Published in final edited form as:

Methods Enzymol. 2019 ; 628: 191–221. doi:10.1016/bs.mie.2019.06.016.

Design of an Automated Capillary Electrophoresis Platform for Single-Cell Analysis

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Abstract

Single-cell analysis of cellular contents by highly sensitive analytical instruments is known as chemical cytometry. A chemical cytometer typically samples one cell at a time, quantifies the cellular contents of interest, and then processes and reports that data. Automation adds the potential to perform this entire sequence of events with minimal intervention, increasing throughput and repeatability. In this chapter, we discuss the design considerations for an automated capillary electrophoresis-based instrument for assay of enzymatic activity within single cells. We describe the key requirements of the microscope base and capillary electrophoresis platforms. We also provide detailed protocols and schematic designs of our cell isolation, lysis, sampling, and detection strategies. Additionally, we describe our signal processing and instrument automation workflows. The described automated system has demonstrated single-cell throughput at rates above 100 cells/hour and analyte limits of detection as low as 10^{-20} mol.

Keywords

Chemical cytometry; single-cell; capillary electrophoresis; enzyme activity measurement; cell array; fluorescence detection; laser-based lysis

Introduction

Across biomedical research fields the move towards single-cell analysis has been a source of innovation. Single-cell studies have led to rapid advancements within the fields of genomics, proteomics, transcriptomics, and metabolomics (D. Wang & Bodovitz, 2010). For example, single-cell genomics enabled the genotyping of single acute myeloid leukemia cells to investigate clonal diversity and evolution in cancer metastasis and relapse (Paguirigan et al., 2015). Non-destructive single-cell proteomic analysis has interrogated the shifting protein expression within *Xenopus laevis* embryos at multiple stages of development (Lombard-Banek, Moody, Manzini, & Nemes, 2019). Through single-cell metabolomics, neuroblastoma specific metabolites and therapeutic drugs were detected in circulating tumor

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cells (Hiyama et al., 2015). A subset of the techniques encompassed by single-cell analysis is referred to as chemical cytometry. Cytometry is the process of analyzing and characterizing single cells, while chemical cytometry analyzes the chemical composition of single cells (Dovich & Hu, 2003). Chemical cytometry typically utilizes high-sensitivity analytical tools such as mass spectrometry, single-cell RNA-sequencing, and capillary electrophoresis (CE) to quantify the contents within a cell. In particular, CE is well suited for use in chemical cytometry as it is capable of achieving detection limits as low as single molecule (Chen & Dovichi, 1996), sampling volumes on the scale of a single cell (Li, Sims, Wu, & Allbritton, 2001), and performing high efficiency separations (Jorgenson & Lukacs, 1981).

Common amongst chemical cytometry are the key steps of isolating single cells, releasing cellular components, interrogating the cell contents, and analyzing single-cell data (Figure 1B). For a CE-based chemical cytometry system these steps are accomplished by placing a single cell in proximity to a capillary or microchannel inlet, delivering the cell contents into the capillary or microchannel lumen, separating cellular components by electrophoresis, and detecting the cell contents. A complete CE-based chemical cytometry instrument requires a cell holder, capillary, high voltage power supply, detection system, and inlet and outlet reservoirs. These minimal components can be augmented with additional modules to add functionality. Examples include: adding a microscope to enable cell imaging prior to electrophoretic analysis; implementing additional lasers for rapid cell lysis, terminating reactions, and photoactivating cellular probes; incorporating a cell array for high-throughput analysis; and including automation software to control motorized components (Mainz, Wang, Lawrence, & Allbritton, 2016; Mainz, Serafin, et al., 2016; Proctor & Allbritton, 2018; Proctor et al., 2014; Proctor, Sims, & Allbritton, 2017; Proctor et al., 2016; A. H. Turner et al., 2016).

Our lab has built a fully automated system (Figure 1C) that has demonstrated single-cell throughput at rates above 100 cells/hour (Dickinson, Armistead, & Allbritton, 2013), limits of detection as low as 10^{-20} mol, and theoretical plate numbers as high as 2×10^5 (K. Wang, Jiang, Sims, & Allbritton, 2012). Versions of this instrument have enabled the study of: AKT activity in rheumatoid arthritis synoviocytes (Mainz, Serafin, et al., 2016), sphingosine kinase activity in natural killer cells (Dickinson et al., 2015), phospholipid signaling in immortalized chronic myelogenous leukemia cells (Proctor et al., 2017), epidermal growth factor receptor activity in respiratory epithelial cells (A. H. Turner et al., 2016) and other measurements. In this review we describe the fully automated system used in our lab.

1.1 The Chemical Cytometry Instrumentation

The base platform of our system incorporates an inverted microscope, an automated xy-stage, a charge coupled device (CCD) camera, and pulsed Nd:YAG laser (Figure 2). An inverted microscope is preferred since it enables cell imaging, accommodates fluidic cassettes, permits access to the cells, and is compatible with automation. Further the open format of the stage supports the capillary, optical-detection train and other hardware such as a microinjection pipette (Cohen et al., 2008; Dovichi & Hu, 2003; E. H. Turner et al., 2008). An automated microscopy xy-stage facilitates highly reproducible positioning of the

sampling tray to permit sequential and rapid cell positioning below the capillary inlet. A CCD camera enables cell tracking for bright-field and/or fluorescence imaging prior to cell lysis while a focused microbeam delivered by the pulsed Nd:YAG laser permits controlled single-cell lysis (McNamara, Difilippantonio, Ried, & Bieber, 2017; Salmon & Waters, 2011). Table 1 highlights critical system components and specifications. Component substitutions can be made depending on experimental requirements and have been discussed in previous work (Dickinson et al., 2013; Hellman, Rau, Yoon, & Venugopalan, 2008; Jiang, Sims, & Allbritton, 2010; Lai et al., 2008; Mainz, Serafin, et al., 2016; Mainz, Wang, et al., 2016; McNamara et al., 2017; Phillips, Bair, Lawrence, Sims, & Allbritton, 2013; Proctor & Allbritton, 2018; Proctor et al., 2014, 2017, 2016; Rau, Quinto-Su, Hellman, & Venugopalan, 2006; A. H. Turner et al., 2016; Vickerman, Anttila, Petersen, Allbritton, & Lawrence, 2018). In subsequent sections, we describe each of the major instrumentation components and their operation.

