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Chemical Fixation to Arrest Phospholipid Signaling for Chemical Cytometry

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Abstract

Chemical cytometry is a powerful tool for measuring biological processes such as enzymatic signaling at the single cell level. Among these technologies, single-cell capillary zone electrophoresis (CZE) has emerged as a powerful tool to assay a wide range of cellular metabolites. However, analysis of dynamic processes within cells remains challenging as signaling pathways are rapidly altered in response to changes in the cellular environment, including cell manipulation and storage. To address these limitations, we describe a method for chemical fixation of cells to stop the cellular reactions to preserve the integrity of key signaling molecules or reporters within the cell and to enable the cell to act as a storage reservoir for the reporter and its metabolites prior to assay by single-cell CZE. Fluorescent phosphatidylinositol 4,5-bisphosphate reporters were loaded into cells and the cells were chemically fixed and stored prior to analysis. The reporter and its metabolites were electrophoretically separated by singlecell CZE. Chemical fixation parameters such as fixative, fixation time, storage solution, storage duration, and extraction solution were optimized. When cells were loaded with a fluorescent C6- or C16-PIP₂ followed by glutaraldehyde fixation and immediate analysis, $24 \pm 2\%$ and $139 \pm 12\%$ of the lipid was recoverable, respectively, when compared to an unfixed control. Storage of the cells for 24 h yielded recoverable lipid of $61 \pm 3\%$ (C6-PIP₂) and $55 \pm 5\%$ (C16-PIP₂) when compared to cells analyzed immediately after fixation. The metabolites observed with and without fixation were identical. Measurement of phospholipase C activity in single leukemic cells in response to an agonist demonstrated the capability of chemical fixation coupled to singlecell CZE to yield an accurate snapshot of cellular reactions with the probe. This methodology enables cell assay with the reporter to be separated in space and time from reporter metabolite quantification while preserving assay integrity.

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Keywords

capillary electrophoresis; phospholipid; phospholipase C; diacylglycerol; chemical cytometry; chemical fixation

1. INTRODUCTION

Phospholipids are crucial components in all living cells, serving as second messengers for signal transduction and as structural components for cell membranes [1,2]. Several lipid metabolic pathways exist in cells regulating cellular biology, although measuring the ebb and flow of information within these pathways has proven a challenge due to difficulties in analyzing lipid metabolites in single cells. One example is the G-protein coupled phospholipase C (PLC) pathway which serves a critical role in production of the lipid second messengers diacylglycerol (DAG) and inositol trisphosphate (IP₃) by metabolism of phosphatidylinositol (4,5)-bisphosphate (PI[4,5]P₂) [3,4]. This pathway acts to regulate multiple cellular functions, such as proliferation, mitogenic signal transduction, and response to external stimuli *via* its influence on downstream effectors, such as protein kinase C and intracellular calcium [1,5,6]. Moreover, multiple other metabolites upstream and downstream of PI(4,5)P₂ are known to be of importance in cellular function, but studies have been limited by a lack of appropriate technologies to make measurements in individual cells. By virtue of their diverse roles in cellular biology and dysregulation in disease states, measurement of the activity of lipid metabolizing enzymes, including PLC, would be of great utility in understanding the molecular mechanisms driving many cellular behaviors.

However, measurement of metabolites within the PLC pathway has proven very challenging because of their relatively low abundance in cells, the polar nature of the lipids, and the highly acidic head group [7]. Traditionally, radiolabeling of lipids followed by either thin-layer chromatography (TLC) or high performance liquid chromatography (HPLC) has been used [8,9], but both TLC and HPLC suffer from low resolution, require large numbers of cells, and are of low sensitivity. Mass spectrometry has been used to monitor various lipid metabolites [7], but suffers from limited applicability for primary cells because of the need to derivatize these lipids and an inability to distinguish between isomers that are prevalent in phospholipid metabolism. To best quantify the low concentrations of PI(4,5)P₂ lipids and metabolites, an analytical technique capable of isomer resolution with the sensitivity to detect very low levels of lipid, preferably at the single cell level, is required.

Chemical cytometry uses highly sensitive analytical techniques which are ideally suited for measuring small quantities of analytes in single cells, such as single-cell capillary zone electrophoresis (CZE). Single-cell CZE was pioneered by Jorgenson and Kennedy in the early '80s and has enabled unique and powerful assays of the metabolic processes of single cells [10]. Single-cell CZE is often coupled with laser-induced fluorescence detection (CZE-LIF) for ultralow limits of detection of cellular components [11,12]. The benefits of single-cell analysis have become increasingly apparent in recent years as heterogeneity among cells in a population has been identified [13–15]. A variety of cellular metabolites have been assayed using single-cell CZE, including thiol-containing compounds [16], small molecules

such as glucose [17], dopamine [18], and amino acids [19], as well as measures of enzyme and peptidase activity [2023]. Single-cell CZE has also been used to study different classes of lipid metabolism in cytosolic lysates and single cells [24], such as sphingolipids [25,26], glycosphingolipids [27], and phosphatidylinositides [28].

