Information processing in eukaryotic chemotaxis

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During the growth phase, Dictyostelium discoideum eukaryotic amoebae live as single cells and hunt their food, bacteria, by moving along the gradients of folic acid (chemotaxis), a by-product of bacterial metabolism. We investigated the range and fidelity of folic acid detection using microfluidic devices with static linear gradients. Chemotactic response was quantified by measuring the amount of information cells acquired about the gradient by observing their response, and comparing it to the amount of information that can be acquired about the gradient, based only on the spatial distribution of cell receptor occupancy. We observed the chemotactic response for gradients as small as 0.2% across the cell body, in the regime where relative difference in receptor occupancy front-to-back on the cell body is about 0.002% with 99% of the receptors occupied on average. For concentrations lower than the dissociation constant, the chemotactic response is found to be completely limited by receptor-ligand binding fluctuations. However, for concentrations higher than the dissociation constant, the fidelity of the response is higher than what is possible based on receptor-ligand binding fluctuations alone, i.e. there is an apparent violation of the data processing inequality. We consider possible mechanisms that could account for the observed sensitivity range and fidelity of information transmission. This failure of application of contemporary information theory also encourages us to search for alternative definitions of information in living matter.

Abbreviations: FA, folic acid; cAMP, cyclic adenosine monophosphate; CI, chemotactic index

Introduction

During the vegetative growth phase, eukaryotic amoebae Dictyostelium discoideum (Dicty), live solitary lives and hunt bacteria using their ability to move along the gradients of chemotactants (chemotaxis); in this case most notably folic acid (FA), a by-product of bacterial metabolism [1, 2]. In their natural environment, amoebae are tasked with locating their food source, bacteria, in order to survive. This task becomes more challenging when one considers that bacteria make colonies with population sizes spanning several orders of magnitude, and consequently, chemotactant gradients and concentrations that can possibly vary by several orders of magnitude. The goal of this work is to systematically investigate the detection range of FA signal by Dicty and analyze it within the framework of Shannon’s information theory [3]. The location of bacteria (transmitter) is encoded into the spatial and temporal concentration distribution of chemotactant(s) in the environment (noisy communication channel) and the amoeba (receiver) has the task of decoding the signal and finding the source. The system is viewed as a noisy communication channel, consisting of two sets of noise sources: a) the external noise, arising from fluctuations in receptor-ligand binding between FA and its cell-membrane receptor, and b) the internal noise, due to chemical reactions within the cytoplasm of the cell (Fig. 1). It has been argued [4, 5] that it is plausible that there is sufficiently noiseless amplification of the signal downstream from receptor binding events (e.g. a single excited receptor can activate multiple G-proteins) and therefore receptor-ligand binding noise alone could account for the major contribution to the overall noise in the chemotacting system.

We examined the response of the population of Dicty amoebae to static linear FA gradients, established in an agarose-gel based microfluidic device [6]. These gradients were achieved by maintaining fixed concentrations of FA on opposite sides of a microfluidic channel. A linear gradient was established by diffusion through the agarose gel in the steady state. Cell migration was then recorded using time-lapse optical microscopy and cell tracks were recorded and analyzed on a computer. Chemotaxis was quantified by multiple measures, most notably the mutual information of the process, in the spirit of previous work on cAMP chemotaxis [7]. The mutual information \( I_{ext}(\theta_{grad}, \theta_{res}) \) between the gradient direction \( \theta_{grad} \) and response direction \( \theta_{res} \), quantifies the amount of knowledge gained about the gradient direction \( \theta_{grad} \), by observing the response of the cell motion \( \theta_{res} \). The probability distribution of the entire population was measured and used to estimate the total information gained i.e. it represents the amount of information acquired with all noise sources included. The information transmission in the external stage between the gradient direction and the direction of the receptor response \( I_{ext}(\theta_{grad}, \theta_{res}) \) represents the information acquired when only one particular noise source is considered – fluctuations in the receptor-ligand binding on the cell surface. This was calculated using the theory derived in [7]. The comparison between these two quantities, total and external mutual information, estimates the extent to which the noise in the receptor-ligand binding contributes to the overall noise or signal degradation in the chemotacting system.

