverse gradient conditions, respectively. This relationship was found by using computed fits to develop linear approximations of Eqs. 9 and 11 for low values of κ . Equation 14 is accurate to within 1% for chemotactic indices less than 0.3. However, CI values given by the formula are less accurate for more directed motion; for perfectly directed motion, CI should be 1 but Eq. 14 incorrectly yields 3/4.

It has previously been suggested that the filter assay cannot distinguish chemotaxis from chemokinesis (Kim and Wu, 2011). The checkerboard assay, though, has been used in measurements of MDA-MB-231 chemotaxis in response to EGF (Sun et al., 2005), with about 50% more cells responding in forward gradients than no-gradient conditions. However, analysis discussed earlier in this paper predicts only a 20% increase, which may indicate that the gradient actually present in the filter assay is even stronger or longer-lasting than our calculations predict. Hindrance of diffusion by cells can sharpen gradients (Hjortø et al., 2015), as can cellular uptake of chemoattractant (Scherber et al., 2012). Repeating the assay using different numbers of cells can indicate whether the presence of the cells is a factor: a varying degree of chemotaxis would indicate that the cells affect the concentration or spatial distribution of the chemoattractant.

Previous analysis assumed that the filter had a negligible effect on the diffusion of the chemoattractant (Lauffenburger and Zigmond, 1981). This assumption is no longer valid as filters now typically have pores occupying only 5–10% of their surface. Filters with low porosity hinder diffusion, leading to a long gradient duration. Moreover, Bignold has shown (Bignold, 1987) that filters with low porosity more clearly distinguish chemotaxis from chemokinesis presumably because, at lower plating densities, chemotaxis would be needed to reliably guide cells to pores.

3.5. Gradient sensing in under-agarose and agarose spot assays

Figure 10 shows the flux of cells moving into the area under the gel in the under-agarose and agarose spot assays. In both of these assays, the effect of direction sensing on the measurement is much smaller than in the filter assay. MDA-MB-231 chemotaxis cannot be measured with these assays because the gradients are too weak to cause a sustained increase in cell migration above what would be observed if the chemoattractant was present at a uniform concentration. Direction sensing increases flux of these cells by 20% for only 1 h in the under-agarose assay and for 0.3 h in the agarose spot assay; for longer periods (e.g., overnight incubation) cell motion would be random, as the gradient would have fallen to be shallower than cells could sense. In contrast, neutrophil chemotaxis can be induced in these assays because direction sensing by the cells increases cell flux by at least 20% for assay

durations of about 2 h for the under-agarose assay and 0.5 h for the agarose spot assay; even so, distinguishing chemotaxis and chemokinesis requires observing cell orientations or tracks.

Cells can become motile upon sensing chemoattractant even in the absence of a gradient so thorough measurements require matching observations in forward gradient conditions, at the same time-varying concentration but with no gradient or with the gradient direction reversed. Generally, in the under-agarose assay more cells crawl out of the well up the gradient than down; likewise, in the agarose spot assay, more cells typically crawl under the chemoattractant spots than under the control spots. In these assays, the “chemotaxis” and “control” conditions differ not just in gradient, but in concentration: in the under-agarose assay, the concentration of chemoattractant varies by an order of magnitude across the cell-containing well (Lauffenburger and Zigmond, 1981; Uden et al., 1986), and, in the agarose spot assay, the concentration near the chemoattractant spots is orders of magnitude higher than near the control spots (Szatmary et al., 2014). Differences in migration can arise due to differences in concentration so confirming that cells are undergoing chemotaxis in these assays requires observing the orientations or tracks of cells.