A difficulty inherent to chemical cytometry methods is that analyzing dynamic metabolic processes can be extremely challenging [29,30]. Cells are sensitive to small changes in the environment, such as temperature, salt concentration, or pH. Signaling pathways can be rapidly altered as a cell copes with a stressor [31]; thus, cytometry techniques must not perturb the cells prior to sampling or artifacts can be generated in the measurement. This can be problematic when the cells originate from a biopsy or surgical specimen and are transported to a distant site to conduct the assay. A method for halting cellular reactions in all cells simultaneously would be of great utility for chemical cytometry measurements of single cells. The Dovichi group pioneered the use of formalin fixation for analysis of glycosphingolipids for cell preservation prior to single-cell CZE [32–35]. When exposed to fixative, amino groups of proteins are amine-crosslinked, which terminates cellular reactions [36–39]. Depending on their size, molecules that are not physically cross-linked can either be entrapped within the cross-linked framework or diffuse out of the cell. Lipids without amino groups, such as the phosphatidylinositide lipids, are not cross-linked by common fixatives but are frequently retained within the cross-linked framework, leaving them accessible for interrogation by an appropriate analysis technique [40–43].

A goal of this work was to explore whether chemical fixation might be coupled to assay of phospholipid metabolism in single cells followed by single-cell CZE. Multiple fixatives were assayed for their impact on the phospholipid probe retention and metabolism. Parameters optimized include the fixation time, extraction solution, and cell storage solution time prior to the CZE assay. Electrophoretic separation buffers capable of resolving hydrophobic phosphatidylinositides from fixed cells were evaluated. The ionophore ionomycin increases free intracellular calcium which stimulates PLC activity. The metabolism of PI(4,5)P₂ to DAG by PLC was characterized in basal and agonist-stimulated cells, both fixed and unfixed. Lastly, formation of DAG was characterized in stored, fixed, single cells via single-cell CZE revealing the heterogeneity in PLC activity in a population of cells.

2. MATERIAL AND METHODS

2.1 Materials

Bodipy-labeled phosphatidylinositol 4,5-bisphosphate with a 6-carbon or 16-carbon acyl chain (C6-PIP₂ or C16-PIP₂), bodipy-labeled phosphatidylinositol 3,4,5-trisphosphate with a 6-carbon or 16-carbon acyl chain (C6-PIP₃ or C16-PIP₃, chemical structures shown in Supplemental Figure S1), H1 histone protein, active SHIP2 lipid phosphatase, and SHIP2 reaction buffer were purchased from Echelon Biosciences (Salt Lake City, UT). Roswell Park Memorial Institute media (RPMI-1640) was procured from Cellgro (Manassas, VA). Fetal bovine serum (FBS) was bought from Atlanta Biologicals (Flowery Branch, GA). Penicillin/streptomycin was purchased from Gibco (Grand Island, NY). Glutaraldehyde was obtained from Sigma-Aldrich (St. Louis, MO). Formaldehyde was purchased from

ThermoScientific (Waltham, MA). The glyoxal-containing fixative Prefer was procured from Anatech Ltd (Battle Creek, MI). Ionomycin was obtained from EMD Millipore (Billerica, MA). All other chemicals were purchased from Fisher or Sigma.

2.2 Cell Culture

K-562 cells (human chronic myelogenous leukemia lymphoblasts) were obtained from the American Type Culture Collection (ATCC) [44]. Cells were propagated in RPMI-1640 media supplemented with 10% FBS, penicillin (100 units mL⁻¹), and streptomycin (100 µg mL⁻¹) and were maintained in a humidified atmosphere of 37 °C in 5% CO₂ and passaged into fresh media every 3–4 days. Cells were not used beyond passage #10 from the original ATCC stock.

2.3 Generation of C16-PI(3,4)P₂ Standard

C16-acyl chain phosphatidylinositol 3,4-bisphosphate, C16-PI(3,4)P₂, was generated by reaction of 10 µM C16-PIP₃ with 78 nM active SHIP2 phosphatase in SHIP2 reaction buffer for 60 min. Complete conversion from C16-PIP₃ to C16-PI(3,4)P₂ was verified with capillary electrophoresis.