![Fig. 1. The current paradigm for natural mechanism of bacterial search by Dicty: the bacteria (shown as a solid black circle) release folic acid (solid blue circles), which binds to FA receptors on the Dicty membrane, denoted by blue (occupied receptor) or white (unoccupied receptor) circles. These binding events trigger a cascade of intracellular events leading to cell polarization, pseudopod extension and finally cell movement.](image-url)
The external mutual information also provides the upper limit for the amount of information that can be acquired in total $I_{\text{tot}}$, due to the data processing inequality, $I_{\text{tot}} \leq I_{\text{ext}}$.

Furthermore, since the cAMP and FA chemotaxis circuits share almost the same internal G-protein signaling pathway [9], we make comparisons with the data previously obtained from cAMP chemotaxis experiments [7, 10]. In comparing critical parameters, the receptor-ligand binding constant $K_d(\text{FA}) = 150 \text{ nM}$ [11] stands out as a factor of five greater compared to cAMP, $K_d(\text{cAMP}) = 30 \text{ nM}$ [12], whereas the number of receptors on the cell surface is almost the same: 60,000 for FA [11] and 70,000 for cAMP [12]. Therefore, in the view of the aforementioned noise sources, one could argue that since both CAMP and FA signaling pathways share almost identical internal signaling circuit, the internal noise should be comparable in both cases. We note two contrasting results from previous studies with cAMP. First, using flowing microfluidic channels with exponential cAMP gradients, the authors in [7] conclude the existence of a regime where the extracellular noise (fluctuations in receptor-ligand binding) dominates the total noise of the system. Second, the authors in [10] conclude, for the similar range of local cAMP concentrations, but with single-pulse temporal gradients, that the extracellular noise is negligible and that internal noise dominates.

### Results

Experimental results consisted of cell trajectories recorded for 3.3 hours and the distribution of angles $p(\theta_{\text{res}})$ of total displacement vectors was obtained (Fig. 2a). In each experiment the concentration gradient was constant and the FA concentration across the width of a channel was given by $c(x) = c_0 + (\nabla c) x$ (Fig. 5), where $c_0$ is the concentration in the middle of the channel at $x = 0$ and $\nabla c = dc/dx$. Local concentrations varied at most (for highest gradients) by a factor of three within the channel, from the value 0.5$c_0$ at the bottom, to 1.5$c_0$ at the top of the channel. The advantage of this device is that the local concentrations span a much lower range for each experiment and as a result the local concentration is relatively well defined, unlike the span in several orders of magnitude in [13]. Using the recorded data, we calculated the total

![Fig. 2](image_url)

**Fig. 2.** (a) Histogram of angles for the peak response for the gradient $dc/dx = 5.4 \times 10^{-2} [K_d/R]$ and mean concentration $c_0 = 17 K_d$. Each radial step corresponds to 15 data points. (b) Chemotactic index (CI) as a function of the gradient. The error bars denote Standard Error of the Mean. CI for each gradient was obtained by averaging 300 to 700 data points. The control and its spread denote the CI for experiments performed with no gradient with mean FA concentrations of 0 $K_d, 17 K_d$ and 67 $K_d$.

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**Fig. 3.** (a) Comparison of the total $I_{\text{tot}}$ (measured) and external $I_{\text{ext}}$ (calculated) mutual information. The x-axis corresponds to the experiments on the main diagonal of the figure in b. Error bars for $I_{\text{tot}}$ represent Standard Error of the Mean (SEM). The shaded range for $I_{\text{ext}}$ denotes its spread due to the range of local concentrations the cells are exposed to in an experiment. Dotted line denotes the $I_{\text{tot}}$ for control experiments without a gradient. The theoretical maximum for $I_{\text{tot}}$ is equal to $\log_2(2\pi) \approx 2.65$ bits, however, even for much steeper gradients the maximum observed response in [7] for cAMP is 0.5 bits. We note that for $dc/dx \geq 5 \times 10^{-2} K_d/R$ the data processing inequality is strongly violated. (b) $I_{\text{tot}}$ (thick colored bars) and $I_{\text{ext}}$ (contour lines) as a function of mean concentration $c_0$ (units $K_d$) and gradient $dc/dx$ (units $K_d/R$) for all our experimental runs shown with horizontal blue-red bars. Their finite width represents the range of concentrations cells were exposed to in each experiment. Gray shaded area corresponds to combinations of $c_0$ and $dc/dx$ inaccessible to our experiment either due to geometrical constrains of the microfluidic channel, or the low solubility of FA in Development Buffer (max. 0.1 mM). Angled blue-orange lines show $I_{\text{tot}}$ from flowing CAMP experiments of [7] (exponential gradients). Horizontal gray lines correspond to the data for flowing CAMP experiments of [13] (linear gradients) where white colors denotes the range where chemotaxis was detected and dark gray lines where it was not, based on the measurements of component of the velocity in the gradient direction $I_{\text{tot}}$ was not measured). Annotations 1 and 2 show the maximum of the FA response and the response in the most saturated limit ($c_0 = 100 K_d$), respectively.
mutual information (units: bits) and chemotactic index (dimensionless). Chemotactic index (CI) is defined as the total displacement in the direction of the gradient $n$, divided by the total path length: $CI ≡ \frac{\sum_{i=1}^{m} |\vec{r}_i|}{\sum_{i=1}^{m} |\vec{r}_i|}$, where $\vec{r}_i$ is the instantaneous cell displacement during the time step $i$. CI for various gradients in units $[K_d/R]$ (where R is the cell radius, taken as 5 μm) and mean concentrations in units $[K_d]$ is shown in Fig.2b.

