Chapter 6

Isodielectric Separation and Analysis of Cells

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Abstract

Measuring the electrical properties of a cell provides a fast and accessible means of identifying or characterizing cells whose biological state differs from the population as a whole. This chapter describes a microfluidic method for characterizing the electrical properties of cells based upon their convergence to equilibrium in an electrical conductivity gradient. The method, called isodielectric separation, uses the dielectrophoretic force induced on polarizable objects in spatially nonuniform electric fields to deflect cells to the point in the conductivity gradient where their polarization charge vanishes. This equilibrium position encodes the cell's electrical properties and can be used to identify cells that are electrically distinct from a background population, to determine the extent of this difference, and to physically isolate them for further study.

Key words: Electrical separation, Dielectrophoresis, Cell separation, Electrical analysis, Microfluidics

1. Introduction

The intrinsic physical properties of cells have the potential to encode valuable biological information. A wide variety of techniques have been developed to gain access to this information, characterizing and sorting cells according to differences in their size, density, rigidity, impedance, or other physical properties (1–9). The efficacy of these techniques depends on three characteristics of the intrinsic property on which they are based: (1) the property should be easily measurable at a single-cell level; (2) variations in the property should correlate with biologically relevant variations; and (3) it should be possible to identify and isolate cells possessing these variations by some means. One area which has proven to be particularly well suited to satisfying these requirements is electrical methods: techniques designed to characterize and separate cells based upon their intrinsic electrical properties (i.e., their conductivity and permittivity). Developments in microfabrication over the past

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few decades have made the integration of cell-sized electrodes into multifunctional devices routine, and because the electrical properties of a cell depend intimately on the cell's structure and composition, differences in these properties are often biologically relevant. These features have established electric fields as an important means of characterizing, sorting, and manipulating populations of cells down to the single-cell level.

Although electrical methods are tremendously diverse, two widely used categories are those based on measuring the impedance of cells, and those based upon a cell's dielectrophoretic response. While impedance-based methods measure the change in resistance and capacitance between electrodes induced by the presence of one or more cells (10–12), dielectrophoretic methods leverage the force exerted on a polarizable object in the presence of spatially nonuniform electric fields (13). The direction of the dielectrophoretic force depends on the electrical properties of the cell relative to the medium in which it is suspended; a cell with positive net polarizability (e.g., one with higher effective conductivity than its surroundings) will move in the direction of increasing electric field intensity, while a cell with negative net polarizability will move in the opposite direction.

In the characterization and separation of cells, dielectrophoretic methods present an advantage in their ability to use the electrical polarization of cells to both interrogate their properties (specifically, the magnitude and sign of their polarization), as well as to physically move them. This ability to simultaneously interrogate and position cells is one of the more attractive features of dielectrophoretic methods. Unlike methods in which interrogation must be coupled to downstream separation (e.g., impedance cytometry or fluorescence activated cell sorting), dielectrophoresis essentially combines the two steps, leading to potentially faster screens that are further simplified by not requiring cell labeling. We have leveraged these advantages of electrical methods and the ability to position cells in different locations according to differences in their electrical properties by developing a new separation and characterization method called isodielectric separation (IDS) (14). In IDS, cells and particles are dielectrophoretically concentrated to the regions in an electrical conductivity gradient where their polarization charge and the resulting DEP force vanish. Using IDS, we have been able to sort and characterize cells and particles spanning three orders of magnitude in volume and electrical conductivity (15).

Figure 1 illustrates the concept of this method. We create a monotonic gradient in electrical conductivity across the width of a microfluidic channel by injecting one solution of relatively high conductivity containing the cell mixture and a second solution of relatively low conductivity into a device with a diffusive mixer (Fig. 1, left). This mixer generates a smooth monotonic conductivity profile that flows directly into a channel containing electrodes arranged across the diagonal (Fig. 1, right). These electrodes guide the cells