2.4 Capillary Zone Electrophoresis for Separation Buffer Optimization

For the separation buffer optimization experiments, the background electrolyte utilized was NaH₂PO₄, with other components modified as indicated. CZE-LIF was performed on a ProteomeLab PA800 (Beckman Coulter; Brea, CA). Fused silica capillaries [30 µm inner diameter and 360 µm outer diameter (Polymicro Technologies; Phoenix, AZ)] were 30 cm long with a 20 cm effective length. Capillaries were conditioned prior to use by rinsing for 1 h in deionized H₂O, 12 h in 0.1 M NaOH, 1 h in H₂O, 6 h in 0.1 M HCl, and 12 h in H₂O. Prior to each run, the capillary was rinsed with 1 M NaOH, H₂O, and separation buffer for 2 min each by application of 20 psi to the capillary inlet. The standard sample containing C16-PIP₂, C16-PI(3,4)P₂, and C16-PIP₃ was hydrodynamically injected for 5 sec by application of 0.5 psi to the capillary inlet and electrophoretic separation was initiated by application of a negative voltage to the outlet electrode while holding the inlet at ground. Field strength varied for each separation buffer sampled; the working voltage was determined by generation of an Ohm's plot with the highest voltage before Joule heating was observed selected as the operating voltage for each separation buffer. Each experiment was repeated at least three separate times. Electropherograms were plotted and analyzed utilizing OriginLab 9.0 (OriginLab Corporation; Northampton, MA). Resolution, R , between peaks was determined using: $R = [2(t_{m2} - t_{m1})]/(w_1 + w_2)$, where t_m is migration time and w is peak width at the baseline. The number of theoretical plates, N , was determined using $N = 5.54(t_m/w_{1/2})^2$, where t_m is the migration time and $w_{1/2}$ is the peak width at half-height.

2.5 Capillary Zone Electrophoresis for Fixed Samples

CZE-LIF for all fixed samples and single cells was performed on a custom-built system mounted to the stage of an inverted microscope, described in detail in Proctor *et al.* [45]. Fused silica capillaries [30 µm inner diameter and 360 µm outer diameter (Polymicro Technologies; Phoenix, AZ)] were 38 cm long with a 20.5 cm effective length and were

conditioned prior to use as described in section 2.4. Prior to each run, capillaries were rinsed with 1 M NaOH, H₂O, for 5 min each and with separation buffer for 10 min by application of pressure to the capillary outlet. Separation buffer at the capillary inlet and outlet was completely refreshed prior to each electrophoretic run. The composition of the separation buffer was 80 mM NH₂PO₄, pH 6.8 + 15% 2-propanol. A field strength of 210 V cm⁻¹ was used for all separations. Internal standards and bulk analysis samples were hydrodynamically loaded by raising the inlet 3 cm relative to the outlet and holding the capillary inlet in the sample for 10 sec. The inlet was then lowered to the height of the outlet and electrophoresis was initiated by application of a negative voltage to the outlet while grounding the inlet. Single cells were electrokinetically loaded into the capillary inlet by firing a single pulse (5 ns) from an Nd:YAG laser near the cell and simultaneously applying -15 kV to the capillary outlet for 2 sec while the inlet was grounded. After a cell was visually observed to enter the capillary, the HiLyte internal standard diluted in 2-propanol was hydrodynamically injected into the capillary. This sample was incubated for 1 min to achieve extraction of the lipid from the fixed cell by the 2-propanol. After 1 min incubation, the inlet was transferred to fresh separation buffer and electrophoresis initiated as described above. Electropherograms were plotted and analyzed utilizing OriginLab 9.0.

2.6 Optimization of Bulk Fixation of K-562 Cells

Glutaraldehyde (5%) in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.75 mM KH₂PO₄, pH 7.4) was prepared from a stock of 25% glutaraldehyde in water (Sigma-Aldrich; St. Louis, MO), aliquoted, and stored at -20 °C until use. A fresh 5% aliquot was used for each experiment and unused portions were discarded. A 5% solution of formaldehyde in PBS was prepared from a stock ampule of 16% (w/v) solution of paraformaldehyde in water (ThermoFisher Scientific; Grand Island, NY) and was stored at room temperature between uses. The glyoxal-containing fixative Prefer was used without further dilution.

K-562 cells (1 million per each condition) were rinsed with extracellular buffer [ECB; 135 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES), 10 mM glucose, pH 7.4, 37 °C] to remove culture media. All centrifugation of cells was performed at 800×*g*. Equal concentrations of lipid and H1 histone protein were mixed to a final concentration of 50 μM H1 histone, 25 μM C6- or C16-PIP₂, and 25 μM C6- or C16-PIP₃ and incubated in the dark for 10 min at 25 °C to allow the histone:lipid complex to form. The histone:lipid complex was diluted with ECB to a final concentration of 5 μM lipid (2.5 μM PIP₂ and 2.5 μM PIP₃) and was mixed with the K-562 cells. In some instances, only the PIP₂ or the PIP₃ lipid was used, to a final concentration of 5 μM. The cells were incubated with the histone:lipid complex for 10 min at 37 °C. After incubation, cells were rinsed with ECB and transferred to a clean tube to ensure no lipid adsorbed to the tube was sampled. Cells were suspended in 50 μL of fixative per 1 million cells and incubated in the dark for the indicated time at 25 °C. Cells were rinsed twice with 50 μL (per 1 million cells) of PBS + 10 mM glycine to quench any fixative remaining. To quantify the amount of lipid lost from the cells during various wash steps following fixation, the supernatant solution generated by each wash was assayed for the total fluorescent lipid present. Cells were resuspended in 50 μL (per 1 million cells) of storage solution and

