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“Fix and Assay”: Separating in-cellulo sphingolipid reactions from analytical assay in time and space using an aldehyde-based fixative

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Abstract

Chemical cytometry using capillary electrophoresis (CE) is a powerful tool for measuring single-cell enzyme activity. However, these measurements are often confounding as dynamic processes within cells rapidly change depending on environment, meaning that cell handling, transport, and storage can affect signaling pathways and alter results. To meet these challenges, we describe a method utilizing aldehyde fixation to simultaneously terminate cellular reactions across a population, freezing reaction results in time prior to analytical analysis. Fluorescent sphingosine was loaded into cells of different lineages (leukemia and lymphoma cell lines and primary leukemia cells) and allowed to react before fixing. The remaining sphingosine and any products formed were then quantified with chemical cytometry utilizing CE. When cells were loaded with sphingosine followed by glyoxal fixation and immediate analysis, $55 \pm 5\%$ of lipid was recoverable compared to an unfixed control. Storage of fixed cells for 24 h showed no statistical differences in total amount of recoverable sphingolipid compared to samples analyzed immediately after fixation—though there was a difference in recovery of low-abundance products. Sphingosine kinase activity decreased in response to inhibitor treatment compared to treatment with a DMSO vehicle ($21 \pm 3\%$ product formed in inhibitor-treated cells vs $57 \pm 2\%$ in control cells), which was mirrored in single-cell measurements. This “fix and assay” strategy enables measurement of sphingosine kinase activity in single cells followed by subsequent analytical assay separated in space and time from reaction initiation, enabling greater temporal control over intracellular reactions and improving future compatibility with clinical workflow.

Graphical Abstract

TOC Figure: Fixation of fluorescent sphingolipid-loaded cells enables cell metabolism and assay readout to be separated in time and space.

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Conflicts of Interest

There are no conflicts to declare.

Introduction

Sphingolipids are bioactive lipids responsible for modulating a wide variety of cellular functions, including cell proliferation, differentiation, migration, and programmed cell death.^{1, 2} The first sphingolipids were isolated in the late 1800s, but much about their structure and function was unknown until relatively recently—within the past 40 years.¹ Three of the most-studied sphingolipids are ceramide, sphingosine, and sphingosine-1-phosphate, which, along with several other bioactive lipids, comprise a network balancing cellular survival with apoptosis.^{1, 3, 4} Increased concentrations of sphingosine and ceramide relative to sphingosine-1-phosphate direct cells towards senescence while decreased relative concentrations are thought to greatly enhance cellular survival mechanisms.^{1, 5, 6} Ceramide is converted to sphingosine by the actions of ceramidases while sphingosine is metabolized to sphingosine-1-phosphate by sphingosine kinases 1 and 2.⁷ Since sphingosine-1-phosphate supports cell proliferation, sphingosine-1-phosphate levels are tightly controlled by regulating both synthesis (via sphingosine kinases) and degradation (via lyases). An altered balance in the levels of these key sphingolipids is a hallmark of multiple diseases, including multiple sclerosis and cancers such as leukemia and lymphoma, where the concentrations of ceramide and sphingosine are decreased relative to sphingosine-1-phosphate.^{8, 9} Both sphingosine kinase 1 and 2 are over-expressed in many cancers, for example, in T-cell large granular lymphocytic lymphoma, acute lymphoblastic leukemia, and non-Hodgkin lymphomas.¹⁰ Multiple therapeutics target the sphingosine pathway and, in particular, inhibitors directed against sphingosine kinases are in clinical trials.¹¹ Companion diagnostic assays to monitor the sphingosine pathway, particularly formation of sphingosine-1-phosphate and its metabolites at the single-cell level, would be of high utility for personalized medicine in oncology both for optimizing drug treatments as well as tracking therapeutic responses.

Measurements of sphingosine and its metabolites within single cells face a number of challenges due to the poor aqueous solubility of the lipids, low intracellular concentrations of these signaling lipids, and the absence of antibodies directed against the lipids.^{1, 12} Furthermore, these bioactive lipids form a complex metabolic network where the product of each reaction can act as the substrate for additional reactions, producing a plethora of products. A complete understanding of the pathway is likely to require simultaneous measurement of these different products. For this reason, most prior strategies to track sphingolipid signaling in cells have incorporated a separation step. Early separations techniques utilized thin-layer chromatography, but suffered from poor resolution and sensitivity.¹³ High-performance liquid chromatography coupled with mass spectrometry (HPLC-MS) has the ability to resolve sphingosine, sphingosine-1-phosphate, and other metabolites in the pathway; however, the poor detection limits of this method require large sample sizes or pooled cellular lysates as opposed to single cells as a sample input.^{13–15} A technique capable of quantitatively measuring the multiple bioactive sphingolipids in single cells would be of utmost utility in understanding the complex nature of the sphingosine signaling network.