1.2 Assembling and Aligning the Pulsed Laser Pathway

Numerous methods for lysing single cells exist, including: electrical, chemical, mechanical, thermal, and sonic means (Shehadul Islam, Aryasomayajula, & Selvaganapathy, 2017). Our lab has typically employed pulsed lasers to perform cavitation-induced lysis of single cells and subsequent analysis by CE. Pulsed microbeam-based cell lysis creates a plasma on sub- μm scales yielding a mechanical shockwave and cavitation bubble on sub- μs timescales. Cell lysis is achieved by the collapsing cavitation bubble as it moves across the cell (Quinto-Su et al., 2008; Sims et al., 1998). Dilution of the small molecule contents such as ATP, glucose and Mg^{2+} acts to terminate cellular reactions on sub-millisecond time scales, i.e. much faster than most physiologic cells processes (Quinto-Su et al., 2008; Shamir, Bar-On, Phillips, & Milo, 2016; Sims et al., 1998). Whereas, the large molecule cell contents are minimally diluted which yields high sample collection efficiencies when the cellular contents are simultaneously electrokinetically injected into the capillary inlet (Quinto-Su et al., 2008; Sims et al., 1998). The theory and background of laser cavitation and its utility as a cell lysis technique are discussed in previously published work (Hellman et al., 2008; Lai et al., 2008; Rau et al., 2006; Sims et al., 1998).

Proper laser beam alignment and stability are critical to perform cell lysis with the high degree of precision that is required for single-cell analysis. All optical components including the microscope are mounted on a vibration-isolated optical table using base clamps, angle brackets, or screws. The angles and positioning of each component should be secured by tightening the corresponding set screws following each alignment step. Our lab utilizes the following components to deliver the focused microbeam for cell lysis: a frequency-doubled, diode-pumped, passively Q-switched Nd:YAG laser (532 nm, 750 ps pulse, 0.1–10 μJ pulse energy), 532 nm high transmission thin film linear polarizer, iris diaphragm, 45° optical mirrors, Keplerian beam expander, dichroic mirror reflecting <565 nm, achromat objective with 0.80 numerical aperture (NA), and CCD camera protected by a 532-nm notch filter which blocks the pulsed beam from entering the camera (Figure 3) (Dickinson et al., 2013; Dickinson, Hunsucker, Armistead, & Allbritton, 2014; Dickinson et al., 2015; Jiang et al., 2010; Lai et al., 2008; Mainz, Serafin, et al., 2016; Mainz, Wang, et al., 2016; Proctor et al., 2014, 2017, 2016; A. H. Turner et al., 2016).

We prefer using the visible Nd:YAG wavelength for safety reasons since it can be easily visualized during alignment. The polarized laser beam requires processing to tune the energy per pulse, remove light debris, and optimize the beam diameter; this is accomplished using a linear polarizer, iris, and Keplerian beam expander in sequence. The polarizer is used to attenuate the beam as it is rotated permitting only a fraction of the light through. The iris spatially filters the beam to produce a circular beam and remove side lobes. The Keplerian beam expander adjusts the beam diameter to fill the back aperture of the microscope objective lens. Backfilling the microscope objective lens permits the beam to be focused to the smallest possible spot size. The beam characteristics described previously are critical for performing highly reproducible laser-microbeam-mediated lysis of single cells. For additional information on important optical pathway characteristics in pulsed-microbeam systems for cell-lysis, previous publications may be consulted (Hellman et al., 2008; Lai et al., 2008; Rau et al., 2006).

Next, the pulsed laser beam is directed to the filter cube within the microscope and where a dichroic mirror sends the beam up towards and into the microscope objective. To focus the laser beam and visualize samples during cell lysis, our lab utilizes high-quality, high NA objectives to yield small spot sizes with higher energy densities decreasing the amount of energy needed to create the plasma and hence cavitation bubble. Upon exiting the objective, the laser beam is expected to be focused to a sub- μm spot, e.g., $\sim 0.7 \mu\text{m}$ diameter ($0.4 \mu\text{m}$, theoretical diffraction limited) for a 40x objective with 0.80 NA (Rau et al., 2006). At sufficiently high pulse energies in an aqueous medium (ranging from 2 – 8 μJ , reported for 0.8 NA objectives), sub-ns pulses by Nd:YAG lasers lead to optical breakdown of the medium, which in turn generates a plasma and cavitation bubble (Jiang et al., 2010; Lai et al., 2008; Rau et al., 2006). Importantly, pulsed-microbeam cavitation events are known to impact cells tens of μm from the cavitation bubble, which imposes the requirement for cells to be 100 μm apart (Hellman et al., 2008; Lai et al., 2008; Rau et al., 2006).

It is important to wear the appropriate protective eyewear and follow proper beam containment procedures during all laser alignment steps. Please refer to the laser safety section for additional information (Section 1.4). The steps for mounting, modifying and directing the Nd:YAG laser are as follows:

1. Securely mount the laser head to the optical table and adjust to the lowest possible energy during alignment.
 - a. Note: To further reduce the risk of injury, the laser should be operated in continuous wave mode during alignment.
2. Align the beam parallel to the surface plane of the optical table using a nonreflective ruler or target marker.
3. Adjust and readjust the position of the target marker as needed such that the beam spot is always at the same height above the table as it travels across the table.
 - a. More detailed information on laser alignment strategies can be found elsewhere (“Lasers | Edmund Optics,” n.d.).

4. Center the beam through a polarizer, iris diaphragm, and beam expander.
 - a. Note: Take care to ensure the beam enters the center of these optical components to avoid imparting unwanted scattering and optical aberrations.
5. Adjust the iris diameter to remove light noise around the beam prior to entering the beam expander.
6. Adjust the beam expander to completely backfill the rear aperture of the microscope objective.
 - a. Note: This is important to achieve the smallest possible laser spot size on the sample. Additional information on spatial filtering of laser beams has been previously discussed (“Understanding Spatial Filters | Edmund Optics,” n.d.).