incubated at 4 °C for the indicated time. After storage, cells were re-suspended and transferred to a clean tube to ensure that lipid extracted in the following step was coming from the cells and not the tube walls. After centrifugation, cells were re-suspended in 5 μL (per 1 million cells) of extraction solution and incubated for 1 min to extract lipid from the fixed cells. Complete separation buffer was added to a final volume of 50 μL per 1 million cells and the solution was centrifuged at $14,000\times g$ to pellet cell debris. The supernatant was collected, flash frozen in liquid nitrogen, and stored at $-80\text{ }^{\circ}\text{C}$ until immediately prior to CZE analysis. For determination of the amount of lipid adsorbed to storage vessel walls, the storage tube was rinsed 2 times with PBS and 5 μL 2-propanol was added for 1 min to remove lipid metabolites from the wall. 45 μL of complete separation buffer was added, the total volume was collected and flash frozen in liquid nitrogen, and samples were stored at $-80\text{ }^{\circ}\text{C}$ until immediately prior to CZE analysis.

2.7 Optimized Fixation Procedure for Bulk and Single-Cell Analysis

K-562 cells were serum-starved overnight in RPMI-1640 media containing 0.2% FBS. Cells (2 million per each condition) were rinsed with ECB to remove culture media. Cells were centrifuged and re-suspended in low-serum media. Ionomycin-challenged cells were incubated with ionomycin (5 μM) for 1 min at 37 °C. Ionomycin (5 μM) was then added to all buffers contacting these cells until the fixation step. Control cells were incubated (1 min at 37 °C) with a volume of DMSO in ECB equivalent to that used for the ionomycin addition. DMSO at the same vol/vol concentration was then added to all buffers contacting these cells until the fixation step. Cells were rinsed with ECB to remove culture media. Equal volumes of lipid and H1 histone protein were mixed to a final concentration of 50 μM H1 histone and 50 μM C16-PIP₂ and incubated in the dark for 10 min at 25 °C to allow the histone:lipid complex to form. The histone:lipid complex was diluted with ECB to a final concentration of 10 μM C16-PIP₂ and was mixed with the K-562 cells. The cells were incubated with the histone:lipid complex for 5 min at 37 °C. After incubation, cells were rinsed with ECB and transferred to a clean tube to ensure no lipid was adsorbed to the tube wall. Cells were suspended in 50 μL of 5% glutaraldehyde in PBS and incubated in the dark for 10 min at 25°C. Cells were rinsed twice with PBS + 10 mM glycine to quench any fixative remaining. For unfixed cell controls, the fixation and rinse steps were eliminated. A two-factor ANOVA for both ionomycin treatment and fixation was used to compare the different treatments. For single-cell analysis, fixed cells were stored in storage solution (80 mM NaH₂PO₄, pH 6.8) and stored at 4 °C until analysis. All samples were analyzed within 7 hours of fixation, from four independent experiments. For bulk lysis of fixed and unfixed cells, cells were re-suspended in 5 μL of 2-propanol and incubated for 1 min. Complete separation buffer was added to a final volume of 50 μL per 2 million cells and the solution was centrifuged at $14,000\times g$ to pellet cell debris. The supernatant was collected, flash frozen in liquid nitrogen, and stored at $-80\text{ }^{\circ}\text{C}$ until immediately prior to CZE analysis.

Single-cell analysis chambers were prepared by using poly(dimethylsiloxane) (PDMS, Sylgard 184; Dow Corning; Midland, MI) to glue a silicon O-ring to a #1 glass coverslip (Fisher Scientific; Pittsburgh, PA). Fixation of cells to be used for single-cell analysis was performed as described above for bulk cell analysis up through the point of storage at 4 °C. Between 25,000 and 50,000 fixed cells were added to 500 μL of storage solution in the

single cell chamber and imaged on the inverted microscope of the single cell CZE-LIF system. A single cell was selected for analysis if it was more than 50 μm away from any other cells and was loaded into the capillary as described in section 2.5. After the cell was loaded into the capillary, the HiLyte internal standard (mixed isomers) diluted in 2-propanol was hydrodynamically loaded (3 cm height difference for 10 sec) into the capillary inlet and incubated for 1 min. The inlet was then immersed in CZE separation buffer and electrophoresis initiated. Remaining fixed single cells were stored in the dark at 4°C until immediately prior to CZE analysis.

