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Research paper

Image-based analysis of primary human neutrophil chemotaxis in an automated direct-viewing assay

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ABSTRACT

Multi-well assays based on the Boyden chamber have enabled highly parallel studies of chemotaxis—the directional migration of cells in response to molecular gradients—while direct-viewing approaches have allowed more detailed questions to be asked at low throughput. Boyden-based plates provide a count of cells that pass through a membrane, but no information about cell appearance. In contrast, direct-viewing devices enable the observation of cells during chemotaxis, which allows measurement of many parameters including area, shape, and location. Here we show automated chemotaxis and cell morphology assays in a 96-unit direct-viewing plate. Using only 12 000 primary human neutrophils per datum, we measured dose-dependent stimulation and inhibition of chemotaxis and quantified the effects of inhibitors on cell area and elongation. With 60 parallel conditions we demonstrated 5-fold increase in throughput compared to previously reported direct-viewing approaches.

1. Introduction

While modified Boyden chambers (Boyden, 1962) in multi-well format are the current standard for highly parallel studies of chemotaxis (Frevert et al., 1998; Schepetkin et al., 2007; Vishwanath et al., 2005), direct-viewing approaches, where cells migrate on a horizontal surface, have particular advantages for quantitative studies, such as perturbation of function by drug candidates. The concentration profile experienced by cells in a Boyden chamber is unknown and may be influenced by cells as they traverse membrane pores (Zicha et al., 1991). In contrast, the concentration gradient produced in direct-viewing devices can be verified using fluorescent dyes (Zigmond, 1977; Zicha et al., 1991; Abhyankar et al., 2006). Furthermore, the geometry of modified Boyden chambers is not conducive to microscopic study of cells during chemotaxis, which precludes the use of high content analysis.

Direct-viewing methods for *in vitro* cell migration studies have a long history covering a wide variety of approaches, each with its own set of trade-offs in gradient parameters, optical clarity, ease of use, and cost. Zigmond (Zigmond, 1977) and Dunn (Zicha et al., 1991) chambers improved on the optics of the under agarose assay (Nelson et al., 1975), while all three are more precise than the glass capillaries used by Ketchel and Favour (1955). Microfluidic approaches (Jeon et al., 2002; Wang et al., 2004; Tharp et al., 2006; Heit et al., 2008; Keenan and Folch, 2008; Meyvantsson and Beebe, 2008), in turn, have enabled a new level of spatiotemporal control, including defined gradient profiles of various shapes (Wang et al., 2004; Tharp et al., 2006) and fast switching between profiles (Irimia et al., 2006b). However, none of the direct-viewing approaches reported to date are suitable for highly parallel studies; the largest number of parallel conditions reported to date is twelve (Kanegasaki et al., 2003).

Many microfluidic gradient generators rely on continuous flow to maintain a constant gradient profile over time (Jeon et al., 2002; Irimia et al., 2006a; Mosadegh et al., 2007; Atencia et al., 2009). Since this demands a unique set of input sources

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for each experimental condition, highly parallel studies using continuous flow are intractable. Furthermore, exposing cells to flow can influence experimental results (Walker et al., 2005; Beta et al., 2008). Devices that do not employ flow must provide means to protect the concentration gradient from disturbances; nano-porous membranes (Abhyankar et al., 2006), hydrogels (Diao et al., 2006), valved compartments (Frevert et al., 2006), and fluid-level equilibration (Kanegasaki et al., 2003) are among those that have been used successfully.

The objective of our work was to develop a method that would provide the rich information of direct-viewing chemotaxis studies in an automated format with throughput comparable to Boyden-based plates. To this end a device was developed where the gradient was kept stable by shunting disturbing flows through a low resistance path. This simple design enabled the construction of large arrays of microfluidic units in which cell and reagent addition could be automated via surface tension-driven passive pumping (Walker and Beebe, 2002; Meyvantsson et al., 2008). Automation allowed highly parallel experiments to be run to study dose-dependent stimulation and inhibition of chemotaxis as well as chemoattractant and inhibitor effects on cell morphology.

2. Methods

2.1. Design, fabrication, and general operation

Each microfluidic unit has five components as shown in Fig. 1b. The attractant and cell port are both 2 mm deep; their volumes are approximately 2.85 μl and 8.86 μl, respectively. The gradient channel is 85 μm deep, 1 mm long, and 2 mm wide, but fans out slightly on the source end; the gradient channel volume is around 200 nl. The source and shunt channels are 3.42 μl and 9.38 μl, respectively. The microfluidic channels were injection molded in cyclo-olefin polymer, and sealed with a 0.25 mm thick film of the same material via laser welding. The internal surfaces of the microfluidic channels were made hydrophilic by tissue culture treatment. Unless otherwise stated a 96-tip CyBiWell (CyBio, Jena, Germany) automated liquid handling system was used for dispensing. The plate had reservoirs in between the microfluidic units and at the edges that were filled with phosphate buffered saline (PBS, Thermo Fisher Scientific) to minimize evaporation.

