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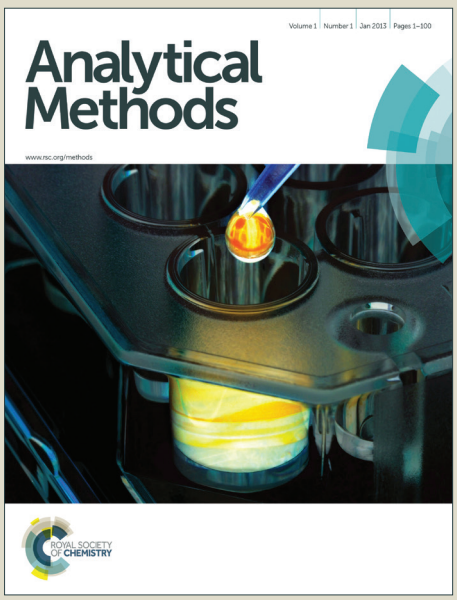

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A lipidomics demonstration of the importance of single cell analysis

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Abstract:

Typical lipidomics data are obtained from collective cell lysis encompassing the digestion of large groups of cells or tissues, leading to inaccuracies and averaging of sample data. Here we describe a novel approach of obtaining single cell information with results demonstrating the importance of single cell analysis. We report obtaining distinct heterogeneity of adipocytes *in vitro* using nanomanipulation-coupled nanoelectrospray mass spectrometry of living individual cells from the same culture plate.

Full text:

There is a great need for single cell analysis for chemical profiling of biological materials in order to build an accurate model of cellular function. Currently, the typical application of obtaining a chemical profile of biomaterials consists of mechanical or chemical digestion of large groups of cells. Following digestion, organic extractions are performed to obtain the targeted biochemistry for use in analysis such as western blots[1], chromatography[2], and electrospray ionization mass spectrometry (ESI-MS). [3] The inherent problem with these multiple cell analysis methods is that the data obtained is representative of an ensemble average of the population, which can obscure variations within individual cells comprising the population. [4] In addition, the entire sample of cells analyzed are sacrificed in the process and cannot be monitored afterwards, which is important for certain progressive diseases such as cancer. It has been established that individual cells and their lipid contents are not homogenous[5-8], so it is

imperative to study them at the single cell level to better understand lipidomic processes and cellular functions.[9]

Several single cell technologies have emerged[10], but still have significant drawbacks. Flow cytometry, capillary electrophoresis, printed microarrays, and microfluidic devices separate hundreds of cells down to the single cell level, but often need to be coupled to more detailed analytical methods for lipid information such as mass spectrometry (MS). [4, 11, 12] In addition, these methods expend the sample, can be less applicable to adherent cells[11], and do not allow for further analysis of the same sample in following days. Matrix-assisted laser desorption/ionization (MALDI)-MS has been used for single cell metabolomics, however this method often requires lengthy analysis times and low mass matrix interferences have been known to be problematic for metabolites.[4] Fluorescence microscopy, while sensitive and noninvasive, is limited in its ability to distinguish among many biochemical species and provides little to no structural information.[4] In many cases the addition of the fluorophore itself can change the chemistry within cells by stopping a biological function.[4]

Here we describe a novel approach to obtaining single cell chemical information that negates the averaging problem of collective cell lysis and leaves the remainder of the cell culture intact by using nanomanipulation-coupled nanoelectrospray-ionization mass spectrometry (NSI-MS). Nanomanipulators are particularly useful for manipulating particles and cells[13-17] with precision for positioning on the nanometer scale. In this work, a nanomanipulator was utilized to perform whole cell extractions by dissolving single adipocytes containing lipid droplets, which are known to contain triacylglycerols[18] (TAGs), in a tailored extraction solvent with a nanospray emitter followed by mass analysis using NSI-MS. The high sensitivity of NSI-MS is especially useful for the low volumes (fL-nL) [4, 11, 14] of individual cell extractions, and directly injecting the

sample into the mass spectrometer avoids lengthy chromatographic separation. Furthermore, the culture plate was able to be extracted upon for multiple days due to the non-invasiveness of the nanomanipulator. Figure 1 displays a differentiated single cell adipocyte extraction containing large lipid droplets (>10 µm). After aspiration of extraction solvent, the emitter was transported to the mass spectrometer and analyzed using a mass range of *m/z* 100-1100. This range encompassed common lipids species such as diacylglycerols, phospholipids, and TAGs, however the main species obtained from the large lipid droplet extractions yielded TAGs (*m/z* 750-950) as identified using LIPID MAPS online tools for lipid research[19]. Details of the specific MS conditions used are provided in supplementary information.