1.3 Laser Cavitation Test

Following an initial coarse alignment, it is likely the laser will still be off-axis. Minor adjustments to optical path components should be made to ensure that the pulsed laser spot is visible on the computer screen, within the microscope objective field-of-view. Never view the laser pulses directly. Make sure to only view the pulse position on-screen via the live feed from the CCD camera. Once the fine alignment has been completed, the final position of the lysis laser spot can be marked on the computer screen using a temporary marker, and/or the data analysis software can be modified to indicate the location of the lysis laser spot on-screen (See Section 5.4). Once the fine alignment has been completed, beam tubes and/or enclosures should be used to enclose the laser beam along its pathway to minimize the risk of injury due to reflected or misaligned beams. The efficacy of laser-induced plasma formation can be assessed empirically using an ink ablation test on glass coverslips. Our lab utilizes #1 glass coverslips with silicon O-rings glued to the top surface to aid in fluid retention during long experiments (Figure 4). The steps for performing an ink ablation test are as follows:

1. Using a permanent marker (e.g., Sharpie) place an ink spot on a glass coverslip and cover with a small quantity of water.
2. Position the coverslip on the xy-stage such that the marked region is within the objective field of view.
3. Adjust the microscope focus to 50–100 μm above the surface of the glass slide.
4. Switch the laser from continuous wave to single-pulse mode once the sample is properly positioned.
5. Fire laser pulses of increasing energy, until the ink is visibly removed from the top surface of the glass slide following a single laser pulse
 - a. Note: It is recommended to start at 1 μJ with increasing steps of 0.5 μJ , for a 532 nm beam, 750 ps pulse width and an objective NA of 0.80. Be careful not to fire the pulsed laser within the glass slide itself or the glass will crack.

1.4 Laser Safety

The primary concern of laser safety is preventing eye injury; secondary concerns are skin injuries and fire hazards. Risk of injury and/or damage depend on several factors including: the wavelength of laser light, laser power, whether the beam is continuous wave or pulsed, pulse width, and whether the exposure is direct or from diffuse reflections. In order to reduce the risk of injury when aligning the pulsed laser path, it is important to wear appropriate personal protective equipment (PPE). This includes laser safety glasses specific to the wavelength of lasers chosen. Additionally, never view the beam directly or a beam reflection as this can lead to irreversible eye damage. Instead, the laser beam location can be indirectly identified using a piece of low reflective material such as black cardboard, paper, and/or a laser viewing card for the respective emission wavelength. When working with microscope-based instrumentation, make sure to never look through the microscope eyepiece when firing the pulsed laser to avoid irreversible eye damage. Our lab has found it useful to either remove the eyepieces completely or cover the eyepieces to avoid accidental viewing of the pulsed laser during experiments. Ensure all beam blocks are properly positioned to prevent accidental exposures from all possible angles. To protect users, secondary laser blocks in the optical pathway and an instrument case that covers all of the optical components may be used. Additional information on the biological risks associated with radiation from UV, visible, and IR lasers, engineering controls, and safety protocols have previously been detailed (Kandari, Raizada, & Razzak, 2010; “Lasers | Edmund Optics,” n.d.; Smalley, 2011).

2.1 Microwell Fabrication

Prior to analysis, individual cells are placed in predetermined locations using an array of microwells fabricated from glass and epoxy photoresist. Cells are added to the microwell array and settle by gravity onto the array surface and into microwells. Excess cells that are not trapped are removed by washing. Single-cell capture efficiency is optimized by tailoring the microwell dimensions and the total number of cells loaded onto the array. We typically use a 10×10 array of microwells that are 20 µm deep and 30 µm in diameter with an inter-well spacing of 100 µm (Dickinson et al., 2013). Isolating single cells using microwells has previously been reviewed in detail (S.-H. Kim, Lee, & Park, 2013; Lindström & Andersson-Svahn, 2011).

Although microwells are commonly fabricated using polydimethylsiloxane (PDMS), our group utilizes microwells fabricated from a photoresist since PDMS adsorbs a wide range of analytes (Gokaltun, Yarmush, Asatekin, & Usta, 2017; Zhou, Ellis, & Voelcker, 2010). We typically fabricate microwells using 1002F photoresist since it possesses stronger adhesion to glass and lower autofluorescence when compared to SU-8 photoresist (Pai et al., 2007). The 1002F photolithography process is similar to that for SU-8. Both the SU-8 data sheet and our published papers can be used for guidance, but processing parameters should be optimized for each unique set of features (Detwiler, Dobes, Sims, Kornegay, & Allbritton, 2012; Gach, Sims, & Allbritton, 2010; R. Kim et al., 2018; Lorenz et al., 1997; Marc, Sims, Bachman, Li, & Allbritton, 2008; McPherson & Walker, 2012; Ormoff, Wang, & Allbritton, 2013; Pai et al., 2007; Shah, Hughes, Wang, Sims, & Allbritton, 2013; “SU-8 2000:: MicroChem,” n.d.). Optimization may also be required when using equipment in different

facilities. Table 2 provides a guideline for choosing and preparing the appropriate photoresist formulation based on desired feature height. Abbreviated fabrication steps are as follows (Figure 5):

1. **Substrate Pretreatment:** The substrate, usually a glass slide, is cleaned with acetone and isopropyl alcohol then dried with an air stream. The glass is further cleaned by plasma-treatment.
2. **1002F Layer:** A spin coater is used to evenly coat the substrate with photoresist, where varying photoresist viscosity and spin speed can be used to achieve the desired photoresist thickness. For the described 30 μm deep microwells, 1002F 10 photoresist is spin coated at 500 rpm for 10 s and then 1100 rpm for 30 s.
3. **Soft Bake:** A soft bake is performed on either a hot plate or a convection oven to evaporate solvent from the photoresist. A soft bake of 30 min at 95 °C is used for a 30 μm thick 1002F 10 layer.
4. **Exposure:** Photoresist is exposed to near-UV light (350–400 nm) through a chrome-backed photomask, where the required exposure energy can vary based on the thickness of the 1002F layer. It is important to place the chrome side of the photomask in direct contact with the photoresist to minimize light refraction and achieve sharp features. A dose of 600 mJ is used for a 30 μm layer of 1002F 10.
5. **Post-Exposure Bake:** UV light initiates chemical cross-linking within the light-exposed regions of the polymer. For the array of microwells previously described, a post exposure bake is performed for 8 min at 95 °C.
6. **Development:** Non-crosslinked photoresist is removed with SU-8 developer (1-methoxy-2-propanol acetate). A two-min development is required for an array of microwells 30 μm in depth.
7. **Hard Bake:** The microwell array is rinsed with isopropyl alcohol, dried under an air stream, and cured overnight at 150 °C to further crosslink the photoresist and promote adhesion to the substrate.
8. **Plasma Treatment:** The final array is exposed to a plasma generated from air for five minutes to increase surface hydroxyls on the 1002F and improve its wettability immediately prior to adding cells to the microwell array.

