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Direct Lipid Profiling of Single Cells from Inkjet Printed Microarrays

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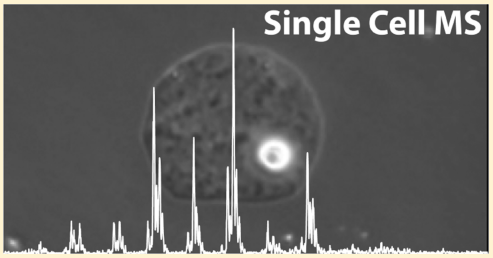
Supporting Information

ABSTRACT: The on-demand printing of living cells using inkjet technologies has recently been demonstrated and allows for the controlled deposition of cells in microarrays. Here, we show that such arrays can be interrogated directly by robot-controlled liquid microextraction coupled with chip-based nanoelectospray mass spectrometry. Such automated analyses generate a profile of abundant membrane lipids that are characteristic of cell type. Significantly, the spatial control in both deposition and extraction steps combined with the sensitivity of the mass spectrometric detection allows for robust molecular profiling of individual cells.



Variable expression of cellular components ranging from genes to metabolites is a well-known phenomenon leading to heterogeneity in cell populations.[1−4] Traditionally, cellular analysis is performed on a pool of cells; however, this can eliminate information regarding cell−cell variability as the resulting data is an average across all cells. Thus, it is important to have methods available that permit analysis of individual cells one at a time to determine their functionality and their identity. Analysis of these single cells can enhance the detection and diagnosis of cell-based diseases and provide an insight into many important physiological processes that occur in individual cell populations.[5] Recent reviews of single cell analysis have revealed that many current techniques either require an external label targeting a specific molecule (e.g., fluorescence) or lack molecular specificity (e.g., Raman spectroscopy).[5−7] By providing the simultaneous analysis of individual cellular components without the need for external labels and with excellent molecular sensitivity and specificity, mass spectrometry (MS) is an alternative method well suited for single cell analysis.[8]

Lipids are ideal targets for the direct analysis of single cells due to their high concentration near the cell surface (i.e., the cell membrane). They are also important physiologically, performing vital roles in cell functions including growth and differentiation,[9] apoptosis,[10,11] and phagocytosis,[12] among numerous others. Lipid profiles can also provide a unique fingerprint for organisms such as bacteria and allow species and even subspecies identification.[13] Moreover, changes in lipid composition are a characteristic of several disease states,[14,15] suggesting that lipid profiles could potentially be used as an identifier of diseased cells.

Bioprinting is an emerging technology for the controlled deposition of living cells, which has been demonstrated using a wide range of techniques including inkjet printing.[16−18] This drop-on-demand technique is attractive for the purpose of single cell analysis as it allows for the precise placement of individual cells and cell-based microarrays with excellent repeatability and without the need for contact with the substrate.[19] Moreover, it has been suggested that bioprinting has great potential for the development of 3D cell-based drug-screening assays.[20] Herein, we describe the use of liquid extraction surface analysis (LESA), a new robotic-based liquid microjunction extraction technique directly coupled with chip-based nanoelectrospray ionization,[21] in combination with recently described bioprinting technology[22] for the preparation and lipid analysis of single cell arrays.

EXPERIMENTAL METHODS

Three different murine cell lines were obtained from the American Type Culture Collection (ATCC): C2C12 (skeletal muscle, mouse, ATCC CRL-1772); PC12 (adrenal pheochromocytoma, rat, ATCC CRL-1721); and L929 (fibroblast, mouse, ATCC CCL-1). Cells were suspended at 6×10^6 cells/mL in a bioink formulation containing a 0.05% w/v gellan gum (CP Kelco) microgel suspension in serum-free Dulbecco's Modified Eagle Medium (DMEM), supplemented with two biocompatible nonionic polymeric surfactants: Poloxamer 188 (Lutrol F68, Sigma) at 0.1% v/v and a fluorosurfactant (Novec

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FC4430, 3M) at 0.05% w/v. Cell suspensions were then loaded into Xaar-126 piezoelectric inkjet print heads housed in a custom printing apparatus. Details on cell printing using this bioprinter and the bioink formulation are reported elsewhere.[22]

Cells were printed onto standard glass slides that had been marked (on the reverse side to cell deposition) with a 4 × 11 array of circular regions ~3 mm in diameter. Cell patterns consisting of either a single droplet, or squares containing 9 (3 × 3 square) or 100 (10 × 10 square) droplets, were printed within these marked regions and subsequently dried under N_2. The number of cells in each individual droplet followed the expected Poisson distribution; i.e., the average number of cells per drop is 1, and on average, 37% of the droplets will contain a single cell.[22] Arrays were imaged using a Zeiss Axiovert 40 CFL inverted light microscope, and the number of cells in each printed pattern was counted using ImageJ software. Cell counting is performed by "clicking" on the image of the cells that leaves a marker to indicate that cell has been counted while a counter records the number of "clicks" and hence number of counted cells in a given array position.