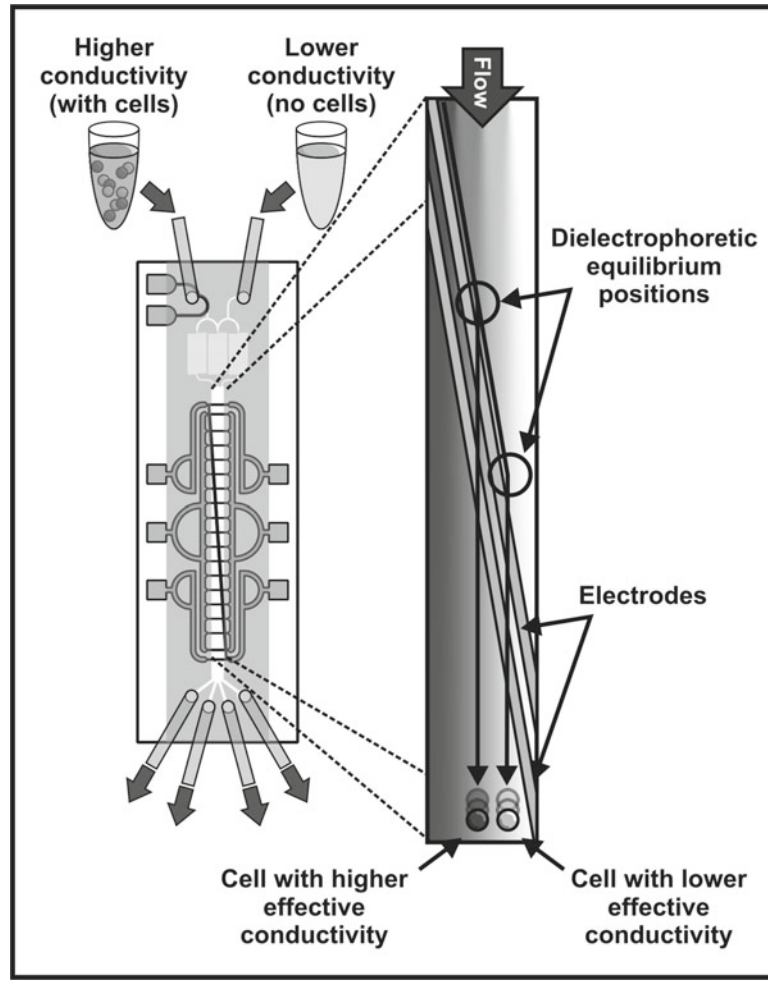

Fig. 1. Analytical separations using IDS. The device is loaded with liquid of higher and lower electrical conductivities, with cells suspended in the higher conductivity. These liquids establish a conductivity gradient across the width of a separation channel, containing electrodes across its diagonal. The cells flow through this channel and are dielectrophoretically deflected by the electrodes across the conductivity gradient until they reach their dielectrophoretic equilibrium positions, where they pass over the electrode barrier. Finally, the cells or particles flow to an observation region, where the cells and particles are imaged to determine their spatial distributions, and to outlets, where fractionated samples may be collected.

in the direction of decreasing medium conductivity—a one-sided approach to equilibrium—until the DEP force becomes sufficiently small that it is overwhelmed by hydrodynamic drag and the barrier is breached. The cells then continue downstream unobstructed for collection. Sampling cells from different portions along the channel width thus segregates cells according to their electrical properties.

Although a wide variety of dielectrophoretic techniques for characterizing and sorting cells have been developed, a common limitation of these approaches is their high sensitivity to the size of a cell. While for some applications this may be desirable, cell size

can vary considerably depending upon factors that one may not be interested in (e.g., cell cycle phase) and thus has the potential to overwhelm more subtle phenotypic differences. Because IDS is an equilibrium separation method, this sensitivity to size is circumvented; cells with the same electrical conductivity and permittivity will approach the same equilibrium position independent of their volumes. For similar reasons, the equilibrium position is also insensitive to density, rigidity, etc. This makes IDS specific only to electrical properties and thus an excellent tool for either electrical cell separation or characterization. This chapter describes the application of IDS to the separation and characterization of populations of the budding yeast *Saccharomyces cerevisiae* according to differences in the electrical properties of their cell envelope.