2.2 Fabrication of the Buffer Channels and Mounting Cassette

Cells alter signaling in response to changes in their microenvironment, including changes in temperature, pH, and salt concentration (Kültz, 2004). Therefore, it is important that living cells remain in a physiologic buffer (135 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM HEPES, 10 mM glucose, pH 7.4) prior to assay (laser-based cell lysis). However, physiologic buffers rarely provide ideal electrophoretic separation conditions. CE chemical cytometry platforms must therefore be designed such that rapid switching can occur between physiologic and separation buffers.

Our system enables the capillary to move readily between the physiologic and electrophoretic buffers without mixing of the two buffers. The system consists of two distinct buffer channels fabricated from two slabs of PDMS bonded to separate glass coverslips. PDMS is polymerized per manufacturer recommendations and channels are formed using a scalpel (Figure 6B). A thin layer of uncured PDMS is used as adhesive to bond the cured PDMS and glass coverslip. The physiologic buffer channel (channel dimensions 3.5 cm x 0.5 cm x 1.5 mm), which is positioned such that it contains the cell microwell array, is separated from the electrophoretic buffer channel (channel dimensions 3.0 cm x 0.5 cm x 1.5 mm) in the x-direction by an air-filled channel (0.5 cm x 1 mm x 1.5 mm) (Dickinson et al., 2013; Mainz, Wang, et al., 2016). The relatively narrow dimensions of the air channel (1 mm) permit the capillary to traverse between the physiologic and electrophoretic buffers without inter-mixing of the two buffers, as the difference in surface energies of the PDMS and buffers prevents the buffers from wetting the air channel (Dickinson et al., 2013).

A 3-D printed cassette secures the two slabs of PDMS rigidly to the microscope stage and maintains their relative positioning as the two slabs are offset in the z-direction by 130 μ m (Figure 6A, 6D). This height difference is most important when hydrophobic additives (e.g., ethanol or detergent) are used in the electrophoretic buffer, as the step difference further prevents buffer mixing. To ensure that the capillary inlet contacts the buffers in each channel despite the height difference between the two buffer channels, channels are overfilled with buffer solutions. The surface tensions of the buffers enable overfilling without spilling over the channel walls. Buffer flow is controlled using fluid-dispensing lines (i.e. buffer inlets) and vacuum lines (i.e. buffer outlets) such that the physiologic buffer maintains a 1 mm/s flow velocity and the electrophoretic buffer maintains a 2 mm/s flow velocity (Figure 6A, 6C) (Dickinson et al., 2013). We wrap the fluid-dispensing lines in heat tape to warm the physiologic buffer to 37 °C at the buffer inlet.

3.1 Fluorescence Detection

Fluorescence detection has demonstrated great utility in various chemical cytometers (Dickinson et al., 2013; Krylov et al., 2000; Lapainis, Scanlan, Rubakhin, & Sweedler, 2007; Shehaj, de la Vega, & Kovarik, 2015). With its low background signal and high selectivity, fluorescence detection is capable of achieving single molecule detection (Chen & Dovichi, 1996), making it extremely well-suited for chemical cytometry applications. When designing a fluorescence detection apparatus there are three principal domains to be considered: excitation light, emission light, and detection.

3.2 Excitation

When selecting a light source for use in fluorescence, several factors must be considered: wavelength for optimal analyte excitation, light output stability, beam collimation, and monochromaticity. Solid-state or diode lasers achieve all necessary performance metrics and are available in a wide range of wavelengths and have been our light source of choice. We use a single-mode fiber optic to convey the light from the laser to the initial optical components, which confines the output light to a single spatial mode ensuring the excitation light can be uniformly and tightly focused onto the capillary lumen. In an epifluorescence

excitation/emission arrangement, the excitation light is pointed onto a dichroic beam splitter to direct the excitation light onto the back aperture of a microscope objective. The objective then focuses the light onto the capillary lumen as well as captures emitted fluorescence from analytes moving through the capillary and traversing the beam. A high NA objective enables the focused beam size to match the diameter of the capillary lumen while permitting high efficiency capture of fluorescence light. Eye damage may occur even when using a continuous wave laser; the safety considerations of section 1.4 Laser Safety must be rigidly followed. To ensure proper alignment, we designed an engineering solution which mounts a plate statically above the microscope stage (Figure 7Ai) to which both the capillary window holder (Figure 7Aiii) and three-dimensional compact flexure stage and epifluorescence assembly (Figure 7Ci, 7Cii) are affixed. This assembly facilitates μm -scale adjustment in the lateral and longitudinal axes with respect to the capillary lumen while independently maintaining capillary position.

3.3 Emission Pathway and Light Detection

The fluorophore-emitted light is collected through the microscope objective (Figure 7Cii) and directed through the dichroic beam splitter to the detector. As shown in Figure 7B, a multimode fiber is coupled to a collimator following the beam splitter. A multimode fiber is preferred due to its larger core diameter and greater NA and hence higher light capture efficiency relative to a single-mode fiber. Our lab uses a photomultiplier tube (PMT) for fluorescence detection as it provides a large dynamic range and possesses the high sensitivity required for single-cell measurements.