Chemical cytometry utilizes ultrasensitive analytical techniques to quantify chemical species within single cells.¹⁶ Capillary electrophoresis (CE) is ideally suited for chemical cytometry since CE requires low sample volumes (pL–nL), provides a large peak capacity due to high separation efficiency, and offers extremely low limits of detection (subattomole). Capillary electrophoresis coupled to laser-induced fluorescence detection (CE-LIF) has been used to quantify enzyme activity of many classes of enzymes in single cells, including protein kinases and phosphatases,^{17–20} proteases,^{21, 22} and lipid-metabolizing enzymes, such as phosphatidylinositol kinases and phosphatases,^{23, 24} and glycotransferases.^{25, 26}

Chemical cytometry utilizing fluorescent sphingosine reporters has been used to quantify sphingolipid signaling in multiple cell types, including cultured cell lines and primary patient samples.^{27, 28} In this strategy, cells were loaded with the fluorescent sphingosine and the cells remained metabolically active until they were sequentially assayed using CE-LIF, achieving throughputs reaching 50 cells h⁻¹. A consequence of this serial assay strategy, however, was that the reaction time for the fluorescent lipid was unique for each cell. The different reaction times for the fluorescent sphingosine with the intracellular enzymes in each cell complicated attempts to understand the signaling heterogeneity within the cell population. A method to simultaneously terminate the chemical reactions within all cells and at the same time preserve the reactants and products within the cell for subsequent assay would greatly increase the versatility and impact of the CE-LIF-based assay. Dovichi and colleagues used formalin-based fixation of cells loaded with fluorescent glycosphingolipid to stop the chemical reactions of these lipids within cells and to measure their intracellular metabolism.^{25, 26, 29–31} Building on this strategy, Proctor and colleagues described a method for analyzing phosphatidylinositol lipids within fixed cells, demonstrating that glutaraldehyde was the best fixative to reliably preserve the phosphatidylinositol metabolites within the framework of a cross-linked cellular environment.²⁴ However, unlike the glycosphingolipids and phosphatidylinositol lipids, sphingolipids possess a free amine group that might be irreversibly cross-linked to cellular proteins by the fixative, making these analytes unavailable for downstream analysis.

The work described herein focuses on investigating the feasibility of utilizing CE-LIF in a “fix and assay” format to analyze metabolism of fluorescent sphingosine in single, fixed cells with a goal of developing a strategy to enable a common start and stop time for the reactions in all cells. Three fixatives were surveyed to determine their ability to entrap the sphingosine and its metabolites within fixed cells after completion of a designated intracellular incubation time. The fixation time, lipid-extraction conditions, and lipid losses were assessed for intracellular sphingolipid measurements. Once these parameters were optimized, metabolism of sphingosine into downstream bioactive metabolites was measured in single, fixed cells to reveal the signaling heterogeneity in cell populations treated with a sphingosine kinase inhibitor. This work lays the foundation for further development of chemical fixation to halt sphingolipid metabolism in a cellular population and then to provide a snapshot of the signaling heterogeneity in single cells using chemical cytometry.

Experimental

Materials

Sphingosine fluorescein (SF; #S-100F) and sphingosine-1-phosphate fluorescein (S1PF; #S-200F) were purchased from Echelon Biosciences (Salt Lake City, UT). Roswell Park Memorial Institute Media (RPMI-1640; #10-040-CV) was obtained from Cellgro (Manassas, VA). Fetal bovine serum (FBS; #S11150) was procured from Atlanta Biologicals (Flowery Branch, GA). Penicillin/Streptomycin (#15140-122) and Dulbecco's Phosphate Buffered Saline (DPBS; #14190-144) were from Gibco (Grand Island, NY). Poly(dimethylsiloxane) Sylgard 184 Silicon (PDMS; #2085925) was obtained from Dow Corning (Midland, MI). Prefer (#410) was purchased from Anatech, Ltd. (Battle Creek, MI). 200 proof ethanol (#2701) was from Decon Labs (King of Prussia, PA). Sphingosine Kinase Inhibitor 2 (SKI II; #10009222) was procured from Cayman Chemical Company (Ann Arbor, MI). Sodium chloride (#S7653), potassium chloride (#P9333), magnesium chloride (#M1028), calcium chloride (#21115), glucose (#G7021), Trypan Blue (#T8154), and 25% glutaraldehyde (#G5882) were purchased from Sigma-Aldrich (St. Louis, MO). Fixable Viability Dye eFlour 660 (FVD 660; #65086414), 30% human serum albumin (HSA; #12667), Dextran 40 (#A22490100), 16% paraformaldehyde (#AA433689L), monosodium phosphate (#BP329), 1-propanol (#A414), methanol (#A412), 2-propanol (#A416), 1-methoxy-2-propanol (#AA41547AK), and microscope coverglass (#12-544-F and #12-545-102) were from Fisher Scientific (Pittsburgh, PA).

K-562 and U-937 Cell Culture

FBS was heat-inactivated prior to use by incubating at 56 °C for 25 min. Heat-inactivated samples were aliquoted into 50 mL volumes at stored at -20 °C until use. K-562 cells (human chronic myelogenous leukemia lymphoblasts; #CCL-243) and U-937 cells (human histiocytic lymphoma; #CRL-1593.2) were obtained from the American Type Culture Collection (ATCC).^{32, 33} K-562 and U-937 cells were grown in RPMI-1640 media supplemented with 10% FBS, 100 units mL⁻¹ penicillin, and 100 µg mL⁻¹ streptomycin. Cells were maintained in a humidified atmosphere of 37 °C and 5% CO₂ and passaged into fresh media every 3–4 days. Cells were not used beyond passage #10 from the original

ATCC stock and cell lines were independently validated with short tandem repeat (STR) analysis at the DNA Analysis Facility at Duke University.