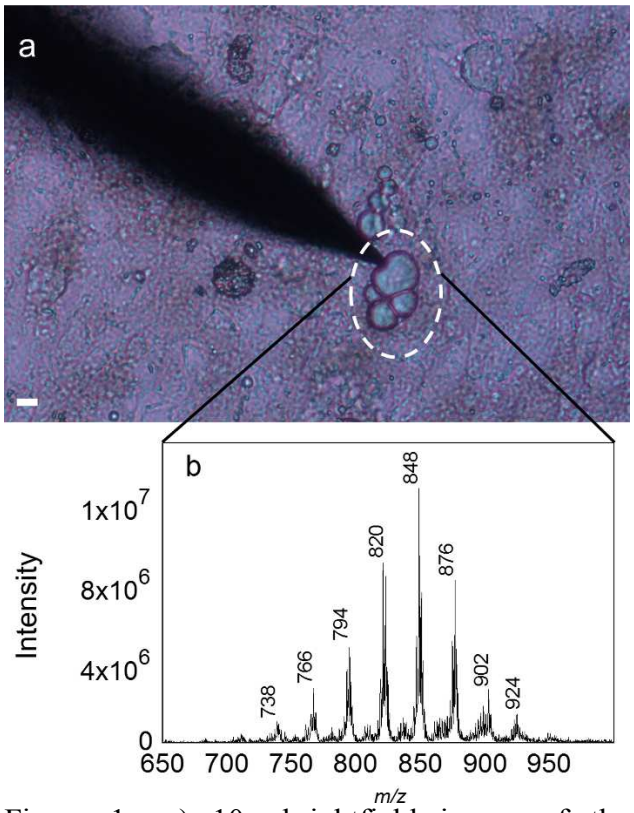

Figure 1. a.) 10x brightfield image of the nanospray emitter in contact with a large lipid droplet of a single adipocyte. b.) Mass spectrum obtained from the extraction. 10µm scale bar.

After extracting adipocytes containing large lipid droplets, early development adipocytes containing small lipid droplets (<1µm) were targeted from the same culture plate to determine if heterogeneity was present (Figure 2). The mass spectrum obtained from a single adipocyte having large lipid droplets is easily distinguishable from one containing small lipid droplets in that the smaller lipid droplet adipocyte contained a higher number of signals with varying

intensities including lipid species with lower mass to charge ratios such as *m/z* 760. The TAG distribution from the early versus late development adipocytes is also distinctive. In the large