3.4 Alignment

Signal loss can occur at every physical junction in an optical pathway when proper alignment is not achieved. A straightforward method for pathway alignment follows the light from the excitation source linearly to the detector. The excitation light intensity can be readily monitored at the front of the objective, thus isolating the entirety of the excitation light path for troubleshooting. The objective ideally generates an illumination spot with dimensions that match the diameter of the capillary lumen. If the spot size is larger than the capillary lumen, light scatters at the interface of the liquid and glass walls due to the large refractive index mismatch, thereby greatly increasing light noise. If the spot size is smaller than the capillary lumen, not all present fluorophores are excited resulting in submaximal signal. To achieve correct alignment of the fluorescence detection apparatus, the following procedure is recommended:

1. Place a capillary, with polyimide coating removed to form a window, in the capillary holder.
2. Maintain the flow of a concentrated ($10\ \mu\text{M}$) fluorophore solution, such as 6-carboxy fluorescein, through the capillary; this can be accomplished using a syringe and needle with tubing that has an internal diameter that matches both the syringe needle and capillary outer diameters.

- a. Note: It is advisable to only use this capillary for alignment as residual fluorophore may adsorb onto the capillary walls increasing the signal noise for separations.
3. Adjust the PMT signal using preamplifier or voltage controls to avoid signal saturation.
4. In the referenced design (Figure 7), adjust the flexure stage to align the laser spot with the capillary lumen, this occurs when the monitored fluorescence signal reaches a maximum.
5. Iterate through adjusting each fiber optic coupling followed by readjusting the flexure stage until all couplings have been aligned and the monitored fluorescence signal reaches a maximum.

4.1 Capillary Electrophoresis

CE exploits differences in electrophoretic mobility (shape, charge, and other attributes) to physically separate analytes in a mixture (Landers, 1996; Lukacs & Jorgenson, 1985; Weinberger, 1993). When coupled with optimally aligned fluorescence detection, CE can detect fluorescent compounds at the sub-attomole level or lower, which is sufficient for analyte detection in single cells (Galievsky, Stasheuski, & Krylov, 2016). A challenge in utilizing CE is the determination of a suitable electrophoretic separation buffer, as a single formulation is not broadly applicable to multiple sample types. Often a systematic screen through various parameters is needed to determine an optimal buffer for a given analyte separation. Our method of screening buffers is thoroughly described in Proctor et al. (Proctor, Wang, Lawrence, & Allbritton, 2019) and additional strategies can be found elsewhere (Bekri, Leclercq, & Cottet, 2016; Landers, 1996; Weinberger, 1993). Peak attributes to assess during the screen are: peak resolution, separation efficiency, and reproducibility (in peak area and migration time).

A computer-controlled power supply is used to apply up to 30 kV across the capillary with the option of switching between positive and negative polarity to ensure the ability to electrophoretically separate a wide variety of species. We determine the separation voltage by plotting the current as a function of voltage over a wide range of voltages (suggested in 2 kV increments) and the highest voltage at which this relationship is linear is used. At greater voltages, the heat created by molecular friction is no longer dissipated and the temperature within the capillary begins to rise (Xuan & Li, 2005). In this Joule heating range, temperature gradients yield convective mixing and the buffer may vaporize to form bubbles in the capillary lumen. All of these processes greatly diminish the electrophoretic separation efficiency and resolution.

4.2 Capillary Selection and Preparation

The most common type of capillary used in CE is made from fused silica coated with a polyimide coating that renders the capillary flexible for facile positioning and handling. Fused silica is available in multiple dimensions (Table 4), enabling precise selection of a capillary with the optimal properties. Good capillary preparation is essential for reliable chemical cytometry separations. The inner surface of the fused silica presents silane groups

that interact with ions and other additives to alter the charge on the capillary wall during electrophoresis (Whatley, 2001). To help with reproducibility, a single capillary should be used for a given buffer formulation as the fused silica surface is dependent on previous treatments and can be permanently modified after exposure to certain compounds (e.g., surfactants or dynamic coatings). When cutting a new capillary to length, burn and remove a small amount of the polyimide coating (<5 mm) from the inlet to provide a clean opening (Figure 8A). Either an improper cut (Figure 8B) or failing to remove the polyimide coating (Figure 8C) will leave jagged pieces of silica and/or coating that can trap sample or cellular debris at the inlet, leading to irreproducible sample loading and capillary clogging.

4.3 Capillary Inlet Housing and Inlet Positioning

Installation of a capillary onto the system requires precise positioning of the detection window and the capillary inlet and outlet. The detection window must be held in an exact, stationary position to ensure consistent detection for every sample. To accomplish this, a permanent mount made of non-conductive Delrin is positioned in front of the collection objective (Figure 8D, 8E). The mount has a v-groove milled down the center to hold the capillary and a hinged door is closed to hold the capillary in place with the detection window positioned in front of the objective. The door has two rubber cushions on either side of the detection window to securely hold the capillary in place without breaking it. The capillary inlet is held with an x-, y-, and z-micromanipulator to enable exact positioning of the capillary over a single cell. Once a capillary is installed, the inlet is positioned as follows:

1. Use coarse adjustment in the x-and y-plane to move the capillary to the laser lysis position. It is helpful to mark the computer monitor at this position to enable reproducible positioning of the capillary every time.
2. Adjust the microscope to focus on a cell. The capillary may appear as a shadow above the focal plane.
3. Raise the focal plane of the microscope 50 μm above the cell. The cell will now be well out of focus.
4. Use fine adjustment of the x-, y-, and z-axes to bring the capillary into sharp focus and position the lumen at the precise location of the laser lysis marking. Lock all 3 positions to hold the capillary in place.
5. Return the focal plane to focus on the cell. No further adjustments will be necessary unless the capillary is moved.

4.4 Capillary Outlet Housing and Safety Considerations

The outlet end of the capillary is placed in a Delrin and polycarbonate housing that is moved along a vertical optical rail (Figure 8F, 8G). The high voltage electrode is mounted in the outlet along with a buffer vial and the assembly is air tight so that pressure can be applied during wash steps. The non-conductive Delrin and polycarbonate serve to protect the user from the high voltage electrode. Care must be exercised as nearby conductive materials can cause arcing from the electrode and may injure users or damage the equipment. As such, the outlet housing should never be handled while a voltage is applied. Teflon or other non-conductive screws should be used to construct the housing, which must be located at a

sufficient distance from the microscope to prevent arcing. Finally, the outlet housing is mounted on an optical rail for manual movement in the z-direction so the outlet can be moved independently from the inlet to enable tasks such as gravity-driven sample injection at the inlet.