Total mutual information ($I_{tot}$) between the gradient direction $\theta_{grad}$ and the direction of the response $\theta_{rec}$ was calculated by segmenting the real interval $0 \leq \theta_{rec} < 2\pi$ into $m$ bins of equal width. The bin size was chosen as $m=14$ in our case for all runs, because the $I_{tot}$ data with that bin size correlated well with the data for CI (compare Fig.2b and Fig.3a) for which no binning was used. Then, the fraction of total displacement angles $n_j$ ending up in the bin $\theta_{rec,j} \leq \theta < \theta_{rec,j+1}$ was counted and $I_{tot}$ was computed in [14]:

$$I_{tot}(\theta_{grad}, \theta_{rec}) = \sum_{j=1}^{m} n_j \log n_j + \log m [1]$$

with the error due to finite number of data points estimated as $(m-1)/2N$ [15], where $N$ is the total number of data points used for each calculation (between 300 and 700). The maximum value for $I_{tot}$ is $\log_2(2m) = 2.65$ bits, however even in the experiments with much steeper gradients the maximum observed response is 0.5 bits [7]. Experimental values for total mutual information for different gradients were compared to the theory for the external mutual information, which quantifies the amount of information gained about the gradient direction, based solely on the (theoretical) knowledge of receptor occupancy distribution. For shallow linear gradients, the external mutual information is estimated in [7] as:

$$I_{ext}(\theta_{grad}, \theta_{rec}; x) = \frac{N}{4 \ln 2c(x)} \left( \frac{\nabla c}{1 + c(x)} \right)^2 [2]$$

where $c(x)$ is the concentration measured in units of $K_d$, $\nabla c$ is the gradient measured in units of $K_d/R$ and R is the cell radius (taken to be 5 μm). The equation is applicable only for shallow linear gradients, where dimensionless small parameter $\epsilon \equiv R \nabla c/(K_d + c_0) \ll 1$, where $\nabla c$ is a gradient in units [nM/μm] and $c_0$ is a concentration in units μM; otherwise one has to resort to numerical simulations. The design of our microfluidic device ensured it was applicable to use the analytical expression for $I_{ext}$ (Eq.2), as in all our experiments this small parameter was in range: $0.0003 \leq \epsilon \leq 0.0065$. In Fig.3a we compare the total and external mutual information for the same data as in Fig.2b. Fig.3b shows the chemotactic response $I_{tot}$ as a function of both mean FA concentration and a gradient. We performed three experiments with the same gradient $\nabla c = 10^{-1} K_d/R$ and for mean concentrations $c_0 = 3.3$, 100 and 333 $K_d$. The signal was found to diminish with increasing concentration. Finally, we performed four additional experiments: two for the mean concentration $c_0 = 33.3 K_d$ and two for $c_0 = 333.3 K_d$ and in both cases decreasing the gradient diminished the signal. Therefore, the signal sensing depends both on the absolute value of mean concentration and the gradient.