2.8 Statistical Analyses

GraphPad Prism software, v.7 (La Jolla, CA) was used for all statistical analyses. For analysis of variance when comparing different fixative parameters, a one-factor ANOVA was used and followed with Tukey's test for multiple comparisons. For analysis of variance when comparing the bulk lysates of fixed and not fixed cells, with and without ionomycin treatment, a two-factor ANOVA was used and followed with a Holm-Šidák test for multiple comparisons. The non-parametric Kruskal-Wallis test for analysis of variance was used to analyze differences in metabolite formation between single cells with and without ionomycin treatment. Median values with the first and third quartiles (calculated in Microsoft Excel) are presented for all single cell data.

3. RESULTS AND DISCUSSION

3.1 Optimization of electrophoretic separation buffer

Numerous products can be generated when $\text{PI}(4,5)\text{P}_2$ is metabolized in cells. These products include diacylglycerol (DAG), phosphatidylinositol (PI), three isomers of phosphatidylinositol-phosphate (PIP_1s), three isomers of phosphatidylinositol-bisphosphate (PIP_2s), and phosphatidylinositol-3,4,5-trisphosphate (PIP_3) [46]. In order to best understand which reactions are occurring in a cellular reaction environment, a separation technique capable of resolving all possible products is required. CZE possesses the separation capacity to resolve all of these different lipid forms including the isomers [46–48]. A previous report detailed the use of a sodium phosphate separation buffer to separate the products of a six-carbon acyl chain PIP_2 (C6-PIP_2) reaction [49]; however, this separation buffer failed to separate C16-PIP_2 and its metabolites. Two isomers, the $\text{C16-PI}(3,4)\text{P}_2$ and $\text{C16-PI}(4,5)\text{P}_2$ were not well resolved, with a resolution of 1.29 ± 0.05 (Figure 1A). To achieve robust baseline separation of all possible C16-PIP_2 reaction products, including isomers, the separation buffer composition was optimized, including salt concentration, pH, and additives. A sample mixture with ($\text{C16-PI}(3,4)\text{P}_2$, $\text{C16-PI}(4,5)\text{P}_2$, and C16-PIP_3) was utilized for the separation buffer screen (Supplemental Information). Two separation buffers (200 mM NaH_2PO_4 , pH 8.3 +15% 2-propanol and 80 mM NaH_2PO_4 , pH 6.8 + 15% 2-propanol) demonstrated improved resolution relative to the starting separation buffer (Figure 1B–D). The high salt separation buffer (200 mM NaH_2PO_4) showed a 2-fold improvement in resolution between the two PIP_2 isomers ($R_{\text{PIP}_2\text{s}}$), but a 1.6-fold reduction in resolution between $\text{C16-PI}(4,5)\text{P}_2$ and C16-PIP_3 (R_{PIP_2-3}) with baseline separation of all analytes. Compared to the original separation buffer, the low salt separation buffer (80 mM NaH_2PO_4) also showed a 2-fold improvement in $R_{\text{PIP}_2\text{s}}$, coupled with a 2-fold improvement

in R_{PIP_2-3} . The low-salt separation buffer provided the best resolution of the different phosphatidylinositides and was used for subsequent separations.

3.2 Determination of appropriate fixative

Prior work suggests that phosphatidylinositides are retained within the cell framework following aldehyde fixation since these lipids do not participate in the cross-linking reaction and prefer the cellular milieu to that of the surrounding high-salt aqueous buffer (Figure 2A) [40–43]. Formaldehyde is the simplest aldehyde, working on fast time scales in thin samples to cross-link amino groups [36]. Glutaraldehyde is also fast-acting and is reported to generate more extensive cross-linking than formaldehyde [50]. Glyoxal, a milder fixative than formaldehyde and glutaraldehyde, offers less extensive cross-linking and is considered the least hazardous of the three fixatives due to its high evaporation point [39,42]. To determine which of these fixatives would be optimal for preserving the PIP_2 metabolites within the cells, the fluorescent lipid was loaded into and incubated within the cells followed by addition of fixative (Figure 2B). The amount of lipid retained in the fixed cells after all wash steps was quantified by solubilizing the cells in bulk and analyzing the resulting supernatant with CZE-LIF (labeled “extraction” in Figure 2B). For each fixative, a short acyl chain lipid (C6- PIP_2) and a longer, more hydrophobic acyl chain lipid (C16- PIP_2) were evaluated to determine the optimal conditions for both the long- and short-chain lipid metabolites.