LESA-MS analysis was performed using a TriVersa NanoMate (Advion Biosciences, Inc.) coupled to a QTRAP 5500 triple quadrupole mass spectrometer (AB Sciex, Concord, ON, Canada). To minimize sample oxidation, the cooling fan was turned off and the source housing was covered to eliminate airflow over the cells. The extraction solvent was 4:2:1 IPA/MeOH/$CHCl_3$ (v/v/v) with 20 mM NH_4OAc. All solvents used for mass spectrometric analysis were LC/MS grade. For analysis of microarray spots containing greater than 10 cells, 2 μL of solvent was aspirated from the reservoir followed by dispensing 1.5 μL onto the area of interest. Solvent was held on the surface for 7 s to facilitate extraction before 2 μL was aspirated back into the pipette tip analysis. Analysis of microarray spots containing 1−5 cells was performed as described above but with a total solvent volume of 1.5 μL and dispense and aspiration volumes of 1.2 and 1.8 μL, respectively. A spray voltage of 1.3 kV was applied to the pipette tip, and backing gas pressure was 0.6 psi. Phosphatidylcholine (PC) and sphingomyelin (SM) lipids were detected using a precursor ion scan (PIS) of m/z 184.1 at collision energy of 40 eV. Cholesterol esters were detected using a PIS of m/z 369.4 with collision energy of 25 eV. Ceramides were detected using a PIS of m/z 264.3 at a collision energy of 35 eV. All spectra were acquired using a scan rate of 200 $Da.s^{-1}$, and 100 or 200 scans (acquired in MCA mode) were summed for the analysis of either 10−200 cells or 1−5 cells, respectively. This resulted in acquisition times of 4.15 and 8.30 min for scans between m/z 400 and 900 for 10−200 and 1−5 cells, respectively.

RESULTS AND DISCUSSION

Cell microarrays were prepared by deposition onto glass substrates through inkjet printing using commercially available piezoelectric print heads (Figure 1a). The number of cells deposited at each array point (a single cell or up to 100 cells) was controlled by changing the number of printed droplets (Figure 1b). Analysis of these printed cells was then performed by LESA-MS by lipid extraction into a liquid microjunction formed between the surface of the sampling area and the tip (Figure 1c) and subsequent chip-based nanoelectrospray ionization.

An important attribute of this approach is the ability to analyze cells in the open environment. However, when

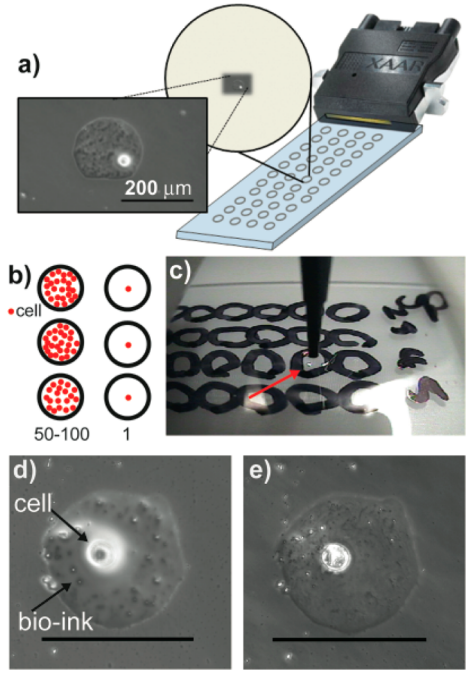

Figure 1. Bioprinting, LESA, and optical images of single printed cells. (a) Schematic of the inkjet printing of cells into microarray spots, including a typical optical image of a single printed cell in one of the circular array spots. (b) Schematic representation of the microarrays spots for LESA analysis. (c) A conductive LESA pipette tip dispenses extraction solvent onto a microarray spot (indicated by arrow) containing printed cells. Following extraction, the solvent is aspirated and analyzed by chip-based nanoelectrospray ionization. The numbers "50−100" and "1" represent spots containing 50−100 cells and 1 cell, respectively. (d−e) Typical optical images of a single inkjet printed L929 cell on a glass slide (d) before and (e) after LESA analysis. Scale bars represent 200 μm.