Fixation Procedure for Bulk and Single-Cell Analyses of K-562 and U-937 Cells

A 5% solution of formaldehyde 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 solution of 16% (w/v) paraformaldehyde in water. 5% glutaraldehyde in PBS was prepared from a stock solution of 25% glutaraldehyde in water, aliquoted, and stored at –20 °C until use. A fresh 5% aliquot was used for each experiment and unused portions were discarded. The glyoxal-containing fixative Prefer was used without further dilution, unless indicated otherwise.

K-562 cells or U-937 cells (1 million per each condition) were centrifuged to remove culture media. All centrifugation was performed at 300g for 1 min unless otherwise specified. For SKI II-treated cells, cells were incubated with 25 μM SKI II in culture media for 60 min at 37 °C. 25 μM SKI II was then added to all solutions contacting the cells until the fixation step. Control cells were exposed to an equal volume of DMSO in culture media equivalent to that used in the SKI II-treated cells. DMSO at the same volume/volume concentration was then added to all solutions contacting the cells until the fixation step. Cells were resuspended in 10 μM sphingosine fluorescein in culture media and incubated for 30 min at 37 °C. After incubation, cells were centrifuged and resuspended in extracellular buffer (ECB; 135 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 10 mM glucose, pH 7.4, 37 °C) to rinse cells and remove culture media. To ensure no lipid was adsorbed to the surface of the tube, cells were transferred to a fresh tube prior to centrifuging to remove the supernatant. Cells were suspended in fixative (formaldehyde, glutaraldehyde, or glyoxal) and incubated 5 min at 25 °C. Cells were rinsed twice with PBS + 10 mM glycine to quench remaining fixative. For unfixed cell controls, the fixation and PBS + glycine rinse steps were not performed. Fixed cells were stored for up to 24 h in storage solution (30 mM NaH₂PO₄, pH 7.3) at 4 °C. All single-cell samples were analyzed within 5 h of fixation, from 4 independent experiments. For bulk lysis of fixed and unfixed cells, cells were resuspended in 10 μL of 1-propanol and incubated for 1 min. An equal volume of electrophoretic separation buffer was added and the solution was centrifuged at 12,000g to pellet cell debris. The supernatant was collected, flash frozen in liquid nitrogen, and stored at –80 °C until immediately prior to electrophoretic analysis.

Single-cell analysis chambers were prepared by using PDMS to glue a silicon o-ring to a round #1 glass coverslip. Fixation of cells for single-cell analysis was performed as described above up to the point of storage at 4 °C. Between 25,000–50,000 fixed cells were added to 600 μL of the 30 mM NaH₂PO₄, pH 7.3 storage solution in the single-cell chamber and imaged on the inverted microscope of the single-cell CE system (described below). 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 below in the single-cell CE section. Remaining single cells were stored in the dark at 4 °C until immediately prior to electrophoretic analysis.

Fixation Procedure for Bulk and Single-Cell Analysis of Primary Cells

De-identified blood samples from patients with chronic lymphoblast leukemia (CLL) were obtained from the University of North Carolina Hematolymphoid Malignancies Tissue Procurement Facility (TPF) under an institutional IRB-approved protocol in accordance with the Declaration of Helsinki. All experiments were performed in accordance with the Guidelines of the University of North Carolina, and approved by the ethics committee at University of North Carolina. Informed consents were obtained from human participants of this study. The tumor cells were enriched by centrifugation with Ficoll-Hypaque followed by removal of the nucleated cell layer prior to storage at the TPF. Primary samples were transported to the lab on dry ice and immediately stored in liquid nitrogen until use. To thaw primary cells, 1 mL of 4 °C thaw mix (0.08 g mL⁻¹ Dextran 40 and 5% HSA in DPBS) was mixed with 1 mL of freshly-thawed cells and 8 mL of 4 °C culture media (RPMI-1640 + 10% FBS). Cells were centrifuged at 300g for 7 min. Supernatant was discarded and the cell pellet gently dislodged prior to adding 10 mL of 4 °C culture media and centrifuging again. Cells were resuspended in 5 mL of 4 °C culture media and kept at 4 °C until use. For experiments, cells were used within minutes of completing the thawing procedure. Cell viability was assessed using trypan blue staining.

Prior to use, CLL cells (1 million viable cells/condition) were centrifuged to remove culture media. All centrifugation was performed at 300g for 1 min unless otherwise specified. Cells were resuspended in 80 µM sphingosine fluorescein in culture media with 1:500 FVD-660 and incubated for 60 min at 37 °C. After incubation, cells were centrifuged and resuspended in 37 °C ECB. To prevent measurement of adsorbed lipid, cells were transferred to a fresh tube prior to washing. Cells were then suspended in undiluted glyoxal-based fixative and incubated 5 min at 25 °C. Cells were rinsed twice with PBS + 10 mM glycine to quench the remaining fixative. For unfixed cell controls, the fixation and PBS + glycine rinse steps were not performed. All single-cell samples were analyzed between 32 min and 4 h of fixation. For bulk lysis of fixed and unfixed cells, cells were resuspended in 10 µL of 1-propanol and incubated for 1 min. An equal volume of electrophoretic separation buffer was added and the solution was centrifuged at 12,000g to pellet cell debris. The supernatant was collected, flash frozen in liquid nitrogen, and stored at -80 °C until immediately prior to electrophoretic analysis. Single cells were analyzed as described in the procedure above for K-562 and U-937 cells. Single CLL cells were only analyzed if they were FVD 660-negative, indicating that they were viable prior to the addition of the fixative.