In these assays, chemotaxis depends on the initial concentration of the chemoattractant: a concentration that is too low causes the magnitude of the gradient to be shallow, but a high concentration may lead to receptor saturation. To obtain the results shown in Figure 10, for each assay and cell type we repeated our calculations for various initial chemoattractant concentrations. We found that using initial concentrations of $40K_d$ in the under-agarose assay and $30K_d$ in the agarose spot assay maximized the duration for which neutrophil flux was at least 20% relative to a random motility control. For MDA-MB-231 cells, initial concentrations of $3K_d$ in the agarose spot assay and $100K_d$ in the under-agarose assay maximized directed motility, but for durations much shorter than is typically required to study these cells.

4. Discussion

4.1. Overview

We used our model to predict when chemotaxis response is robust enough to be measured by counting cells in each of the assays considered. Unlike previous mathematical analyses of these assays, we do not just calculate the chemoattractant gradients, but also predict how much cell migration would be observed. We showed that neutrophils are more than 20 times as sensitive to gradients of formyl peptides than MDA-MB-231 cells are to EGF or CXCL12. To exhibit directed motility, MDA-MB-231 cells (and similar cells) require steep gradients because they are relatively insensitive; also, they require the gradients to be stable over time, as they migrate slowly. Consequently, although directed neutrophil migration can be induced in all of the assays considered, we found that one should not expect these assays to readily demonstrate chemotaxis for MDA-MB-231 and other weakly-responsive cells. For example, in the filter assay, 2–4 times as many neutrophils pass through the filter when exposed to a gradient than when no gradient is present; in contrast, only 10% more MDA-MB-231 cells pass through the filter when subjected to a gradient. Furthermore, in all other combinations of cells and assays that we considered, no more than 10–20% additional cells

are counted as having migrated when a gradient is present than in a random motility control. Hence, distinguishing chemotaxis from chemokinesis requires careful controls. An ideal control in all these assays would subject cells to no gradient, or equal and opposite time-varying gradients, but the same time-varying chemoattractant concentrations. This control should be repeated for each chemotaxis measurement; instead, with the filter assay, this control is typically used just for calibrating a measurement, if it is performed at all. Unlike the filter assay, in the under-agarose and agarose spot assays the gradient direction cannot be reversed while maintaining the same time-varying concentration; for example, in the under-agarose assay, if chemoattractant were added to the cell well instead of the chemoattractant well, the cells would be subjected to completely different concentrations and gradient slopes. As thorough controls are typically not done with the filter assay, and not even possible with the under-agarose and agarose spot assays, it is likely that chemotaxis is over-reported, especially chemotaxis of cells that, like MDA-MB-231 cells, move slowly and are relatively insensitive to gradients. Findings obtained using these assays should be confirmed by subjecting cells to stable gradients, e.g., in Zigmond (1977) or Dunn (Zicha et al., 1991) chambers, or in microfluidic devices (Wu et al., 2013; Boneschansker et al., 2014); cell migration should be characterized by cell orientations or tracks.

4.2. Issues with interpreting assay results

Erroneous interpretation of results obtained from chemotaxis assays is an impediment to identifying the pathways that transduce gradient signals; our guidelines enable one to identify possible examples of this. For example, to determine which signaling molecules MDA-MB-231 cells use to sense EGF gradients, Price et al. (1999) used the filter assay to measure chemotaxis of these cells treated with inhibitors of these signaling molecules. However, they only tested the effects of these agents in forward gradient conditions and therefore did not adequately show that direction sensing in particular was disrupted. It is unlikely that direction sensing alone was affected; the agents inhibited cell movement to a greater extent than our model predicts when only gradient sensing is impaired.

Use of the agarose spot assay may also be hindered by misinterpretation of results. In the first published use of the assay, EGFR tyrosine kinase inhibitors decreased the number of MDA-MB-231 cells that migrated under the spot by 50% (Wiggins and Rappoport, 2010); this difference was attributed to decreased chemotaxis. However, we show that it is implausible for gradient sensing to contribute to more than 10% of the cumulative number of cells that migrate under the spot; thus, in this case, it is likely that the inhibitor was acting primarily by preventing stimulation of random motility, not by impairing direction sensing. A scratch assay performed with and without chemoattractant present could also reveal effects on stimulation, and possibly with less effort than the agarose spot assay requires.