2. Materials

The composition of the medium (i.e., liquid) used to establish the conductivity gradient is generally application specific, depending both on the types of cell being studied and the parts of these cells one is interested in (e.g., cell envelope, cell membrane, or cell interior). Additionally, the range of the conductivity gradient may be adjusted as appropriate for the range of electrical conductivities represented by the population of cells being studied. The following guidelines are representative of those we have used to sort and characterize populations of the budding yeast *S. cerevisiae* according to differences in the electrical properties of their cell envelope.

2.1. Separation Buffers

1. High-conductivity buffer: starting with 40 ml deionized water (conductivity of ~5.6×10^{-8} S/m at room temperature), add 1.5 ml phosphate-buffered saline (conductivity of ~1.5 S/m at room temperature; Gibco, Carlsbad, CA) and 0.2 ml bovine serum albumin for the higher conductivity separation buffer (conductivity of ~0.065 S/m).
2. Low-conductivity buffer: for the lower conductivity buffer, add 0.2 ml bovine serum albumin to 40 ml deionized water (conductivity of ~0.01 S/m). The intermediate conductivity can be achieved by mixing these solutions 1:1. For mammalian cells, osmotically balanced solutions can be prepared by replacing deionized water with a solution of deionized water containing glucose at a concentration of 300 mM.

2.2. Device Fabrication

1. Clean-room facilities capable of photolithography and metal deposition.
2. Six inch Pyrex wafers and silicon wafers (Bullen, Eaton, OH).

3. SU8 2015 (MicroChem, Newton, MA) and NR7-3000P (Futurrex, Franklin, NJ) photoresists and developers.
4. Poly(dimethylsiloxane) (Dow Corning, Midland, MI).
5. Customized printed circuit board (ExpressPCB).

2.3. Additional Equipment and Instrumentation

1. Three glass luer-lock syringes (1000 series, Hamilton Company, Reno, NV).
2. PEEK tubing (1561), luer adapters (P-618, P-135), ferrules (P-200), and fittings (P-235); (all available from IDEX Health and Sciences, Oak Harbor, WA).
3. Syringe pump (KD Scientific 200, Holliston, MA).
4. Function generator (33220A, Agilent, Palo Alto, CA).
5. Fluorescence microscope with 5× objective and camera.

3. Methods

3.1. Device Fabrication and Assembly

The device consists of two parts: a microfluidic channel that encloses the liquid and the cells, and which is made of PDMS cast onto an SU-8 mold, and a glass substrate containing patterned electrodes. Although processing parameters may vary for different facilities, those listed here provide general guidelines. Consult the MSDS and follow appropriate protocols for the use and disposal for of all chemicals listed here.

Molds for microfluidic channels

1. Dehydration bake the clean silicon wafer(s) on a hot plate at ~200°C for ~30 min. Dispense ~6 ml of SU8-2015 photoresist on the center of the wafer and ramp to 500 rpm at 100 rpm/s, hold for 5–10 s, then ramp to 2,250 rpm at 300 rpm/s and hold for 30 s. This achieves a film thickness of ~20 μm, defining the depth of the microfluidic channel (see Note 1).
2. Prebake the wafer(s) using a slow ramp on a hot plate from 60 to 95°C, hold at 95°C for 2 min; then allow the wafer to cool to room temperature. UV expose the wafer through the flow chamber mask, using a total dosage of ~120 mJ/cm^2 at 365 nm. For the postbake, repeat the parameters of the prebake (see Note 2).
3. Develop the wafers for 3–5 min using PM acetate. To remove any residual developer and to dry the wafer afterwards, we perform a 30 s spin while spraying with PM acetate, a 30 s spin while spraying with isopropanol, and a 30-s spin dry (see Note 3).
4. Before applying PDMS to the wafer to mold devices, the wafer should be silanized; place it in a vacuum chamber along with

three to four drops of HMDS and allow it to set (with vacuum on) for approximately 30 min (see Note 4).