Capillary Electrophoresis of Cell Lysates

Capillary electrophoresis coupled with laser-induced fluorescence detection (CE-LIF) of bulk lysate samples was performed on a custom-built system mounted on top of an inverted microscope, described in detail in Mainz, *et al.*¹⁸ Briefly, a 3D-printed housing held a sample/buffer chip, electrodes, and a buffer inlet and outlet securely to the automated stage of a Ti-E microscope (Nikon; Melville, NY). CE buffer (27 mM NaH₂PO₄, pH 7.3 + 10% 1-propanol; 25 °C) was continually supplied to the chip using a syringe pump (KD Scientific, Holliston, MA) and removed via vacuum at the outlet. The sample/buffer chip was fabricated of PDMS adhered to a glass coverslip (45 mm × 50 mm × 1.5 mm). The CE buffer channel was 3 cm long, 0.5 cm wide, and 1.17 mm in depth. Six sample wells were

spaced 0.5 cm apart and were made with a 2 mm diameter biopsy punch through 1.17 mm thick PDMS. Sample wells held 8 μ L of sample without wetting neighboring wells. Fused silica capillaries [30 μ m inner diameter, 360 μ m outer diameter (Polymicro Technologies; Phoenix, AZ)] were 40 cm long with an effective length of 4 cm. Prior to use each day, capillaries were rinsed for 10 min each with 1 M NaOH, H₂O, and electrophoretic buffer. Capillaries were stored with both ends in H₂O after rinsing with 1 M NaOH and H₂O for 10 min each. Lysate samples were hydrodynamically loaded into the capillary inlet by lowering the capillary outlet 6.8 cm below the inlet for 10 s. Total sample volume was 0.45 ± 0.01 nL, which includes 0.37 nL due to the hydrodynamic loading and 0.08 ± 0.01 nL due to the spontaneous fluid displacement that occurred as the capillary was moved from sample into the electrophoretic buffer. Electrophoretic buffer composition was 27 mM NaH₂PO₄, pH 7.3 + 10% 1-propanol and a field strength of 500 V cm^{-1} was used for electrophoretic separations. Electrophoresis was initiated by applying a negative voltage to the outlet electrode while holding the inlet at ground. Electropherograms were plotted and analyzed utilizing OriginLab 9.0 (OriginLab Corporation, Northampton, MA).

Capillary Electrophoresis of Single Cells

Capillary electrophoresis of single cells was performed on a custom-built system mounted to the stage of an inverted microscope, described in detail in Proctor, *et al.*³⁴ Fused silica capillaries [30 μ m inner diameter, 360 μ m outer diameter (Polymicro Technologies; Phoenix, AZ)] were 38 cm long with an effective length of 20.5 cm. Capillaries were conditioned and stored daily as described in the above section. Composition of the electrophoretic separation buffer was 27 mM NaH₂PO₄, pH 7.3 + 10% 1-propanol and a field strength of 316 V cm^{-1} was used for separations. Electrophoresis was started by applying a negative voltage to the outlet while the inlet was held at ground. 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 -10 kV to the capillary outlet for 2 s while holding the inlet at ground. After a cell was visually observed to enter the capillary, 1-propanol was hydrodynamically loaded into the inlet for 10 s by raising the inlet 3 cm with respect to the outlet. This sample was incubated for 1 min to achieve extraction of the lipids from the fixed cell. After the 1 min incubation, the inlet was lowered into electrophoretic buffer and electrophoresis was initiated by applying a negative voltage to the capillary outlet. Electropherograms were plotted and analyzed using OriginLab 9.0.

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 on the bulk cell lysates, a one-factor ANOVA was used and followed with Tukey's test for multiple comparisons. The non-parametric Mann-Whitney test for analysis of variance was used to analyze the differences in metabolite formation between single cells with and without SKI II treatment. Required sample sizes were estimated using a power analysis (GPower 3.1, <http://www.gpower.hhu.de/> with $\alpha = 0.05$ and $1 - \beta = 0.95$), based on prior data.²⁷

Results and Discussion

Identification of cell fixatives compatible with retention and release of fluorescent sphingolipids from cells