analyzing printed cells that had been exposed to ambient laboratory conditions for up to several hours prior to analysis, oxidation of unsaturated cellular lipids was observed and was found to increase with exposure time. This phenomenon was attributed to ambient ozonolysis as previously observed for lipids on surfaces[25] and resulted in the formation of lower (aldehdyes and carboxylic acids) and higher mass (secondary ozonides) ions (Figure S1 in Supporting Information). While ambient ozonolysis has proven analytical capability, it was important to reduce the extent of oxidation such that lipid profiles could be used for cell-type identification. This was achieved primarily by eliminating airflow over the printed cells.

To demonstrate lipid profiling from printed cells, initial analyses were conducted on microararys containing up to 100 cells per spot. Detection of various lipid classes present in the cell membrane including PC, SM, cholesterol esters, and ceramides was achieved by employing class-specific precursor ion scans (PIS) on a triple quadrupole mass spectrometer.[26] Due to their high abundance in the cell membrane and their ease of ionization, we focused on the detection of PC and SM lipids. These were readily detected in positive ion mode as $[M + H]^+$ ions by employing an m/z 184.1 PIS. The corresponding spectra (Figure 2a−c) acquired from the three cell types allowed for the identification of 23 PC and SM lipids as $[M + H]^+$ ions (Table S1 in Supporting Information). In addition, several lyso PC (LPC) lipids, namely, LPC (16:0) and LPC (18:1) with $[M + H]^+$ at m/z 496 and 522, respectively, were

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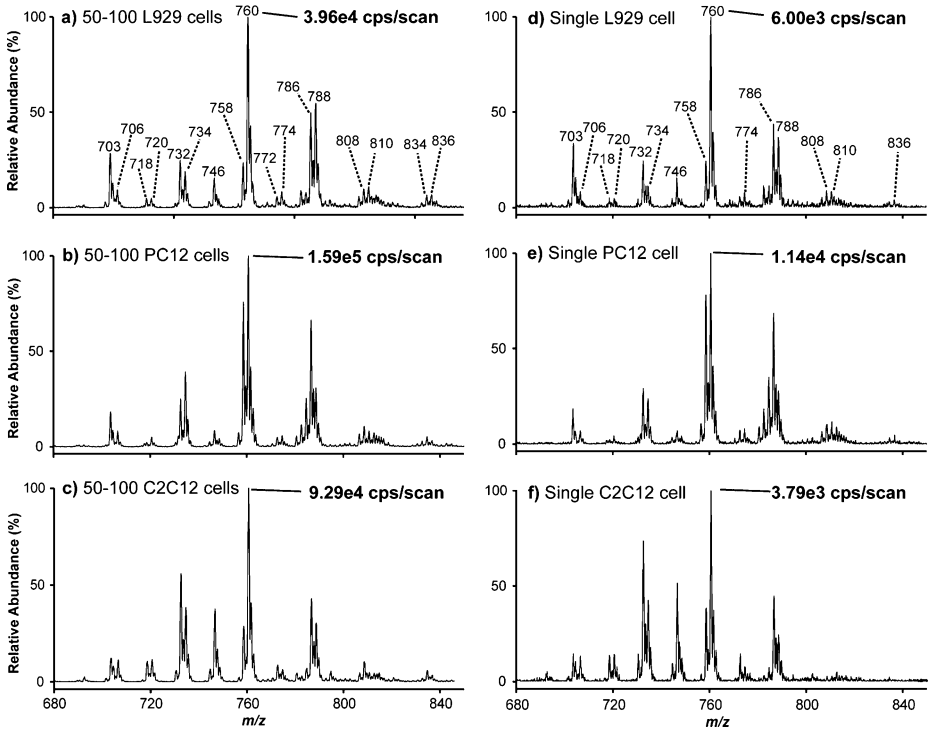