Electrodes

1. Perform a dehydration bake on the clean Pyrex wafer(s) at ~120°C for ~30 min and apply photoresist adhesion promoter (HMDS).
2. Coat the wafer(s) with a 1–2 μm layer of NR7-3000P negative photoresist (Futurrex). For a 6 in. wafer, we allow the resist to spread for 6 s at 750 rpm, followed by a 30-s spin at 2,500 rpm.
3. Prebake wafer(s) on a hot plate at 155°C for 90 s. UV expose the wafer through the electrode mask. The UV dosage should be optimized; we use an exposure energy of ~100 mJ/cm^2 at a wavelength of 365 nm. Bake (on a hotplate) at 120°C for 2 min.
4. Gently agitate the wafer(s) in Resist Developer RD6 (Futurrex) for 25–30 s to develop, then rinse with deionized water and spin dry.
5. Deposit metal layers (100 Å Ti, 2,000 Å Au) on the resist-patterned wafers and then immerse them in acetone to lift off the metal film overlying the photoresist (see Note 5).
6. Dice the wafers to separate the individual dies.

Device assembly

1. Create the flow chamber by casting PDMS (10:1 base to curing agent ratio) on the patterned silicon wafer to a thickness of ~4 mm and allowing it to cure (see Note 6).
2. Remove the cured PDMS and carefully cut out a chamber. Punch holes for each of the inlets and outlets (0.06 in. diameter) so that tubing can be press-fit into the device to load and collect samples (see Note 7).
3. Align the chamber to an electrode chip and bond by exposing the contacting surface to oxygen plasma for ~1 min (see Note 8). Form electrical contacts to the chip, and seal around the bonded PDMS with an insulating epoxy to prevent leaking (see Note 9).

3.2. Sample Preparation and Loading

1. Before preparing or loading the cells, prime the device using deionized water. To do this, connect tubing to one of the inlets while leaving the other inlets and outlets open. Manually driving liquid through the device will cause it to fill and leave droplets at the inlets and outlets. Continue to perfuse the device with water until any internal bubbles have been removed (see Note 10).
2. Wash the sample in the highest conductivity separation buffer, and adjust the cells to the desired concentration (see Note 11). If visual characterization of the cell's equilibrium positions is

desired, they can be fluorescently labeled; this should be done prior to washing and resuspending cells in the high conductivity medium.

3. Load the cell suspension and the lower conductivity separation buffers into separate glass syringes, removing any bubbles from the syringes. Using a luer-lock adapter, attach tubing to the syringes; place the syringes into the pump and activate the pump so that liquid flows through each syringe at a rate of ~5 μl/min. Once the tubing is filled with liquid (indicated by small droplets forming at the ends of the tubing), insert the tubing into the device (see Note 12).
4. Verify (using a microscope) that the sample is entering the device with the high conductivity buffer, and that the fraction of the channel containing the lower conductivities does not contain any cells. If bubbles are present, allow the device to run until they have been removed (see Note 13). Once the sample loading appears to be steady, you are ready to reduce the flow rate and begin the experiment. A total flow rate (i.e., combined flow through all syringes) of 4.5 μl/min (±50%) is appropriate for most applications (see Note 14).
5. Connect the function generator to the leads of the PCB and adjust the settings to appropriate values for your sample. For *S. cerevisiae* in a conductivity gradient spanning 0.01–0.065 S/m, the decade of frequencies from 0.1 to 1 MHz should generally be appropriate (see Note 15).

3.3. Sample Characterization and Collection

1. Visual characterization of a sample consists of recording the position along the channel width where cells localize. For example, if the sample contains two differently labeled populations, the positions at which these samples pass through the electrode barrier gives their effective conductivities relative to each other at the particular electric field frequency being used. Figure 2 illustrates a typical experimental setup for this type of experiment.
2. Spectral characterization is performed by recording shifts in the equilibrium position of cells as the frequency is varied (see Note 16). The general concept behind characterizing labeled populations by varying the operating conditions is illustrated in Fig. 3.
3. In many applications, it is not practical (or possible) to label different populations for imaging, and it is necessary to collect the sample for follow-up studies. The sorted fractions of cells may be collected directly from the device's outlets in a variety of ways, depending upon the volume of the sample and the number of cells that are needed (see Note 17).