4.3. Model benefits and limitations

In our model, directed motion increases continuously with increases in the gradient magnitude. In contrast, previous models simply note whether the magnitude of the gradient is above a threshold, or neglect the effect of the magnitude altogether. The model by Tranquillo et al. (1988) is most similar to our model, but it treats cell motion as having separate directed and random components; in contrast, in our model, increasing DFRO shifts

cells from behaving purely randomly to being perfectly directed. Another limitation of that model is that its use is restricted to studying the density of large numbers of cells; the way that random motion is represented prevents that method from being used in models of motion of discrete cells. Although our model currently focuses on cell motion past a boundary, we are extending it to determine motion of an ensemble of cells on a surface.

As explained in Sec. 2.5, data suitable for calibrating a model such as this one are scarce, which is why we focus on neutrophil and MDA-MB-231 behavior. Dendritic cells have been observed to be directed in response to 0.4% gradients (Haessler et al., 2011), while Zigmond's data shows neutrophils sensing 1–2% gradients; this suggests that dendritic cells have a higher sensitivity than neutrophils do, and would thus exhibit stronger direction sensing in assays. MTLn3 cells exhibited higher chemotactic indices than MDA-MB-231 cells, but these measurements were made in a time-varying gradient (Raja et al., 2010); we did not model MTLn3 motion because response to time-varying gradients is not suitable for calculating sensitivity, but one could expect them to be more sensitive than MDA-MB-231 cells. Any of these cells could have higher or lower sensitivities to other chemoattractants; likewise, sensitivity of cells to gradients can be affected by drugs and signaling molecules.

We model cell motion as being driven by the difference in fractional receptor occupancy (DFRO) across the cell surface, but actual gradient sensing is more complex than this single factor. For example, measurements of neutrophils in a microfluidic treadmill show that cell response depends on temporal variations in chemoattractant concentration (Aranyosi et al., 2014). However, there is not enough data for various concentrations and gradient slopes to calculate an adjusted neutrophil sensitivity based on temporal variation in concentration. DFRO and mathematically equivalent terms are adequate to relate gradient conditions to motion of neutrophils (Herzmark et al., 2007), dendritic cells (Haessler et al., 2011), and MDA-MB-231 cells (Wang et al., 2004). Because there is not enough information to enable accounting for additional factors, it is appropriate to account for DFRO alone in predicting cell migration in the assays we consider.

4.4. Conclusion

In summary, we have developed a model to predict effects of gradient sensing on cell migration in the filter, under-agarose, and agarose spot assays, and have developed the following guidelines for the use and interpretation of these assays. Chemotaxis of many cell types, from sensitive immune cells to MDA-MB-231 cells and similar slow and insensitive cells, can be measured using the filter assay provided that each measurement of cell migration in a forward gradient is matched with a reverse gradient measurement to distinguish directed and random motility. Cells can sense the gradient best with an initial concentration of $2K_d$ on one side of the filter. The agarose spot and under-agarose assays are not suitable for inducing chemotaxis of slow and insensitive cells such as MDA-MB-231 cells, but these assays are useful for inducing chemotaxis of neutrophils and other cells that are highly sensitive to gradients. Counting the number of migrated cells in these assays is potentially useful for screening for effects of drugs on direction sensing, but findings should be confirmed with methods such as determining the chemotactic index. Direction sensing by neutrophils may be maximized if initial concentrations of approximately $40K_d$ and $30K_d$ are

used in the under-agarose and agarose spot assays, respectively. The filter, under-agarose, and agarose spot assays are frequently used in ways that do not conform to these guidelines; hence, chemotaxis may be systematically over-reported.