Figure 2. Precursor ion scans (PIS) on inkjet printed cells. (a–c) Typical m/z 184.1 PIS obtained from printed cell microarray spots (50–100 cells per spot) of L929 (a), PC12 (b), and C2C12 (c) cells. Spectra were produced from 100 summed PIS. (d–f) Typical m/z 184.1 PIS on printed cell microarray spots (1 cell per spot) of L929 (d), PC12 (e), and C2C12 (f) cells. Spectra were produced from 200 summed PIS. Values in the top right of each spectrum correspond to the average intensity in counts per second (cps) per scan of the base peak.

also observed (data not shown). Cholesterol esters and ceramides (containing the d18:1 sphingosine base) could also be detected from printed cell arrays (Figure S2 in Supporting Information). For example, ions with nominal m/z values of 640, 642, 666, and 668 are assigned as $[M + NH_4]^+$ ions of cholesterol esters containing 16:1, 16:0, 18:2, and 18:1 fatty acids (Figure S2a in Supporting Information). Protonated ceramides are observed at m/z values of 510, 538, 566, 592, 622, 648, and 650 and are assigned as protonated d18:1 ceramides with 14:0, 16:0, 18:0, 20:1, 22:0, 24:1, and 24:0 fatty amide chains, respectively (Figure S2b in Supporting Information).

The capability to prepare microarrays containing single cells allowed us to investigate the potential of LESA-MS for single cell analysis. Figure 2d–f shows representative spectra acquired from individual inkjet printed L929, PC12, and C2C12 cells. LESA-MS analysis on these single cells allowed the detection of the same PC and SM lipids with similar relative abundances as observed for the spectra acquired on microarray spots with up to 100 cells, albeit with slightly lower signal-to-noise. Single cell spectra were reproducible for all cell types investigated and highlight the excellent sensitivity afforded by this approach. As expected, the precursor ion signal decreases when single cells are analyzed as indicated by the average ion counts provided in Figure 2. To highlight the typical signal-to-noise ratio achieved from the analysis of 50–100 cells versus single cells, an enlarged m/z 680–758 region acquired from L929 samples is provided as Supporting Information (Figure S3). The effect of the extraction process on printed single cells is shown in Figures 1d and 1e. The images suggest that the LESA process has extracted some of the bioink material from the surface as well as some cell material (evident from the detection of lipids) with remaining cell material still present on the surface after extraction.

The lipid profiles from each of the three cell types was found to provide a characteristic "fingerprint", allowing identification of cell type using principal component analysis (PCA, see Supporting Information) (Figure 3). PCA is a common method used to reduce the dimensionality of multidimensional data sets (such as mass spectra containing many peaks) into several new variables while maintaining much of the original sample information and variation.[25,26] Hence, it is an ideal method to decipher the unique correlations of the multiple peaks in the LESA-MS spectra of different cell types. The results of this analysis on microarray spots containing up to 100 cells or single cells are shown in Figures 3a and 3b, respectively. The spectral data acquired from printed cells are clearly grouped according to cell type and separated from other cell types, indicating that the respective lipid profiles are different and characteristic, thereby allowing identification based on the "lipid fingerprint". This identification was possible even with data obtained from individual printed cells, and overlays of representative mass spectra are provided as Supporting Information (Figure S4), emphasizing the origin of similarities and differences in these profiles. To demonstrate that the printing and analysis approach did not alter the lipid composition in cell membranes, data points acquired by analysis of a lipid extract derived from nonprinted L929 cells using direct infusion nano-ESI (see Supporting Information) were introduced into the PCA data sets (shown as triangles in Figure 3). These data were found to correlate well with the LESA data of printed cells from microarray spots containing both single and multiple cells, providing evidence that the printing process did not alter the lipid composition of the cells. This is further supported by recent work demonstrating that printed cells retain >95%