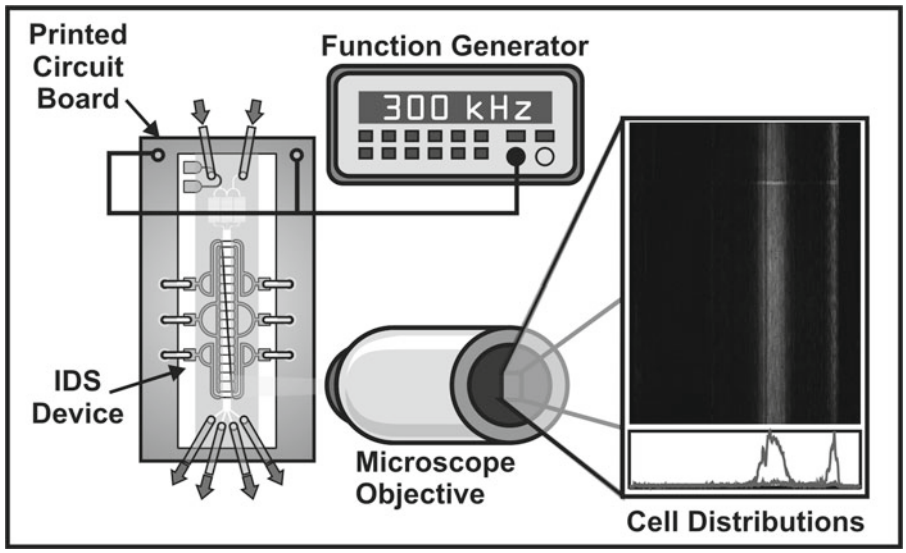

Fig. 2. A schematic layout of the device and accompanying instrumentation.

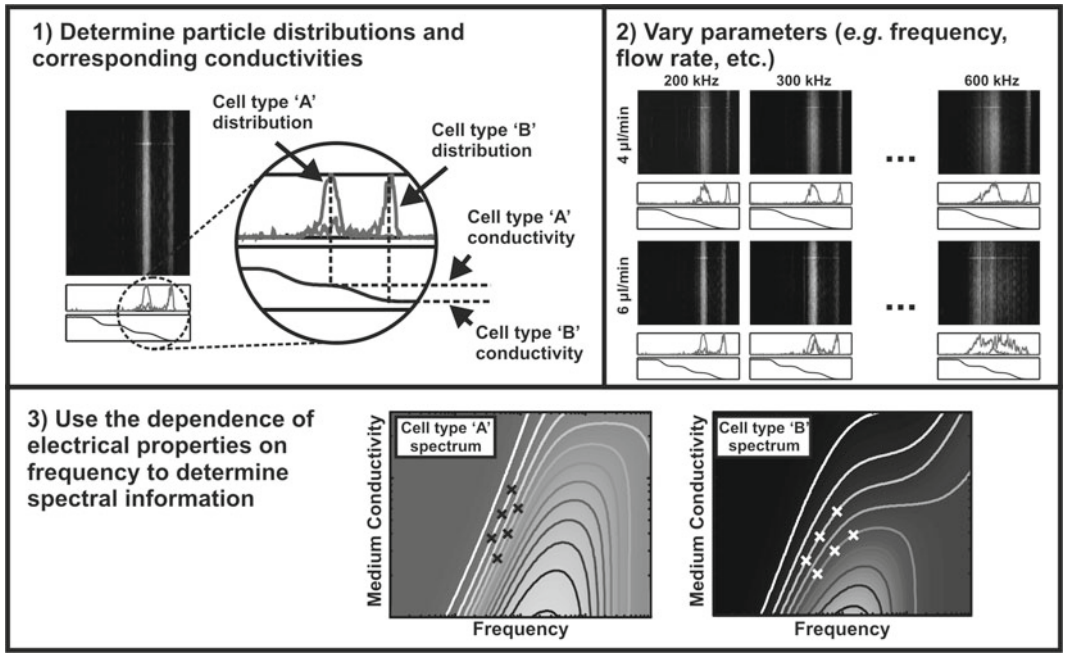

Fig. 3. Typical process for characterizing two populations of cells. Two populations of cells ("A" and "B") are fluorescently labeled and imaged as they pass through the IDS device. The spatial distributions of these cells give the conductivity at which they pass through the dielectrophoretic barrier presented by the electrodes (step 1 above). Varying operating parameters such as frequency, voltage, and flow rate while observing changes in the equilibrium conductivity of the two cell types determine the polarization spectrum of the different cell types (steps 2 and 3 above).