**Discussion**

We analyzed and quantified the motion of Dicty in gradients and mean concentrations varying over the range of six orders of magnitude. In contrast to previous work [7, 13] on cAMP chemotaxis and flow channels, we focus on FA signaling system and look for the similarity to their cAMP response. Dicty showed a wide range of sensitivity, varying across three orders of magnitude in terms of the FA gradient and mean concentration. The most surprising immediate result is that the signal is observed for gradients as low as 0.2% across the cell body, with the concentration as high as 15 μM (100 $K_d$) and a gradient of 3.2 nM/μm (see Annotation 2 in Fig.3b). With receptor-ligand dissociation constant $K_d = 150$ nM, this is indicative of a highly saturated receptor regime. Assuming a steady state of the receptor-ligand binding interaction, the fractional receptor occupancy can be computed as $n = c/(c + K_d)$ and a relative difference in the fraction of occupied receptors front-to-back on the cell body is then computed to be only 0.002%, or on the order of 1-10 receptors difference front-to-back on the cell body while there are about 34,600 receptors (or 99%) occupied on each side of the cell. This contrasts with the regime where cAMP chemotaxis is observed, for concentrations of the chemotactant slightly below $K_d$ for cAMP (Fig.3b). In addition, analyzing our results using mutual information we find an apparent violation of the data processing inequality: the observed response is better than what it ought to be, by considering the fluctuations in receptor-ligand binding as the only source of noise in signal transmission. Assuming the theory for mutual information applied here is appropriate, we conclude that the cells are somehow getting extra information about the gradient and discuss different possibilities of how this could have occurred.

**Detection with an effectively lower $K_d$.** One attempt to explain the violation of the data processing inequality is to take into account the degradation of FA by means of FA deaminase, a FA degrading enzyme, which is present both on the cell membrane and secreted into the extracellular environment. We calculate the extent to which the FA concentration can be reduced by this process. Following up on the previous study of the level of deaminase secretion under same conditions [16], we estimated the deaminase activity (defined as the amount of FA degraded per cell per unit time) for our system. The reported mean value for the deaminase activity from [16] is 35 pmol per 10^6 cells per minute. Assuming a steady-state flat concentration profile of deaminase in our experiment of total volume of 0.15 ml, about 50 cells in total and about 5 hours the cells spent in the chamber (corresponding to the middle of our run), the amount of FA that could possibly be degraded by that time is $5.25 \times 10^{-12}$ mol. On the other hand, the total amount of FA in this entire volume, at 2.5 μM mean concentration is $3.75 \times 10^{-10}$ mol, so the degradation by FA deaminase could account for less than 0.1% of the expected amount of FA. This calculation is summarized in the Table 1. This conclusion was verified experimentally by changing the cell density by a factor of four (from 7 cells/mm^2 to 30 cells/mm^2) for the gradient where we observed peak response and the same result in terms of chemotactic index (0.10 ± 0.02 for lower vs 0.09 ± 0.01 higher density) and total mutual information (0.14 ± 0.02 bits vs 0.14 ± 0.01 bits) was observed. Thus, we conclude that degradation of FA by FA deaminase cannot account for the violation of the data processing inequality.

**Table 1. Summary of the calculation for FA deaminase contribution to the observed results for the case of our best response at 2.5 μM mean concentration.**

<table>
<thead>
<tr>
<th>quantity</th>
<th>value (pmol/cell min)</th>
<th>units</th>
</tr>
</thead>
<tbody>
<tr>
<td>activity</td>
<td>$3.5 \times 10^{-6}$</td>
<td>μmol</td>
</tr>
<tr>
<td>total volume</td>
<td>0.15</td>
<td>ml</td>
</tr>
<tr>
<td>time</td>
<td>300</td>
<td>min</td>
</tr>
<tr>
<td>cell number</td>
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<tr>
<td>FA amount</td>
<td>$3.75 \times 10^{-10}$</td>
<td>mol</td>
</tr>
<tr>
<td>FA amount degraded</td>
<td>$5.25 \times 10^{-13}$</td>
<td>mol</td>
</tr>
<tr>
<td>FA percentage degraded</td>
<td>0.07</td>
<td>%</td>
</tr>
</tbody>
</table>
Detection at a different $K_d$. Previously, it has been suggested that FA receptors can exist in two different states, each with a different affinity for FA [11, 17], based on the curvature of Scatchard plots. First, we consider the possible existence of another state or type of a FA receptor with a different dissociation constant, $K_d = 2.5 \mu M$ and attempt to fit the theory of external mutual information. In this hypothesis, one receptor type/state detects the signal at $c_0 = 150$ nM (1 $K_d$) and the other at $c_0 = 2500$ nM (17 $K_d$), before and after the local minimum on the graphs for CI (Fig.2) and total mutual information (Fig.3). This data fit is shown in Fig.4a.