A common method to terminate cellular reactions in a biological sample is chemical fixation by cross-linking protein amino groups which leads to inactivation of the modified proteins.^{35–37} The fixatives formaldehyde, glutaraldehyde, and glyoxal are aldehyde fixatives that act to form polypeptide bonds when reacting with cellular proteins.³⁸ Formaldehyde, the simplest aldehyde, possesses a single aldehyde available for cross-linking.³⁵ Glyoxal and glutaraldehyde are dialdehydes, with glutaraldehyde possessing three additional carbon atoms between the aldehyde groups relative to glyoxal. The greater distance between glutaraldehyde's reactive groups enables this fixative to participate in more extensive cross-linking than glyoxal.³⁹ Since it participates in less extensive cross-linking and is less toxic than either formaldehyde or glutaraldehyde by virtue of it being less volatile with a decreased risk of inhalation, glyoxal is increasingly preferred in place of these other fixatives.^{40, 41} The ability of these aldehyde fixatives to terminate reactions with a fluorescent sphingosine in cells and entrap the sphingosine and its metabolites within the cellular structure for subsequent assay was assessed. Sphingosine-fluorescein (SF) was loaded into K-562 cells and incubated, followed by addition of one of the fixatives. The amount of lipid retained within the fixed cells after removal of the fixative was quantified by solubilizing the washed cells in bulk and analyzing the supernatants with CE-LIF. Electropherograms of fixed samples were compared to that of an unfixed control to determine whether the same metabolites were observed and to compare their relative amounts (Figure 1A, Supplemental Figure S1). When compared to an unfixed control sample, $24 \pm 8\%$, $40 \pm 2\%$, and $55 \pm 5\%$ of sphingolipid metabolites were recovered from cells fixed with glutaraldehyde, formaldehyde, and glyoxal, respectively. The poorer recovery of fluorescent lipid from the formaldehyde- and glutaraldehyde-fixed cells relative to that of the glyoxal-fixed cells may have been due to the more extensive chemical cross-linking initiated by these fixatives and ensuing entrapment of lipids within the cell or due to loss of membrane components (with fluorescent lipid) from the cell architecture during the fixation step. For this reason, only the glyoxal-based fixation of cells was further characterized.

When compared to unfixed cells, the electropherograms of the glyoxal-fixed cells demonstrated an additional peak relative to that found in the unfixed sample (Supplemental Figure S1A and S1D). Both samples possessed identifiable SF, sphingosine-1-phosphate fluorescein (S1PF), and hexadecanoic acid fluorescein (HDAF) peaks. The fourth peak present in the glyoxal-fixed sample migrated between SF and S1PF. Since both SF and S1PF possess an amine group, it was possible that this extra peak was a by-product of the fixation step.^{40, 42} For this reason, SF and S1PF were incubated with the glyoxal-containing fixative for varying amounts of time and the samples assayed by CE-LIF. Electropherograms of the glyoxal-reacted S1PF demonstrated a single peak that co-migrated with the S1PF standard suggesting that S1PF did not react with glyoxal (Supplemental Figure S2). Electropherograms of SF incubated with glyoxal exhibited two peaks, one co-migrating with SF and one co-migrating with the unknown fourth peak from the cell samples (Supplemental

Figure S2). The amount of this unknown product increased with the incubation time as well as the glyoxal concentration (Supplemental Figure S3). The amount of SF in the reaction mixture decreased in parallel with the increase in the amount of the unknown peak, suggesting that SF was being converted to a product. MALDI-MS of the sample demonstrated two compounds, SF and an SF-glyoxal product identified by its molecular weight with the loss of two H₂O molecules ($m/z = 695.301$; Figure 1B). In all subsequent experiments, the peak area of SF plus the glyoxal adduct (SF-G) was summed and used as a surrogate measure of the SF not metabolized by cellular reactions. Though S1PF also possesses an amine group that could potentially react with the glyoxal, this was not observed, likely due to the proximity to the negatively-charged phosphate group adjacent to the amine (Figure 1B). To verify that fixation with glyoxal terminated cellular reactions, K-562 cells were loaded with SF and incubated; a subset of the sample was fixed with glyoxal at 15 min after start of the incubation. In the fixed cells, the total product amount remained constant over time while that in the unfixed cells continued to increase (Supplemental Figure S4.)

The concentration of glyoxal for cell fixation was optimized by loading cells with SF and then incubating the cells with varying dilutions of the fixative solution in water followed by quantification of the products formed within the cells. For any dilutions of the fixative solution, metabolite recovery varied widely at each fixation time, with relative standard deviations (RSD) as high as 58% (data not shown). When undiluted fixative was utilized (Figure 1C), the RSD of the data was always less than 14%, with the lowest RSD at the 5 min incubation time (5%, 3%, and 4% RSD for SF, S1PF, and HDAF, respectively). All subsequent experiments incubated cells for 5 min in undiluted glyoxal-based fixative. When compared to unfixed control cells loaded with SF, HDAF was recovered from the fixed cells without loss ($102 \pm 4\%$), while both SF and S1PF were recovered at a reduced efficiency of $66 \pm 3\%$ (SF) and $65 \pm 2\%$ (S1PF; Figure 1C) with respect to unfixed cells. The reduced recovery efficiency was present at the shortest fixation times examined (1 min), suggesting that either the fluorescent lipid was lost during the fixative-removal wash or was not extractable from the fixed cells.