5.1 Hardware and Signal Processing

A data acquisition (DAQ) board is an essential component for the automated CE system to convert analog into digital signals for input into the computer, as well as to create analog and digital signals for hardware control. Sampling frequency and resolution needed to analyze a typical electropherogram should be considered when selecting DAQ hardware. It is important to select an analog to digital converter with an effective resolution that will span the expected experimental dynamic range. The maximum dynamic range of a typical fluorescence experiment using a PMT can span up to four magnitudes; thus, an analog to digital converter with 16-bit resolution and above would be appropriate. The sampling rate should be selected based on the smallest peak width in a separation where the minimum sampling rate would be twice the highest frequency signal. For chromatography, it is ideal to sample 10–20 points per peak, which for most experiments lies between 4 and 10 Hz. Just as under sampling will decrease resolution and distort peak shape, oversampling can decrease the signal to noise ratio (Wahab, Dasgupta, Kadjo, & Armstrong, 2016). The DAQ hardware must have enough analog and digital output lines to control and monitor the power supply and pulsed laser (Figure 9A). The quality of the software library for controlling the DAQ programmatically should be considered when selecting a DAQ. Many manufacturers have examples of controlling the DAQ in different programming languages.

Common signal processing routes for chromatography can be applied to chemical cytometry (Dyson, 1998). Filtering high and low frequency noise from the signal can be performed using software or hardware filters. Most programming languages have libraries that include common signal processing algorithms that can be adjusted depending on experimental conditions.

5.2 Microscope control

For automated cell lysis followed by lysate loading into the capillary, each cell must be positioned under the capillary lumen (10–50 μm below the capillary inlet). Cell and capillary position can be acquired using any modest microscope camera connected to a computer. Cell positioning requires a microscope xy-stage. These stages typically move in sub- μm increments but larger increments (1–5 μm) are acceptable if the cell can be reliably positioned under the capillary inlet. Because the camera image can act as a feedback for positioning the cell, encoders for the stage are considered optional. As with the DAQ board, when selecting the camera and motorized xy-stage consider the software libraries for computer control. In addition to libraries provided by the manufacturer, $\mu\text{manager}$ is an open source software that can control most automated microscopes, stages, and cameras (Edelstein, Amodaj, Hoover, Vale, & Stuurman, 2010).

5.3 Serial Loading of Cell Contents into the Capillary

For a typical CE separation, a sample is loaded into the capillary and then separated and detected prior to loading a second sample. However, to increase throughput, single-cell lysate loading into the capillary can be timed so that the next cell is loaded into the capillary before all the contents of the prior sample have been detected. This method of serial injection requires that two conditions are met. First, the slowest moving analyte of a sample must pass the detector before the fastest moving analyte of the next sample; second, the next cell must be loaded into the capillary at a time where there are no analyte bands in front of the detector to avoid peak distortion.

In order to determine the timing, the contribution of multiple sample plugs to the electric field across the capillary needs to be considered. The addition of multiple plugs of sample buffer with each injection, which is generally at a different conductivity than the separation buffer, will change the electric field throughout the capillary. This change in electric field will alter the migration time of the analytes compared to when a single injection occurs in the capillary. To determine the proper timing for serial injection it is best to load the capillary with multiple equally dispersed plugs of sample buffer before the first sample is loaded. Subtracting the time of the fastest moving analyte from that of the slowest moving analyte gives the earliest time that the next cell or sample can be loaded into the capillary. As mentioned previously, it is important to pick a time to load the samples (T_{load}) when no analyte band is in front of the detector to avoid any peak distortion.

5.4 Software Workflow

By programming the hardware to lyse, load, and separate cells in an automated fashion, a greater number of cells can be analyzed than typically possible under manual operation. There are many methods and programming languages that can be used to automate the hardware and collect data. The following protocols outline the main features, workflow, and considerations when programming an automated chemical cytometry instrument.

At the start of any experiment, the position or coordinates of the microwells needs to be determined so that cells can be moved to the appropriate location for cell lysis e.g., 1 cell radius or $\sim 5 \mu\text{m}$ from the pulsed microbeam spot. An example workflow to calibrate the stage and well positions is outlined:

1. Mark the position of the laser spot and then identify the desired location for a cell to be positioned during microbeam-mediated cell lysis.
2. Using the camera feed and motorized xy-stage, determine the coordinates of the xy-stage for each microwell.
3. Record the position of 3 corner wells, determine vectors between the corner well (column = 0, row = 0) and the wells immediately vertical (column = 0, row = 1) or horizontal (column = 1, row = 0) to the corner well.
 - a. Note: These vectors can then be used to determine the xy-position of any given microwell on the array ($x, y = \text{column} \times V_{\text{horizontal}} + \text{row} \times V_{\text{vertical}}$).

4. Record the positions to which the capillary will move for electrophoretic separation (within the electrophoretic buffer channel), the channel openings for either side of the air gap, and any other positions that may be required.

The focal plane across the microarray needs to be calibrated to ensure the lysis laser will be well focused at every microwell. The following is a minimized workflow for calibrating the focus and capillary position:

1. Record the focus z-position for 3 corner microwells.
2. Determine the equation for the plane that passes through these three points.
3. The software should use this equation to determine the z-focus position every time the microwell array is moved to position a cell for analysis.

The final step for creating the automated program is to iterate through the list of microwell addresses (containing cells) to analyze only those cells that are selected by the user. A simplified workflow for initializing each run is provided:

1. Either manually or by script, scan each microwell and record microwell addresses that have a desired single cell for analysis.
2. Use this cell address list to proceed through the workflow highlighted in Figure 9B.
 - a. Note: Depending on the layout of the chip, additional xy-movements may need to be incorporated when moving between the sample reservoir and the electrophoretic buffer reservoir to prevent the capillary from colliding into the channel walls.
 - b. Note: It is recommended that a stop button or command is integrated into the workflow to terminate the voltage, xy-stage movement, laser pulse, and any other motorized hardware in case of a capillary breakage or other system malfunction.

6. Future Perspectives

In the preceding sections, we have outlined the design and use of an automated CE system for use in chemical cytometry applications. The outlined system design is our most recent iteration, but there are unrealized performance improvements to be made in the areas of software capabilities, system-wide automation, and single-cell throughput. By adding multiplexing capabilities to this instrument, it is possible to improve the amount of information gathered from a single cell.