Second, we note a previous systematic analysis of binding of FA and its derivatives to FA receptors [17], where it was found that a methylated FA has a $K_d = 4500 \pm 500$ nM, close to where the peak of our response was observed. Hypothesizing that a methylated FA is being detected in the higher concentration range, using the same receptor we find the data can be fit reasonably well with the total number of receptors $N=150,000$ (see Fig.4b). However, this would require a (hypothetical) methylation of a micromolar amount of FA on the cell membrane. If either of these hypotheses are correct, for a biologically reasonable total number of cell surface receptors on the order of $10^5$, the conclusion is that the majority of the noise in the entire chemotactic response is contributed by the fluctuations in receptor-ligand binding ($I_{ext} \approx I_{tot}$ as seen in Fig.4). The difficulty with both hypotheses, is that no state or receptor type with sufficiently high $K_d$ has been found and no methylation of FA has been detected in the previous work [17], and especially not of the necessary magnitude.

Further, the possibility of receptor-receptor interactions was also ruled out due to previous findings of uniform receptor distributions for either of the major chemotactic receptors, FA receptors [18] and cAMP receptor cAR1 [19, 20]. Assuming the validity of the simple assumptions behind the steady-state theory of receptor-ligand binding, it remains necessary to explain (i) the large dynamic range of FA sensing, (ii) the unusually high fidelity of the response in large mean concentration range, e.g. $50 \, K_d \leq c_0 \leq 150 \, K_d$; see the annotation 2 in (Fig. 3b). The data processing inequality relies on a Markov chain of three variables $\theta_{grad} \to \theta_{rec} \to \theta_{res}$, where arrows indicate an explicit dependence, $\theta_{rec}(\theta_{grad})$ and $\theta_{res}(\theta_{rec})$. Therefore, the violation of data processing inequality points to the existence of an extra communication channel, e.g. $\theta_{res}(\theta_{rec}, \theta_{grad})$. However, none of the existing alternative binding processes are sufficient to explain the observed fidelity of information transmission, i.e. $I_{tot}$ is still too large. There is also a possibility of a more complicated binding mechanism that is more difficult to quantify, e.g. FA receptors possibly being involved in FA transport into the cell. This could serve as a different communication channel, or if a FA transporter is a separate protein, as a separate communication channel for FA detection. Finally, we note that the definition of information applied here is possibly inapplicable in this form (related to the probability distributions of receptor occupancy $\theta_{rec}$ and cell response $\theta_{res}$) and this encourages us to search for alternative measures of information flow.

Acknowledgments

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Materials and Methods

Cell Growth and Cell Preparation. Wild type axenic strain, AX4 cells (provided by Dicty Stock Center, Northwestern University), were grown in shaker culture suspension at 150 RPM in Formedium H5L with glucose culture medium up to the concentration of about $0.5 - 3 \times 10^8$ cells. Development Buffer (DB; DityBase recipe: 5 mM Na$_2$HPO$_4$, 5 mM KH$_2$PO$_4$, 1 mM CaCl$_2$, 2 mM MgCl$_2$; pH 6.5) was chosen as the medium for FA chemotaxis experiments because it is a well-defined medium and is an approximation of a physiological environment due to its low ionic strength [21]. A negative aspect of using DB is cell’s progression into development after 6+ hours (depending on cell density) and eventual loss of FA chemotactic sensitivity. This was circumvented by performing the experiment before the starvation response occurs. Furthermore, the developmental progression will be induced as well in other media lacking vitamins or amino acids essential for growth. Since it was shown that the H5L medium already contains about 0.12 mg/l of FA [22] (=0.3 $\mu M$), the medium was diluted by factor $\geq 30,000 \times$, lowering the background FA concentration in the medium to at most 0.01 nM. This corresponds to about 1 molecule of FA per volume size of a Dicty cell (100 $\mu m^3$). Depending on the cell concentration, 1-5 ml of cells were taken from the shaken culture and DB was added for a total volume of 10 ml (dilution $\geq 2 \times$). The cells were then centrifuged for 40 seconds at 1000 RPM (200 g force), 9.8 ml of supernatant was removed, and 9.8 ml of DB was added to again have the final volume of 10 ml (dilution 50 $\times$); this was repeated once more (another dilution of 50 $\times$). 9.8 ml of supernatant was removed again and finally, 0.2 ml of 1 $\mu M$-sized colloidal particles (at concentration 10$^8$ particles/ml) in DB and 1-5 ml of DB was added, depending on the starting cell concentration (dilution $\geq 6 \times$). The colloidal particles allowed us to monitor unintended convection. The entire procedure took about 20-30 minutes after which the cells were immediately loaded into the microfluidic device with an already established gradient.