For the C6 acyl chain lipid metabolites, most of the lipid was lost into the fixation solution itself (Figure 2C). After the initial loss of lipid, little was lost in the subsequent steps. When compared to an unfixed control sample, $23 \pm 6\%$, $24 \pm 2\%$, $36 \pm 8\%$ of C6 metabolites were extracted from the cells for formaldehyde, glutaraldehyde and glyoxal fixation, respectively. While the fixed cells did retain some of the lipid, the relatively polar nature of the C6 lipids enabled most of these lipids to diffuse into the bulk fixative solution. For the C16 lipid metabolites, there was no difference in the amount of lipid extracted from the fixed cells when compared to the non-fixed control ($P = 0.2019$; Figure 2D). Approximately 10% of lipid was lost during the three steps prior to extraction. The more hydrophobic C16 acyl chain likely helped retain the lipid metabolites during fixation and wash, as partitioning into the aqueous, higher salt solution is energetically unfavorable for hydrophobic compounds. The C16 chain also increases the lipid size and may act to hinder diffusion through the cross-linked protein network within the cells. Glutaraldehyde was selected as the fixative in subsequent studies due to its fixation efficiency, ability to release entrapped phosphatidylinositol molecules in response to extraction, and its long-term stability in solution [51].

3.3 Optimization of fixation time

Due to their small volume and large surface-to-volume ratio, the fixation time for single mammalian cells is expected to be rapid [38]. Extensive cross-linking during fixation must be avoided since this may interfere with lipid extraction. To determine the optimal fixation time, glutaraldehyde was added to cells for 1, 5, 10, or 20 min followed by washing steps and fluorescent lipid extraction (Supplemental Figure S8). For the fluorescent C6 lipid metabolites, significantly more lipid was extracted after 1 min of fixation than at the longer

fixation times ($P < 0.05$). For fixation times of 10 min or less, the amount of C16 lipid extracted from the cells was not statistically different (Figure 2D). However, fixation for 20 min showed a significant decrease in the amount of fluorescent lipid that could be obtained from the cells ($P < 0.05$). Based on these results, a fixation time of 10 min was used for all subsequent experiments.

3.4 Selection of extraction solution

Selection of an appropriate solution to release or extract the fluorescent lipids from the fixed cell is key to obtaining maximal and unbiased lipid metabolite recovery. The extraction solution should also recover all metabolites in the same relative abundance as present in the unfixed cells, act on rapid timescale (minutes or less), and have minimal impact on electrophoretic separations. Surfactants, such as sodium dodecyl sulfate (SDS) or Brij, in which lipids are highly soluble; non-ionic surfactants such as NP-40 or Tween-20, commonly used in biological applications to solubilize lipids; or organic compounds in which lipids are soluble, such as ethanol or propanol were all candidate reagents for the extraction buffer.

Cells were loaded with fluorescent lipid, fixed, rinsed, and the lipid extracted by incubating the cells with an extraction solution for 1 min (Figure 3). For both the C6 and C16 lipid metabolites, there was a wide range of lipid recovery efficiencies, depending on the extraction solution used. For the C6 lipid, the poorest performer (the CZE separation buffer for the lipids) recovered 18× less lipid than the highest performer (NP-40). This difference was even more dramatic for the C16 lipid, where the worst performer (CZE separation buffer again) recovered 84× less lipid than the highest performer (SDS). For the C6 lipid metabolites, there was no statistical difference in the amount of lipid extracted using the top four extraction solutions; however, two of these solutions had adverse effects on the CZE separation when used as the sample solution, yielding a single peak containing all metabolites (10% NP-40) or broadening the peaks and reducing resolution between metabolite peaks (Tween 20). In contrast, neither Triton X-100 or 2-propanol significantly impacted the quality of the CZE separation for the C6 lipid. For the C16 lipid, there was again no statistical difference in the top four extraction solutions, based on the total amount of lipid recovered. However, both the Triton X-100 and SDS severely impacted CZE separation quality such that only one or two peaks were present (SDS) or peaks were greatly broadened (Triton X-100). Both the 2-propanol and the 1-propanol had minimal impact on the CZE separation when used as the sample buffer. For the current studies, 2-propanol was selected as the extraction solution for both the C6 and C16 lipids since lipid from fixed cells was efficiently recovered with little impact on subsequent electrophoretic separations.

3.5 Storage of fixed samples

Fixation has the potential to terminate chemical reactions within a cell enabling the products and remaining substrate in that cell to be preserved for analysis at a later time. The buffer in which the sample is stored after fixation and prior to reporter quantification is of paramount importance. Uncross-linked metabolites, such as the lipids, must remain entrapped or stored within each cell. Judicious selection of the storage buffer solution can be used to create an environment external to the cells that is energetically unfavorable for lipid metabolites.

Three aqueous buffer solutions were assessed for their ability to act as a storage buffer for the fixed cells: two high salt neutral pH buffers (extracellular buffer [ECB] and phosphate buffered saline with glycine [PBS-glycine]) and the aqueous component of the optimal CZE buffer (80 mM NaH_2PO_4 , pH 6.8). Cells were loaded with fluorescent lipid, fixed, and stored in one of the three solutions for up to 24 h at 4 °C (Supplemental Figure S9). After storage, the storage solution was removed and the fluorescent lipid metabolites were extracted with 2-propanol. The storage solution supernatant and the cell-extracted solution were assayed for fluorescent lipids by CZE. Additionally, the wall of the storage vessel was rinsed with 2-propanol and then assayed to determine whether lipid was reversibly adsorbed to the wall during the long storage time.