Acknowledgements:

The authors thank the National Institutes of Health (CA177993, CA224763) and Tyler Hutchens for assistance with figures generated. This work was performed in part at the Chapel Hill Analytical and Nanofabrication Laboratory, CHANL, a member of the North Carolina Research Triangle Nanotechnology Network, RTNN, which is supported by the National Science Foundation, Grant ECCS-1542015, as part of the National Nanotechnology Coordinated Infrastructure, NNCI.

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Figure 1.

CE-based chemical cytometry. **(A)** CE-based analysis of single cells consists of cell collection, cell placement, single-cell analysis, and single-cell data output. **(B)** Minimal steps in the assay of single cells by a CE instrument (from left to right) cell placement in proximity to a capillary lumen, releasing cellular components, separation of cellular contents, and detection of desired analytes. **(C)** Block diagram of our CE-based chemical cytometry instrument.

Figure 2.

Schematics (CAD drawings) and photographic images of our inverted microscope-based chemical cytometry system. **(A)** Schematic of tilted top-down view of the automated xy-stage, fluorescence detection optical pathway, and cassette with cell array and fluidic compartments. **(B)** Photograph of tilted top-down view of the automated xy-stage. **(C)** CAD view of the side of the instrument. **(D)** Photograph of the entire assembly from the side. **(E)** CAD view from the front of the instrument assembly. **(F)** CAD top-down view of the microscope with the xy-stage, and CE employing fluorescence detection optical pathway removed. **Legend:** (i) xy-stage, (ii) capillary and fluorescence-detection system mount, (iii) xyz-micromanipulators, (iv) white LED, (v) custom fluidic cassette, (vi) CE optical path mount, (vii) capillary outlet holder & buffer vial, (viii) optical post, (ix) microscope revolving turret w/ objective, (x) Nd:YAG laser entry port, (xi) coarse/fine focus knobs, (xii) xy-stage mounts, (xiii) microscope filter-sets, (xiv) CCD camera.

Figure 3.

Diagram of the optical path, illustrating the path of the laser beam. Side-on view of the optical path coupled to the microscope assembly, showing the light-path into the objective and major components indicated. (i) Nd:YAG laser head, (ii) laser head table mount, (iii) shutter, (iv) linear polarizer, (v) iris diaphragm, (vi) Keplerian beam expander, (vii) microscope entry port, (viii) CCD camera, (ix) laser line notch filter, (x) dichroic mirror, (xi) objective.

Figure 4.

Diagram of an ink ablation test to validate proper functioning and/or alignment of the pulsed cell lysis laser. **(A)** Images of a marked silicon O-ring coverslip set in the sample tray on the xy-stage; (i) the xy-stage, (ii) the sample tray, (iii) region of ablated ink on the glass coverslip following testing of the pulsed laser. **(B)** Brightfield image of ink that has been ablated from the surface of the O-ring coverslip following a successful cavitation event.

Figure 5. Microwell Production. Microwells are produced using standard photolithography processes. **(A)** Photolithography schematic (i) Substrate is cleaned. (ii) A spin coater is used to evenly coat the substrate with photoresist. (iii) A soft bake induces solvent evaporation. (iv) Photoresist is exposed to near-UV light through a photomask. (v) A post-exposure bake induces crosslinking in photoresist exposed to light in the exposure step. (vi) Unexposed photoresist is removed using 1-methoxy-2-propyl acetate. Remaining features are hard baked to promote photoresist crosslinking and adhesion to the glass substrate. **(B)** Brightfield microscopy image showing an entire microwell array. Scale bar is 200 μm . **(C)** Scanning electron microscopy (SEM) image of fifteen microwells. Scale bar is 200 μm . **(D)** Tilted view SEM image of one microwell. Scale bar is 10 μm . **(E)** Brightfield microscopy showing individual cells, indicated by arrows, trapped in microwells. Scale bar is 30 μm .

Figure 6.

H-Channel Design, Fabrication, and Housing. **(A)** CAD image of the 3D-printed housing to secure the H-channel cassette and buffer inlets and outlets. **(B)** Physiologic buffer channel, air channel, and electrophoretic buffer channel are fabricated by cutting a slab of cured PDMS with a scalpel. Templates can be used if necessary. **(C)** A fully fabricated H-channel. Food dyes were used for visualization. Blue liquid represents the physiologic buffer and red liquid represents the electrophoretic buffer. An array of photoresist microwells is positioned within the physiologic buffer in line with the narrow air channel. **(D)** Schematic showing a cross section view of the physiologic (blue) and electrophoretic (red) buffers offset by 130 μm to prevent buffer mixing. PDMS is outlined with dashed line. The capillary can traverse between the two buffers using the air channel shown in panels A-C. For all panels: (i) buffer inlet (ii) electrophoretic buffer channel (iii) air channel (iv) buffer outlet (v) physiologic buffer channel (vi) capillary (vii) 3D-printed mounting cassette. (*) indicates the location of a microwell array described in Figure 5. All scale bars are 1 cm.

Figure 7.

Example fluorescence assembly. **(A)** CAD of the (i) mounting plate, (ii) capillary and (iii) capillary holder shown in isolation above the microscope stage. These components keep the capillary window stationary during instrument operation. **(B)** CAD of the epifluorescence assembly from the side with light pathway overlaid on the filter cube assembly. Excitation light is carried by a (i) single-mode fiber, emission light is carried by a (ii) multi-mode fiber, and a (iii) dichroic is used to direct excitation light onto the capillary and pass emission light to the detector. **(C)** CAD of the (i) flexure stage detail highlighting the (ii) objective and (iii) filter cube are adjustable with respect to the capillary window. **(D)** Photograph of (i) flexure stage and (ii) the adjustable objective (iii) filter cube assembly.

Figure 8:

CE capillary inlet and outlet (A) Acceptable cut and removal of polyimide coating from capillary inlet: i-hollow capillary lumen; ii-fused silica capillary wall. (B) Unacceptable cut of the capillary. (C) No removal of polyimide coating. CAD model (D) and photograph (E) of the capillary inlet: iii-capillary inlet x-, y-, and z-translational control; iv-detection window housing; v-door to securely hold detection window in position; vi-fused silica capillary. CAD model (F) and photograph (G) of the outlet housing: vii-air tubing for pressurized rinsing; viii-high voltage electrode; ix-threaded glass vial for outlet reservoir; x-fused silica capillary.