Optimization of fluorescent sphingolipid extraction from fixed cells

In the above experiments, the fluorescent lipids were released from the fixed cells by incubation of the cells with 2-propanol (Figure 2A), in which both SF and S1PF are soluble but which is known to precipitate cellular proteins, potentially entrapping lipid within protein aggregates within the cells.⁴³ Prior work removing phospholipids from fixed cells also demonstrated that extraction solution optimization was critical for lipid recovery and that some solvents might degrade subsequent electrophoretic separations.²⁴ To optimize the lipid extraction step, the different alcohols (methanol, ethanol, and propanols) were evaluated for their ability to recover the expected fluorescent sphingosine metabolites, act on fast time scales, and minimally impact the subsequent electrophoretic separation. Five organic alcohols as well as the electrophoretic separation buffer (which contains 1-propanol) were screened for their ability to extract sphingolipid metabolites from fixed cells (Figure 2B). All undiluted alcohols displayed a similar efficiency for extraction of the fluorescent lipid from cells. The electrophoretic buffer (10% 1-propanol), however, performed poorly,

extracting less than half the fluorescent lipid compared to the other solutions. Since 1-propanol was already present in the electrophoretic buffer, this alcohol (undiluted) was selected as the extraction solution. Next, the incubation time of 1-propanol with the fixed cells was optimized by varying this time from 30 s to 5 min and measuring the total fluorescent lipid recovered (Supplemental Figure S5). There was no statistical difference in lipid extracted for the various incubation times. A 1 min incubation time was selected since this time was the shortest time to reproducibly perform the extraction procedure.

Optimization of fluorescent sphingolipid retention within fixed cells

The ability to fix cells with stable retention of the lipid within the cell for later quantification offers a number of potential assay benefits. Chief of these is the ability to separate in time and space the intracellular reaction step from the assay or sensing method. This offers convenience, particularly in a clinical setting where instrumentation must often be located far from the site of sample acquisition. For measurement accuracy, the fixed cells must retain the sphingolipid over time and in a state unmodified relative to that present at the time of fixation. For transport and storage of the cells post-fixation, buffer systems that encourage the partitioning of fluorescent sphingolipids into the fixed cellular microenvironment rather than the surrounding aqueous environment were assessed. Given their non-polar nature and high hydrophobicity, the sphingolipids are least soluble in polar media such as salt-containing aqueous solutions.⁴⁴ K-562 cells were loaded with SF, fixed, washed, and then incubated in four salt-containing aqueous buffer solutions (ECB, PBS, 30 mM NaH₂PO₄ (pH 7.3), 80 mM NaH₂PO₄ (pH 6.8)). After 24 h of cell storage, no statistical difference was observed between the solutions with respect to fluorescent lipid recovery from the cells for the 4 tested solutions. Consequently, the 30 mM sodium phosphate solution was selected for further analysis since it is used as the background electrolyte in the electrophoretic buffer and thus minimizes challenges due to sample and electrophoretic buffer mismatch during the separation step.

Electropherograms of lipids extracted from fixed cells at varying times after storage in 30 mM sodium phosphate demonstrated 6 peaks, corresponding to substrate forms (SF and SF-G) and metabolic products (S1PF, HDAF, and two additional unidentified metabolites). There was no statistical difference in the total product present when the total product was expressed as the percentage of total fluorescent lipid during storage times up to 24 h (Figure 3A). However, at all time points, a statistically significant decrease was observed in the percentage of fluorescent lipid present in the product form compared to that for an unfixed control. This apparent product loss may have been due to an inability to detect other glyoxal-lipid adducts or to extract product forms irreversibly cross-linked to cell constituents. Next we sought to determine whether individual products might be selectively lost over time during storage (Figure 3B). The amount of S1PF or total product (S1PF+HDAF+peak 5+peak 6) recovered over time was not statistically different; however, when examined individually, each of the minor products (HDAF, peak 5, and peak 6) underwent a statistically significant change in the apparent percentage of product formed between the 15 min and 24 h measurement times ($p < 0.01$). On average, these products underwent an apparent increase at a rate of 5% per h (1–24 h). The most likely explanation was due to small losses of the S1PF which, while insignificant given the large percentage of S1PF,

resulted in large apparent increases for the minor products. These data suggest that species at low concentration, such as HDAF, cannot be accurately quantified at long storage times using this protocol. The data also suggest that measurement of S1PF formation is likely accurate only when the percentage of S1PF formed dominates formation of other products and when S1PF is formed in relatively large amounts. However, the percentage of loaded SF converted to any product form should act as an overall metric of the metabolism of sphingosine through sphingosine-1-phosphate.

Measurement of inhibition of sphingolipid metabolism

Sphingosine kinase inhibitor 2 (SKI II) is a competitive inhibitor of sphingosine kinases 1 and 2 and reduces the formation of S1PF.⁴⁵ K-562 cells were incubated with or without SKI II and then loaded with SF. The cells were fixed with glyoxal and stored in 30 mM sodium phosphate for up to 24 h. Sample aliquots of the fixed cells were removed over time, the fluorescent sphingolipids extracted, and the supernatants analyzed by CE-LIF to quantify the total fluorescent lipid (Figure 4). No statistical difference in the percentage of recovered fluorescent lipid was observed for the fixed cells with and without inhibitor at any time point. In cells incubated with 25 μ M SKI II, three peaks were observed in the electropherograms, identified as SF, SF-G, and S1PF by their migration times. Cells without the inhibitor possessed 6 peaks as described above. At all time points, the fixed inhibited cells possessed significantly less total product (S1PF+HDAF+peak5+peak 6) when expressed as a percentage of the total lipid recovered compared to that of the fixed uninhibited cells (p-value = 0.0001). This behavior of the inhibitor was readily observed in the fixed cells despite the lipid losses during fixation and storage.