Ideally, sensitivity of cells to gradients would be measured by observing motion of individual cells in gradients that are stable over time. More cell types should be characterized with this level of rigor but it is probably impractical to use such methods for the large number of observations needed for drug screening or to trace gradient-sensing transduction pathways; even so, rigorous measurements can be used to design high-throughput experiments using the filter assay. Because of difficulties in distinguishing chemotaxis and chemokinesis, it would be worthwhile to use the filter assay—with thorough controls—in attempts to replicate previous studies of gradient sensing. Also, the model presented here could be improved by incorporating more experimental data as it becomes available. Eventually, complementary models may be developed that can predict cell sensitivity; these sensitivities could then be used in this model. The filter, under-agarose, and agarose spot assay are among the chemotaxis assays with the lowest cost and highest ease of use. An assay that retained these features, that has gradients as durable as in the filter assay, and that is as accessible to imaging as the under-agarose and agarose spot assays, would thus be of great use for the study of chemotaxis.

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Highlights

- Chemotaxis, cell motion directed by a gradient, can be mistaken for random motion
- We calculated gradients in frequently-used assays and modeled resulting cell motion
- We found that observed cell motion is often mostly due to random motion
- Chemotaxis is likely over-reported, especially for epithelial-derived cancer cells

Figure 1.

Schematic of (a) filter assay, (b) under-agarose assay, and (c) agarose spot assay. In the filter assay, a gradient forms by diffusive transport of chemoattractant from the lower to the upper well; cells respond by crawling through pores in the filter. (b) In the under-agarose assay, a gradient forms by diffusion of chemoattractant from a source well; motion of cells from a starting well is observed, with the difference between the number of cells crawling up the gradient (Field 1) and down the gradient (Field 2) taken as evidence of direction sensing. (c) In the agarose spot assay, a gradient forms at the interface between a spot containing chemoattractant and the medium surrounding it; the number of cells that migrate under the spot, compared with the number that migrate under a control spot lacking chemoattractant, is the typical measure of cell migration.

Figure 2. Overview of modeling approach. We predict cell flux (motion) in chemotaxis assays by using the properties of a given assay to calculate the resulting chemoattractant gradient, which leads to a difference in fractional receptor occupancy (DFRO) across a cell. DFRO biases the orientation distribution of a population of cells, which affects cell flux and thus the number of migrated cells in a chemotaxis assay. The effect of DFRO on orientation bias depends on cell sensitivity; we calibrated our model to find this sensitivity for neutrophils and MDA-MB-231 cells. The calibration procedure differs from regular application of the model by its use of observable cell behavior to determine cell sensitivity (see Sec. 2.5).

Figure 3.

Difference in Fractional Receptor Occupancy (DFRO) for a cell in a gradient with the source to the right. The Fractional Receptor Occupancy (FRO) varies across the length of the cell. In this illustrative example, 4 of the 10 receptors in the low-concentration half of the cell are occupied, while 6 of the 10 of the receptors in the high concentration half are occupied. DFRO is a measure of the strength of the gradient signal acting on the cell; by taking the difference in FRO across the cell, DFRO of 0.2 is found. (For simplicity, in this schematic, differences in FRO between regions on a cell are used to find DFRO; the model actually uses Eq. 2, which treats FRO as continuously varying.)

Figure 4.

Bias in cell angle distributions is characterized by κ . Each pane represents a rose plot of cell angle distributions with respect to the gradient direction (indicated by the arrow): $\kappa = 0$ (a), $\kappa = 0.1$ (b), $\kappa = 0.3$ (c), and $\kappa = 0.5$ (d). Experimental data on orientations or trajectories of directed cell motion is sometimes presented in this form. Random orientation corresponds to $\kappa = 0$. Neutrophils are more sensitive to gradients than MDA-MB-231 cells: $\kappa = 0.1$ is typical for MDA-MB-231 cells in a 4% gradient, but $\kappa = 0.5$ is possible for neutrophils in a much shallower 0.6% gradient. In the analysis here, the effect of chemotactic gradient sensing is modeled as a bias in the orientation distribution of motile cells.