For the C6 acyl chain lipids, there was no statistical difference in the total amount extracted from the cells over time or amongst the three storage buffers (Figure 4A, Supplemental Figure S10 A–C). In each case, very little (< 10%) of the lipid was found in the storage solution or adhered to the vessel wall over the time period examined. All three storage solutions worked equally well for fixed cells containing the C6 fluorescent lipids when cells were stored for times up to 24 h. For the C16 lipid, the recovered fluorescent lipid for cells stored in 80 mM NaH_2PO_4 ($P = 0.001$) and ECB ($P = 0.05$) for 24 h was statistically different from that recovered at 0 h (Figure 4B, Supplemental Figure S10 D–F). The PBS-glycine storage solution showed no statistical difference in the amount of lipid recovered at 0 h and 24 h. Based on these results, the PBS-glycine solution appears to be the better storage solution for fixed cells loaded with the C16 lipid. However, as the relative amounts of lipid recovered were the same over time, any of the three storage solutions tested would be acceptable choices for storage up to 24 h after fixation. Though acceptable for most cases, in this case, the PBS-glycine storage solution resulted in slight peak broadening during CZE, so the background electrolyte storage solution, sodium phosphate, was used for sample storage.

3.6 Comparison of fixed and unfixed cells analyzed in bulk

Comparison of fluorescent lipid recovery from fixed versus unfixed cells was performed to determine whether fixation altered the metabolites produced within the cells. Serum starved leukemic cells (K-562 cells) were incubated with or without ionomycin followed by loading with C16-PIP₂. Ionomycin increases the intracellular free calcium concentration activating calcium-sensitive PLC activity leading to the metabolism of PI(4,5)P₂ to form the lipid DAG [52]. Cells were then incubated with or without glutaraldehyde and the fluorescent lipid metabolites were extracted with 2-propanol and the percentage of C16-DAG with respect to total fluorescent lipid was quantified (Figure 5A). For both fixed and unfixed cells, there was a statistically significant increase in fluorescent C16-DAG formation in ionomycin-treated cells compared to cells not treated with this ionophore ($P = 0.01$). There was, however, no statistically significant difference between fixed and unfixed cells when comparing the untreated cells or the ionomycin-treated cells ($P > 0.05$ in both cases). These results demonstrated that fixation of the cells did not alter the results of the PLC reaction and that fixation is compatible with accurate measurement of fluorescent C16-PIP₂ metabolism to C16-DAG in cells.

3.7 Comparison of fixed and unfixed single cells

To determine if individual cells could be fixed and subsequently analyzed, serum-starved K-562 cells were incubated with or without ionomycin, incubated with C16-PIP₂, and fixed with glutaraldehyde. Cells were stored at 4 °C in the 80 mM NaH₂PO₄ storage solution until individual cells were analyzed by single-cell CZE (stored up to 7 hours, Figure 5B–D). One to four peaks were observed on the electropherograms with the earliest migrating peak identified as C16-DAG (peak 1) and the latest migrating peak identified as C16-PI(4,5)P₂ (peak 4). The two additional peaks were likely the other metabolic products of C16-PI(4,5)P₂, C16-PI and C16-PIP₁, based on their migration times. There was a statistically significant greater production of C16-DAG in ionomycin-treated, fixed cells ($n = 10$) compared to untreated fixed cells ($n = 11$, $P = 0.0024$, Figure 5B). In the ionomycin-treated cells, a median value of 100% of the fluorescent lipid was present as C16-DAG (Q_1 and Q_3 of 52% and 100%). In the untreated cells, a median value of 7% of the fluorescent lipid was present as C16-DAG (Q_1 and Q_3 of 4% and 34%). Storage time after fixation did not appear to affect metabolite quantity over time, as cells analyzed shortly after fixation showed similar patterns to cells analyzed several hours later. In the control cell population, all cells possessed some C16-PIP₂ (11 of 11), whereas in the ionomycin-treated cells, the C16-PIP₂ was completely metabolized to C16-DAG in most cases (7 of 10). In those cases where C16-PIP₂ was not converted fully to C16-DAG, two additional metabolites were present, demonstrating the heterogeneity expected when individual cells are interrogated. These results establish that PLC activity in single cells can be measured after fixation to quantify the amount of C16-PIP₂ metabolites produced in single cells.