Single-cell analysis of fixed cells

To determine whether sphingosine kinase activity could be analyzed in single cells that were subsequently fixed, K-562 cells were loaded with SF, incubated for 30 min, and then fixed with glyoxal before being placed in the 30 mM sodium phosphate storage solution. To quantify the differences in the response of single cells to the sphingosine kinase inhibitor, cells were also pre-treated with SKI II or a DMSO vehicle prior to loading the SF (Figure 5A–C). All single cells were analyzed between 9 min and 5 h of fixation and storage. Electropherograms of control single cells (n = 14) demonstrated 2–6 peaks, identified in order of migration time as: SF, SF-G, S1PF, HDAF, plus the two unknown metabolites (peaks 5 and 6) (Figure 5A). Cells treated with SKI II (n = 12) possessed up to 3 peaks, identified as SF, SF-G, and S1PF (Figure 5B). When expressed as a percentage of total lipid present on the electropherogram, there was a statistically significant difference in the percentage of product formed in single cells treated with SKI II compared to that in untreated control cells (p-value = 0.0001; Figure 5C). As reported previously, significant heterogeneity in the metabolism of SF at the single-cell level was present in both the inhibited and uninhibited cells. However, in both cases, the average result from the single cells closely reflected that of a bulk lysate sample prepared at the same time.

To ensure that this “fix and assay” method applied to other cell types, U-937 cells (a non-Hodgkin’s histiocytic lymphoma) known to exhibit aberrant sphingosine pathway signaling were utilized.³³ A lymphoma cell line was chosen for its differences from a leukemia cell

line—though both are blood cancers, leukemia originates in the bone marrow while lymphomas arise in the immune system and infiltrate the lymph nodes. U-937 cells were treated with SKI II, incubated with SF and then fixed, and the fluorescent metabolites were quantified (Figure 5D–F). Electropherograms from untreated cells ($n = 10$) possessed up to 3 peaks, identified as SF, SF-G, and S1PF (Figure 5D), while electropherograms from SKI II-exposed cells ($n = 10$) showed up to 2 peaks, identified as SF and SF-G (Figure 5E). No other products were seen in either group of cells. There was a statistically significant difference in the percentage of fluorescent lipid converted to product in single, inhibited U-937 cells compared to that in untreated control cells (p -value = 0.0001; Figure 5F). The average percentage of product formation of all of the single cells was nearly identical to that measured from a bulk cell lysate. When compared to the K-562 leukemia cell line, the U-937 lymphoma cell line converted a significantly lower percentage of SF into product (8 – 26% product in single, untreated U-937 cells compared to 33 – 69% product in single, untreated K-562 cells; p -value = 0.0001) and U-937 cells loaded significantly less lipid per cell than K-562 cells (8 ± 5 amol in U-937 cells compared to 19 ± 5 amol in K-562 cells; p -value = 0.0001). U-937 cells are approximately one-third the diameter of the K-562 cells or a 27-fold lower volume than the U-937 cells explaining the lower amount of lipid loaded into the cells. Other attributes of these cells may also have impacted the total SF loaded since the cells are derived from different types of tumors (leukemia vs lymphoma).⁴⁶

Demonstration of “fix and assay” in primary patient samples

To assess the feasibility of the “fix and assay” protocol in primary patient samples, de-identified peripheral blood samples from patients with chronic lymphoblast leukemia (CLL) were obtained, loaded with SF, fixed with glyoxal, and single cells ($n = 9$) analyzed with CE-LIF (Figure 6). Two peaks were observed in the electropherograms, identified as SF and SF-G (Figure 6A). Product forms were not observed in the single cells. When a lysate sample of the CLL cells was analyzed, only 2% of the SF was converted to S1PF, much lower than was seen in either of the two tissue-cultured cell lines. The total amount of recovered fluorescent lipid in each CLL single cell ranged from 1 – 12 amol, with an average of 7 ± 4 amol fluorescent lipid per cell, similar to the U-937 cells and did not correlate with the storage time prior to assay for this data set (Figure 6B). To determine if the ratio of SF to SF-G in each single cell depended on the total amount of lipid present in the cell, the ratio was plotted as a function of total loaded SF (Figure 6C). There was no correlation in the ratio of SF:SF-G as the total lipid content increased ($R^2 = 0.0002$), with an average ratio of 4 ± 1 seen in the CLL cells, 3 ± 1 seen in the K-562 cells, and 3 ± 1 seen in the U-937 cells.

Conclusions

Aldehyde fixation was successfully used to simultaneously halt cellular reactions and measure product formation in cells loaded with a fluorescent sphingosine. Conversion of sphingosine to sphingosine-1-phosphate and hexadecanoic acid was measured in fixed, single cells of multiple lineages, including leukemia and lymphoma cell lines and primary patient cells. Multiple parameters, including type of fixative, fixation time, storage solution, storage time after fixation, and extraction solution identity, were optimized to determine a

fixation procedure that retained lipid reactants and products within the fixed cells until the point of analysis. To validate the “fix and assay” procedure, cells were treated with a sphingosine kinase inhibitor prior to fixation. There was a statistically significant decrease in product formation in inhibitor-treated cells compared to that of control cells. Though there was no statistical difference in the total amount of product recovered from fixed cells, there was a difference in the amount of individual products detected—with lower-abundance products varying more than higher-abundance ones. Further work optimizing the fixation procedure should correct these discrepancies; this future work includes examining alternative fixation solutions, such as utilizing acid-free glyoxal,⁴⁷ to improve the retention of all sphingolipid species within the fixed cells. Furthermore, this work demonstrated the feasibility of fixing cells in an alternative location prior to transport to the analytical lab—enabling the sphingolipid reactions in individual cells to be simultaneously terminated and analyzed at a later time. This provides the advantage of eliminating the variable of reactant incubation time, allowing for a more facile measurement of sphingosine kinase activity within single cells from the same population. Continued work includes additional analysis of primary patient samples from a larger patient cohort utilizing this “fix and assay” protocol to determine sphingosine kinase signaling heterogeneity in different primary patient samples.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1:

Identification of optimal fixative. (A) The total fluorescent lipid recovered from K-562 cells after fixation was compared to an unfixed control. **** $P < 0.0001$; *** $P < 0.001$; ** $P < 0.01$. (B) Chemical structures of lipids. (C) Recovery of SF+SF-G (closed black squares), S1PF (open red circles), and HDAF (closed blue triangles) from glyoxal-fixed cells relative to that of unfixed cells. Cells were incubated with the undiluted fixative for varying times as shown on the X-axis. Data points are the average of triplicate measurements and the error bars represent one standard deviation.

Figure 2:

Extraction of lipids from fixed cells. (A) Schematic of experimental process to obtain extracted lipids from fixed cells. i: Live cells; ii: incubate with SF; iii: SF-loaded cells; iv: add fixative; v: fixed cells; vi: CE-LIF analysis of single, fixed, SF-loaded cells; vii: add extraction solution; viii: CE-LIF analysis of fluorescent lipid in lysate supernatant. (B) Total amount of fluorescent lipid recovered from K-562 cells fixed with glyoxal, washed and then incubated with the indicated extraction solution. * $P < 0.05$. Bar height represents the average of triplicate measurements and the error bars indicate one standard deviation.

Figure 3:

Fluorescent lipid extracted from fixed cells after storage. (A) Percentage of reactants (SF and SF-G) and products (S1PF+HDAF+peak 5+peak 6) extracted from fixed K-562 cells after storage in 30 mM NaH₂PO₄ (pH 7.3) at 4 °C. There is no statistical difference in the total percentage of fluorescent lipid at any of the storage timepoints. There is a statistical difference in the percentage of fluorescent lipid in the unfixed control compared to each of the storage time points ($P = 0.01$). (B) Percentage of individual products extracted from fixed K-562 cells after storage in 30 mM NaH₂PO₄ (pH 7.3) at 4 °C. When looking at each

individual product, there is a statistical difference in the percentage of fluorescent lipid recovered at early timepoints (< 1 h) and later timepoints (> 10 h; $P = 0.01$). Bar height represents the average of triplicate measurements and the error bars indicate one standard deviation.

Figure 4:

Fluorescent lipid products expressed as a percentage of total fluorescent lipid extracted from fixed K-562 cells treated with 25 μM SKI II or a DMSO vehicle. The cells were stored for varying times in 30 mM NaH₂PO₄ (pH 7.3) at 4 °C. At each timepoint, there is a statistical difference in the total product formed depending on treatment with SKI II ($P = 0.0001$).

There is no statistical difference in the percentage of product recovered at any of the storage timepoints within a single treatment condition. Bar height represents the average of triplicate measurements and the error bars indicate one standard deviation.

Figure 5:

Single-cell analysis of fixed K-562 and U-937 cells treated with or without 25 μM SKI II. Electropherogram of a single, fixed K-562 cell treated with (A) a DMSO vehicle or (B) 25 μM SKI II. (C) Percentage of total fluorescent lipid in each K-562 cell present as an SF metabolite other than SF-G (total product) is shown with and without incubation with the inhibitor. Electropherogram of a single, fixed U-937 cell treated with (D) a DMSO vehicle or (E) 25 μM SKI II. (F) Percentage of total fluorescent lipid in each U-937 cell present as an SF metabolite other than SF-G (total product) is shown with and without incubation with the inhibitor. SF = sphingosine fluorescein; SF-G = sphingosine fluorescein-glyoxal adduct; S1PF = sphingosine-1-phosphate fluorescein; HDAF = hexadecanoic acid fluorescein; and Peaks 5 and 6 are unidentified metabolites. For panels C and F, each data point represents a single, fixed cell and the blue star is the average from the bulk lysate of that sample set. The box plot shows the 1st quartile (bottom of box), the median (center line), and the 3rd quartile (top of box) of the data set; the whiskers extend to data points not considered outliers, and data points beyond the whiskers are considered outliers. Solid black diamonds represent cells treated with a DMSO vehicle and open red circles represent cells treated with 25 μM SKI II. **** P = 0.0001.

Figure 6:

Single-cell analysis of fixed primary human CLL cells and tissue cultured cells. (A) Electropherogram of a fixed CLL cell. SF = sphingosine fluorescein; SF-G = sphingosine fluorescein-glyoxal adduct. (B) The total amount of fluorescent lipid in cells as a function of storage time. The x-axis shows the time cells were analyzed after fixation and storage. Each data point represents a single cell. (C) The ratio of SF:SF-G as a function of the total amount of fluorescent lipid in cells. Each data point represents a single cell. Solid black squares are CLL cells, solid red circles are K-562 cells, and open blue triangles are U-937 cells.