4. Once the samples are collected, they are amenable to characterization by any standard molecular or cell biology technique. For example, collecting and counting cells from the outlets provides an alternate means of determining their distribution across conductivity that does not require microscopy. Alternatively, if the population contains genetic variants (or is clonal but exhibits heterogeneous gene expression), the relative abundance of different strains in each sorted fraction can be determined through quantitative PCR, microarray analysis, or sequencing.

3.4. Cleaning the Device

1. After the experiment, the device can generally be cleaned and reused. Rinse the device thoroughly with deionized water; this can follow a rinse with detergent in extreme cases, or can be accompanied by sonication.
2. After rinsing with water, dry the device thoroughly by connecting it to a low pressure supply of nitrogen gas.
3. If the device has become clogged or it is not possible to clean it by rinsing or sonication, the device can be taken apart, the PDMS channel removed, and the electrode chip reused. Solvents such as acetone can generally be used to soften the epoxies used during device assembly, and cured PDMS can be dissolved from the electrode chip (Dynasolve 220; Dynaloy, Indianapolis, IN).

4. Notes

1. Dispense the resist slowly, being careful to prevent any bubbles from forming on the wafer.
2. Do not allow the wafer to cool too rapidly. Also, if the prebake is not sufficiently low, the wafer may adhere to the mask during exposure. If this occurs, extend the prebake.
3. We find that using fresh photoresist (i.e., not approaching the expiration date) it critical in obtaining smooth features that do not easily delaminate from the wafer.
4. Once an SU8 mold has been created, it is possible to create many replicas by recasting the mold in durable plastic (16).
5. In some cases where the features are particularly small or dense, the lift-off process can be accelerated by gently swabbing the wafers with an applicator soaked in acetone.
6. It is important that the PDMS layer be neither too thick nor too thin; if too thick, it may be difficult to image the sample using any microscope objective without a long working distance.

If too thin, it may be difficult to press-fit inlet tubing into the device without the sample or separation buffers leaking.

7. Tubing with smaller inner diameters minimizes the residence time of cells during loading, and will improve the uniformity of the loading concentration.
8. Manual alignment of the chip with accuracy better than ~0.5 mm may be difficult. If this is the case, an automated stage can be used for more precise alignment.
9. We find that custom-designed printed circuit boards and conductive epoxy (CW2400, Chemtronics, Kennesaw, GA) are an effective way to establish electrical connections to the chip.
10. Leaving liquid droplets at the inlets of the device after it has been primed provides an interface for inserting the tubing later without introducing any bubbles.
11. Because of electrostatic and hydrodynamic interactions between cells as they pass through the device, the performance will depend on the concentration at which cells are loaded. For the purpose of characterizing cells at a single-cell level, low concentrations will work best. However, for preparative separations (i.e., those in which appreciable numbers of cells are to be collected) higher concentrations can not only improve the throughput, but the sensitivity of the separation as well. In working with *S. cerevisiae*, we find that concentrations between 1 and 5×10^7 cells/ml work well.
12. It is easiest to insert the tubing without introducing bubbles if there are droplets remaining over the inlets from the priming step (see Subheading 3.2).
13. Because PDMS is permeable to gas, increasing the pressure within the channel can force the removal of bubbles through the channel ceiling and walls. This can be achieved by increasing the flow rate, or by plugging the outlet channel.
14. During long-term operation with cells that sediment rapidly, we recommend mounting the syringe pump vertically and making the tubing connecting the syringes to the device as short as possible.
15. Consideration must be given to the operating conditions used in any experiment to prevent fouling of the device. High current densities, even for a very brief time, may irreparably damage the electrodes; as a general guideline, we find that current densities lower than 10^5 A/m^2 do not damage the electrodes.
16. Device fouling can also result if the frequency is set to a value where the cells have strong positive polarizability. When this is the case, they will be attracted to the surface of the electrodes, where they may become stuck and produce clogging. Mechanical

agitation of the device can be effective in freeing cells that have become stuck in this way.

17. We like to use pipette tips, press-fit directly into the device's outlets. These act as reservoirs, filling with sample that can easily be transferred to other containers with a manual pipettor.

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