The plate is generally used as follows: Starting with a dry plate, 20 μl of cell culture media (RPMI + 10% FBS) containing inhibitors is dispensed at the attractant port. The media fills the source, gradient, and shunt channels via capillary action (see Fig. 1c-f). A cell suspension is dispensed to the cell port where they settle adjacent to the gradient channel. The plate is incubated for 30 minutes for compound treatment (otherwise 10 minutes to ensure cells have settled). To introduce chemoattractant, 3 μl solution is added to the attractant port and flows into the source channel via surface tension-based passive pumping (Walker and Beebe, 2002). The plate is incubated for 2.5 h and subsequently imaged using an automated inverted microscope. This protocol is often modified for specific applications, such as for morphology analysis as described below.

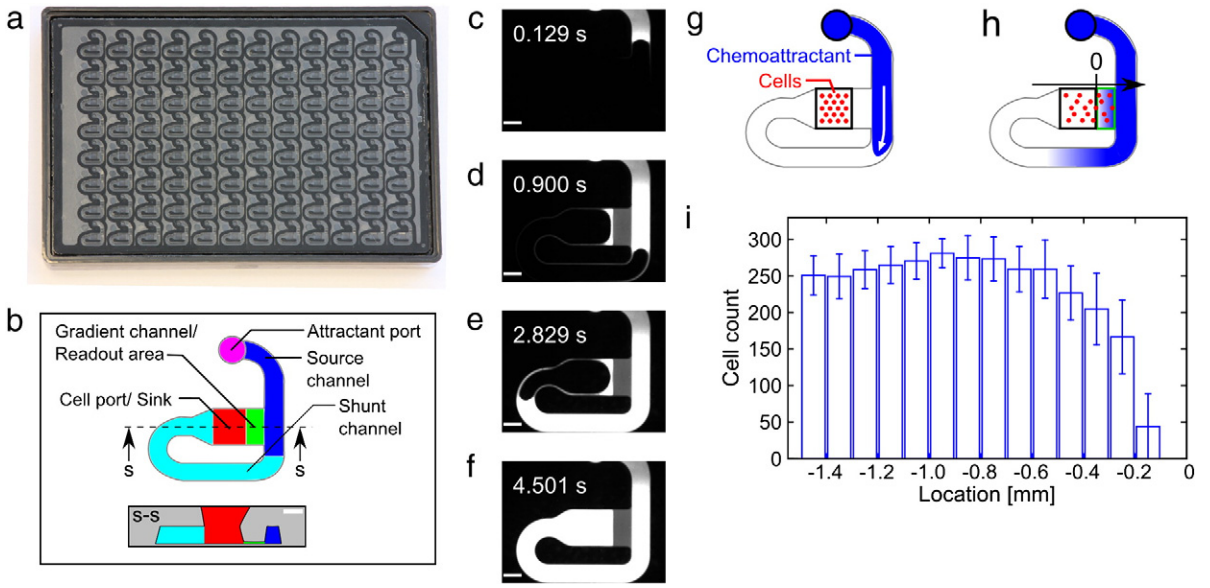

Fig. 1. Direct-viewing plate supports wick-filling and cell patterning. a) The plate (bottom view) has 96 microfluidic units located in a rectangular grid pattern with 9 mm × 9 mm spacing. b) Each unit has five components: an attractant port and source channel where a chemoattractant is provided, a gradient channel connected to the source into which the chemoattractant diffuses to create a gradient, a sink (cell port) located at the other end of the gradient channel, and a shunt channel that helps ensure a stable gradient by diverting flow around the gradient channel. "s-s" shows a cross-section along the dashed line. c–f) A time sequence showing wick-filling. c and d) Since the internal surfaces are hydrophilic, aqueous solutions are drawn into the channels via capillary action. e) Due to the sharp increase in height at the junction between the gradient channel and cell port, the lowest energy path for filling is around the shunt channel. f) Once the shunt channel has been filled, the cell port is filled up from both ends. g and h) A schematic representation of a chemotaxis assay. For morphology assays the cells were seeded in a different way (see text). g) After wick-filling each unit with cell culture media, cells were seeded in the cell port and a chemoattractant solution was loaded in the source channel. h) After a period of time the chemoattractant diffused into the gradient channel and formed a gradient. Chemotactic cells moved into the gradient channel toward increasing concentration. The origin was set to the junction between the cell port and gradient channel. The location of the cells was recorded relative to the origin. i) At the beginning of the assay the cells are distributed in a repeatable pattern in the cell port ($N = 13$; see section 2.2 for locations); bars show mean and standard deviation.