Microfluidics device design. The microfluidic device was designed as an agarose gel containing 3 channels: the static middle channel and two flowing side channels, that represent fixed boundary conditions, were separated by a layer of agarose gel and the gradient was formed by waiting for diffusion of FA to reach a steady state. Reservoirs were connected via Teflon tubing and the steady flow was supplied by a Harvard PHD 2000 syringe pump.
Device Preparation. The 3% agarose gel was formed in the following way. 0.300g of agarose was mixed with 10 ml of DB. The agarose mixture was heated and kept near the boiling point in a microwave oven for 40 seconds total. Agarose was molded by pouring the heated mixture over an inverted PDMS master, which was itself molded from the original Teflon master. After about 2 minutes the agarose solidified, the holes were punched and the chamber was secured between a plexiglas manifold and a glass microscope slide. In this experiment 3% agarose serves as an environment permeable to small molecules, such as water and folic acid, but not permeable to Dicy. Dicy are migrating naturally attached on the glass surface, with 250 μm of static liquid (DB+FA gradient) on top and around them.

Cell recording. At t=0 hours: the gradient formation was started. At t=3 hours: the cells were loaded in the device. Since we noticed that cells were not very mobile when first introduced into the device, we allowed them to adjust to the new environment for about 3.3 hours to establish a good degree of mobility. At t=6.3 hours recording started. At t=9.3 hours: the recording stopped. This time was chosen based on the fact that this is time when one would first observe morphological changes associated with cell-to-cell CAMP signaling during the starvation response (e.g. elongated cells and formation of streams) when the cell density was significantly (10x) higher. Cell motion was recorded using bright field time-lapse optical microscopy, using an Olympus IX71 inverted microscope and a Home Science Tools Mi-DC5000 5.0 Megapixel camera. Snapshots were taken every 30 seconds and cell trajectories were later analyzed on a computer.

Analysis of cell trajectories. We used ImageJ (http://imagej.nih.gov/ij) with ParticleTracker Plugin [24] for automated cell detection and tracking. Particle tracks were analyzed in a custom-made MATLAB code, where the following filtering was applied: the cells that could not be tracked consistently for more than 6 minutes (3% of the total recording time) were discarded and points on the screen that did not move at all were discarded as well; the latter corresponding to dead cells or other artifacts on the glass surface or CCD. Each experimental run was repeated at least 3 times, until 300 to 700 cell trajectories were gathered.

Fig. 5. Numerical 2D time-dependent diffusion simulation for the gradients shown in (Fig. 2 & 3a). (a) The concentration profile intensity of FA at the time before the recording started (~5 hours). The top and bottom box represent two flowing channels with fixed concentrations and the middle box represents the middle static channel with Dickey cells. (b) The concentration of FA at the center of the channel at the same time in units of the mean concentration c0.

The time to reach the steady state was checked by running a 2D diffusion simulation in COMSOL Multiphysics 3.5 (COMSOL, www.comsol.com) and analyzing the gradient in the middle of the channel (Fig. 5). The microfluidic channel containing Dickey cells, also contained 1μm-sized colloidal particles. These were used to monitor the flow rate in the static channel and the measured Peclet number $Lv/D$ (dimensionless number characterizing the ratio of advective versus diffusive effects) was always below 0.3, where $L$ is the channel height (250 μm), $D$ the diffusion constant of folic acid $194 \mu m^2/s$ [23] and $v$ measured average drift velocity of colloidal particles. After loading the cells, the gradient in the middle channel was temporarily lost, however, the time-scale of diffusive refilling of that channel from the bulk of the material above is estimated to about $t \sim L^2/D \approx 5$ min.