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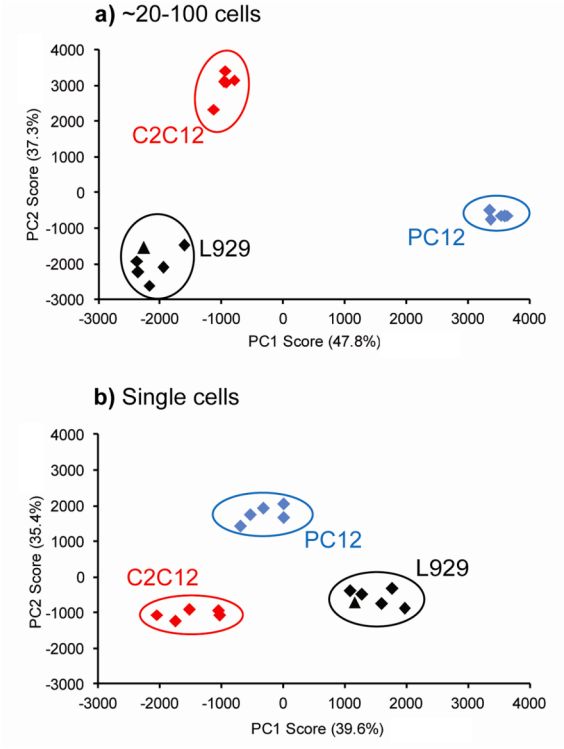

Figure 3. Principal component analysis (PCA) on inkjet printed cells. PCA score plots using PC/SM lipid profiles acquired from 5 microarray spots for each cell type (L929, C2C12, or PC12 cells) containing (a) up to 100 printed cells and (b) a single cell. Triangles indicate the data point obtained by direct infusion of lipid extract from cultured L929 cells.

viability after printing and proliferated at a rate comparable to nonprinted controls.[22]

Analysis of PC and SM lipids from printed cells does not indicate marked differences between cells of the same type (i.e., little heterogeneity is observed). Possible reasons for this may be the preparation of cells under controlled culture conditions and the fact that PC lipids perform primarily structural roles within the cell membrane. Culture conditions are known to influence cellular lipid composition[27] and given that PC12 cells were cultured with a different serum composition than L929 and C2C12 cells, it is important to consider that observed lipid differences may arise from culture conditions rather than being solely determined by cell type. However, given that L929 and C2C12 cells were cultured under identical conditions, the differences observed in lipid profiles of these cells can confidently be attributed to inherent differences in membrane composition independent of the culture medium. With future improvement in sensitivity, it may be possible to detect less abundant lipids that are typically involved in signaling pathways, which may be more likely to show a heterogeneous distribution throughout single cells. Nonetheless, the capability to detect intact biomolecules from single cells makes this a promising approach for cell analysis.

CONCLUSIONS

We have demonstrated that the combination of LESA-MS and drop-on-demand inkjet printing is a viable approach for the chemical analysis of intact single cells. A variety of lipid classes can be detected directly from printed cells, although currently only PC and SM lipids can routinely be detected from single cells. Crucially, lipid profiles were found to provide a characteristic "fingerprint" for each of the investigated cell types, allowing each to be distinguished and identified.

Importantly, the "soft" ionization afforded by LESA-MS allows the detection of intact phospholipids from single cells, representing an advantage over more energetic secondary ion mass spectrometry (SIMS) approaches that have traditionally been used for single cell mass spectrometry. The energetic desorption process of SIMS typically results in the detection of low mass fragments that are characteristic of phospholipid class (i.e., headgroup structure) but do not provide information on individual molecular lipids present in single cells. Mass spectrometric analysis of single cells[28] has also been demonstrated using pulsed laser-desorption approaches (i.e., matrix-assisted laser desorption and related techniques) that provide good spatial resolution and can also give rise to intact ionized metabolites.[4,29] A key advantage of the LESA-MS approach described here, however, is the generation of a stable, prolonged electrospray of up to 15 min from a single-cell extract. This persistent spray affords: (i) averaging of numerous individual spectra from a single extraction, significantly improving sensitivity and reproducibility, and (ii) sufficient time for multiplexed interrogation of ions (i.e., acquisition of multiple different precursor and neutral loss scans), improving the confidence of molecular structure assignments. The excellent spectral quality and reproducibility achieved in this study also suggest shorter acquisition times could be achieved (by either increasing scan speeds or narrowing the mass range) without significant compromise in the ability to differentiate cells based on their lipid profile. In some applications, decreasing acquisition times would hold the advantage of increasing the throughput of the analysis.

Given the ability of inkjet printing to deposit living cells,[22] we envisage that adaptation of this approach to printed living cell microarrays could help to elucidate how individual cells are influenced by their microenvironment and the role of lipids and other molecules in biological heterogeneity.[30] Finally, although the results presented here have been acquired from model cell types, the methods employed could be applied to any cell-based study. The combination of inkjet printing and direct analysis by LESA-MS invites the development of high-throughput single cell assays based on this approach.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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