4. CONCLUSIONS

The dynamic processes of phosphatidylinositol lipid signaling were measured in single cells using chemical fixation prior to single-cell CZE. A single lipid reporter, PI(4,5)P₂, can be converted into multiple products within cells, yielding a fuller view of metabolic activity within cells. By adjusting the salt composition, concentration, pH, and organic modifier of the separation buffer, a CZE separation capable of baseline resolution between the lipid metabolite products, including isomers, was achieved. Furthermore, a method to halt metabolic reactions within all cells in a sample prior to chemical cytometry analysis was developed. By adjusting the chemical fixative, duration of fixation, storage solution, storage time, and extraction solution employed, an optimal method for cell fixation followed by phosphatidylinositol metabolite measurement was developed. To demonstrate the applicability of the described method, PI(4,5)P₂ conversion to DAG by PLC was examined. For cells analyzed in bulk, there was no difference in fixed cells compared to their unfixed counterparts. When the ionophore ionomycin was used to stimulate PLC activity, there was a statistically significant increase in the amount of DAG produced compared to a control population, both for the fixed population and the unfixed control cells. Taken together, these results demonstrate that fixation does not alter the experimental outcome. This was also true when individual cells were analyzed—ionomycin-treated single cells showed statistically significant higher levels of DAG production than control single cells. Chemical fixation of single cells provides the ability to freeze cellular reactions in time, enabling the transport of a sample from the origination site to the analytical laboratory without additional stress to the

cells or loss of analytes. Furthermore, halting all cells at the same point by using fixation eliminates potentially confounding results originating from cells from the same sample being analyzed at different time points. Future work includes exploration into fixation processes utilizing alternative reporters for analysis of different metabolic pathways within single cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- High quality separation of phosphatidylinositol isomers was developed
- Exogenously added fluorescent phospholipid is retained in cells after fixation
- Fluorescent phospholipid was recovered from fixed cells
- Fixation stops cellular reactions for later chemical cytometry of metabolites
- Phospholipase C activity was measured in single, fixed leukemic cells

Figure 1.

Optimization of separation buffer conditions to separate C16 lipid metabolites.

Electropherograms of three analytes separated in different separation buffers; peak 1 is C16-PI(3,4)P₂, peak 2 is C16-PI(4,5)P₂, and peak 3 is C16-PI(3,4,5)P₃. Separation buffers of (A) 32 mM NaH₂PO₄, pH 7.3 + 20% 1-propanol [49], (B) 200 mM NaH₂PO₄, pH 8.3 + 15% 2-propanol, and (C) 80 mM NaH₂PO₄, pH 6.8 + 15% 2-propanol. (D) Resolution (R) and theoretical plates (N) for analyte separations in the different separation buffers.

Figure 2.

Determination of appropriate fixative. (A) Schematic of the fixation process within cells. Example contents within the cell include proteins with free amino groups and the fluorescent phospholipid reporter. When glutaraldehyde is added, the proteins are cross-linked *via* the amino groups while the phospholipids without free amino groups are not impacted. (B) Schematic of the wash steps and solutions in the fixative selection process. Samples assayed for total fluorescent lipid content by CZE are indicated in bold text in the lower part of the

panel. (C) C6 and (D) C16 total fluorescent lipid measured at each step in the fixation process. Error bars indicate one standard deviation from the mean. **** $P < 0.0001$.

Figure 3. Survey of extraction solutions for analysis of fluorescent lipid metabolites from fixed cells. Total amount of (A) C6 and (B) C16 lipid metabolites extracted with the indicated extraction solutions. Error bars indicate one standard deviation from the mean.

Figure 4.

Evaluation of storage buffers for varying storage times for (A) C6 and (B) C16 fluorescent lipid. The total amount of fluorescent lipid is normalized to that extracted after 0 h of storage. Error bars indicate one standard deviation from the mean. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ when compared to the respective 0 h sample.

Figure 5.

Comparison of metabolites from fixed and unfixed cells. (A) Comparison of DAG production in control and ionomycin-treated cells with and without fixation. Cells were extracted in bulk and then assayed, **P < 0.01. Error bars indicate one standard deviation from the mean. (B) Boxplots of fluorescent C16-DAG and C16-PI(4,5)P₂ extracted from fixed single cells, control (*n* = 11) or ionomycin-treated (*n* = 10). Box upper limits represent the 3rd quartile, lower limits represent the 1st quartile, and the solid line indicates the median. Whiskers extend to maximum values not considered outliers, data points outside the whiskers are outliers. Symbols are defined as follows: solid black diamond (C16-DAG in control cells), solid black circle (C16-DAG in ionomycin-treated cells), open red diamond (C16-PI(4,5)P₂ in control cells), open red circle (C16-PI(4,5)P₂ in ionomycin-treated cells), **P < 0.01, ****P < 0.0001. (C) Example single cell electropherogram from a single, fixed basal cell and (D) ionomycin-treated cell. The doublet peak marked with a * is an internal standard, peak 1 is C16-DAG, peaks 2 and 3 are lipid metabolites, and peak 4 is C16-PI(4,5)P₂.