Figure 9.

Instrument workflow and operation. **(A)** Our hardware components and connections. In this arrangement, the DAQ board is connected to the computer via a PCI slot. The high voltage power supply is controlled by a 0–10 V analog signal. The current and voltage are monitored from the power-supply current-monitoring ports, which deliver a 0–10 V output proportional to the current in the capillary. The photomultiplier delivers a 0–15 V signal to the 16-bit analog input port of the DAQ. A hardware timed TTL pulse is delivered to the pulsed laser controller via the DAQ digital output channel. Additional computer connections to the motorized microscope components and camera are made via USB and FireWire respectively. **(B)** Software flow diagram for automated single-cell analysis. Calibration steps that require user input are performed prior to the start of the experiment. Once a list of microwells to be analyzed is defined, the software automates all subsequent steps, stage positioning, cell lysis, cell-content loading into the capillary, electrophoretic voltage application, and analyte detection. The channels used to control the hardware from the DAQ are included in parentheses. Once the time T_{load} has been reached, if there are cells pending analysis, the program stops the voltage and repeats the process until no cells are remaining, then once the final peak has passed the detector (T_{final}) the program stops.

Table 1.

Critical components, sub-components, and component specifications that are commonly employed in the microscope assembly of a pulsed laser-based capillary-electrophoresis-based instrument. Note that several substitutions to the components utilized in our system are included.

Component Name	Specifications/Parameters	Additional Sub-Components
Microscope Frame	Inverted configuration, camera port, stage mounts	Objective (magnification, numerical aperture (NA), air/immersion oil, lens curvature), revolving turret, dichroic mirror (reflect pulsed laser, transmit fluorescence), filter sets (pulsed laser & fluorophore dependent)
XY-Stage	Automated and/or manual operation, programmable (RS232 and/or USB connectors), movement speed (experiment dependent; typically, 1–10 mm/s), bi-directional repeatability (experiment dependent; typically, < 20 µm)	Motion controller, encoder strips, sample-tray inserts
Camera	CCD, exposure times (ms-s), 16-bit processor (higher bit depth = higher dynamic range), pixel array size (larger = better resolution)	Data acquisition/image processing software (Image J, µ-manager), notch filter (must match pulsed laser wavelength)
Pulsed Laser	UV, visible, or IR wavelengths; solid state (e.g., Nd:YAG: 355, 532, 1064 nm), semiconductor diode, gas, excimer. Energy output per pulse (experiment dependent, typically 0.1–20 µJ), pulse widths 100 ps-1 ns.	Laser controller, cooling system (heatsink, water-cooled, air-cooled), polarizer, iris diaphragm, pinhole aperture, precision mirrors, beam expander (Keplerian or Galilean)
Miscellaneous	Optical posts, xyz-micromanipulators, z-stage, optical brackets, optical notch and/or bandpass filters, grounding wires, instrument case (fabricated from an optically opaque material)	Optical table screws, washers, nuts

Table 2.

Preparation of different photoresist formulations. All formulations are listed as weight percentages. After mixing all components, mix on a bottle roller until all resin is dissolved. Note that photoresist resins are inhalation hazards and a respirator should always be worn when handling, especially outside a fume hood. Suggested processing parameters for various feature heights are often provided by the photoresist manufacturer.

Formulation	Layer Thickness (μm)	Resin (wt %)	γ-Butyrolactone (wt %)	Photoinitiator (wt %)
1002F 10	10 – 30	49	46.1	4.9
1002F 50	25 – 100	61	32.9	6.1
1002F 100	100 – 200	64	29.6	6.4
SU-8 5	5 – 15	52	43.0	5.0
SU-8 10	10 – 30	59	36.0	5.0
SU-8 25	15 – 40	63	32.0	5.0
SU-8 50	40 – 100	69	26.0	5.0
SU-8 100	100 – 250	73	22.0	5.0

Table 3.

Typical components that might be used to construct a fluorescence detection pathway. Example specifications are designed for fluorescein-tagged compounds with an excitation wavelength near 488 nm and emission wavelength near 525 nm.

Component name	Selection considerations	Example specifications
Excitation Laser	Single wavelength, low power, continuous wave	Wavelength-488 nm Power-20 mW
Single mode fiber optic	Consistent with laser wavelength	Operating wavelength-405–532 nm
Dichroic mirror	Reflect excitation light and pass emission light	Transmission band-520–800 nm Reflection band-380–490 nm Cut-on wavelength-505 nm
Microscope objective	High NA and compatible with filter cube	Magnification-40x NA-0.65
Multi-mode fiber optic	Consistent with emission wavelength and large core diameter	Core diameter- $25 \pm 3 \mu\text{m}$ Wavelength range-400–550 nm
Photo multiplier tube (PMT)	Large dynamic range and high sensitivity	Wavelength range-300–720 nm Sensitivity-176 mA/W
Fiber optic couplers	Adjustable in three dimensions and low hysteresis	
Collimator	Couples directly to fiber optic	
Filter cube	Compatible with dichroic, couplers, objective, and collimator	

Table 4.

Fused silica capillary parameters.

Parameter	Available Options	Recommendation	Rationale
Capillary OD	90 – >3500 μm	360 μm	Dissipates heat well (reduces Joule heating); less prone to breaking
Capillary ID	1 – >2000 μm	30 – 50 μm	Minimizes physical blockages; high resistance yields low currents
Total Length	User preference	40 cm	Longer yields higher separation efficiencies and resolution
Effective Length	Total length	< 20 cm	Shorter yields higher throughput; limited by instrument design
Detection Window Size	User preference	0.5 cm	Enables facile z-positioning of window; limits window breakage

Table 5.

Data acquisition and software component parameters and considerations.

Component Name	Specifications/Parameters	Additional Parts/Components
Data Acquisition Board	At least 1 analog input channel (16 bit), additional analog digital output channels as necessary to control hardware	Computer ports and/or cables
Software	Capable of controlling hardware	