Figure 5.

Functions relating the behavior of cell populations to the bias, κ , of the cell orientations. The normalized flux of cells across a boundary in a 2D chemotaxis assay (Eq. 7) is defined to be 100% in a random motility condition ($\kappa = 0$), and can increase by a factor of π as κ increases. The percent of cells that are oriented up the gradient (Eq. 10) is 50% for randomly-oriented cells. The chemotactic index (Eq. 11), the ratio of distance traveled up the gradient to total path length, varies from 0 to 100%.

Figure 6.

Percent of neutrophils oriented up the gradient for varying concentrations of fMMM and different gradient slopes; dashed lines with markers indicate data re-drawn from (Zigmond, 1977), while solid lines are fits to the data obtained by Eq. 12. The measurements were made with tenfold (10x) and threefold (3x) changes in concentration across a 1 mm bridge. While at high concentrations there is good agreement between theory and experiment, at low concentrations the model under-predicts the percent of oriented cells.

Figure 7.

Distribution of EGF developed in the filter assay using a $10\mu\text{m}$ thick, 5% porous filter. (a,b) Concentrations as a function of position, for several time points (a) on 1 min increments in the first 5 min of the experiment and (b) on 3 h increments after the 1 h mark. The initial concentration below the filter (left) was set to $2K_d$ and above the filter (right), 0. After the first hour, the concentration difference across the filter is only 20%. (c) Time evolution of the concentration gradient. The filter hinders diffusion, making the gradient inside the filter 20 times steeper than the gradient immediately above the filter.

Figure 8.

Comparison of (a) concentration, (b) chemoattractant gradient slope, and (c) Difference in Fractional Receptor Occupancy (DFRO) for the under-agarose, agarose spot, and filter assays. Values are calculated for a point below the top of the filter in the filter assay, at the edge of the cell well facing the chemoattractant well in the under-agarose assay, and at the interface between the spot and the medium in the agarose spot assay (See Fig. 1). Results are shown for MDA-MB-231 cells in a gradient of EGF.

Figure 9.

Calculated normalized flux of cells due to chemoattractant gradient sensing, relative to a random motility (i.e., no gradient) control, for cell motion through the filter in the filter assay, calculated using Eq. 7. Results are shown for neutrophils, as well as for MDA-MB-231 cells and cells with intermediate sensitivities, responding to EGF. In all cases, the number of cells migrating through the filter decreases per unit time due to the decay of the chemoattractant gradient. In the case of neutrophils, direction sensing has a large effect on directed migration, with two to three-fold increases in the number of migrated cells over a 1–2 h incubation period, which is sufficiently long to observe migration of these cells. However, MDA-MB-231 cells are much less sensitive to gradients, so the increase in cell motility due to gradient sensing, relative to a random motility control, is only 20% after the first 2 h. Moreover, compared to neutrophils, MDA-MB-231 cells migrate more slowly so longer incubation periods are needed for migration to be observed, during which time the gradient continues to fall. Cells with greater sensitivities have a more robust response to gradients. The cells with sensitivity $S = 230$ are as sensitive to gradients as neutrophils are, but exhibit a greater response because the EGF gradient they respond to decays more slowly than the fMLP gradient that the neutrophils sense.

Figure 10.

Calculated normalized flux of cells (Eq. 7) due to chemoattractant gradient sensing, relative to a random motility control, in (a) the under-agarose assay and (b) the agarose spot assay. Fluxes are shown for neutrophils and MDA-MB-231 cells. Direction sensing increases neutrophil flux by at least 20% for an interval of 2 h in the under-agarose assay, and 0.5 h in the agarose spot assay. MDA-MB-231 cells have a lower level of directed migration than neutrophils at almost all time points for both assays. Even though the normalized flux is high for the first few minutes of the measurement, the actual number of cells that move in that time would be low, making it impractical to measure direction sensing for MDA-MB-231 cells in these assays.