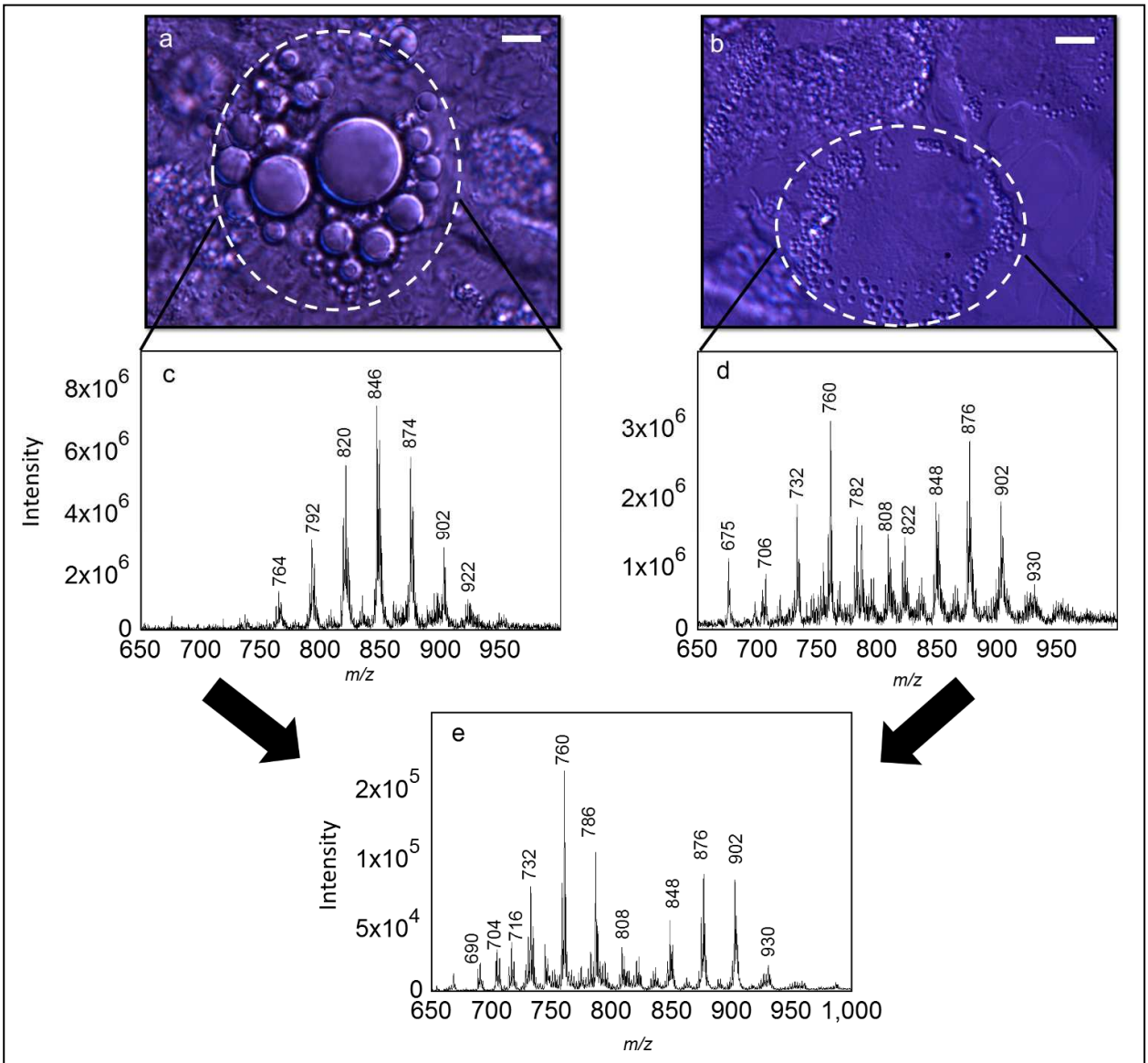

Figure 2. a.) Differentiated human adipocyte containing large lipid droplets. b.) Adipocyte containing small, early development lipid droplets. c.) Mass spectrum obtained from nanoextraction of adipocyte with large lipid droplets d.) Mass spectrum of small lipid droplet adipocyte. e.) ESI-MS spectrum obtained from typical whole flask digestion of multiple adipocytes. Photographs are 100x oil immersion differential interference contrast images, and scale bars shown are 10μm.

lipid droplet extraction, *m/z* 846 clearly has the highest intensity of the large lipid droplet peaks, whereas in the smaller lipid droplet extraction, *m/z* 876 is the highest intensity TAG species. Additionally, the highest intensity ion of the small lipid droplet extraction (*m/*z 760) is absent from the large lipid droplet extraction. We observed heterogeneity even within the distribution of saturated and unsaturated TAGs having the same number of carbons. The large lipid droplet shows a higher intensity of *m/z* 846, while the small lipid droplet extraction has a higher intensity of the saturated form *m/z* 848. The same is true for *m/z* 874 in the large lipid droplets versus *m/z* 876 in the small lipid droplet extraction. In the large lipid droplet extraction, *m/z* 902 TAG shows a much lower relative intensity than the dominant *m/z* 846 peak, whereas in the smaller lipid droplet extraction, the two peaks are nearly equal. The spectra obtained clearly display a definite contrast of lipid information, which would not be discoverable without single cell analytical methods.

To further exemplify the heterogeneity between large and small lipid droplet extractions, we performed a typical collective cell lysis experiment consisting of digestion and chemical extraction of an entire culture flask of adipocytes in varying stages of development using the Folch method.[20] The extract was analyzed with ESI-MS and displays an averaging of the lipid composition obtained from the single cell extractions. This spectrum is further distinguishable from the individual cell extractions in that it has a greater number of ion signals detected, and displays a different distribution of peaks. For both single cell large and small lipid droplet extractions, the *m/z* 902 peak is lower in intensity than *m/z* 874 or 876, but in the ESI-MS spectrum the two display approximately the same intensity. The highest intensity ion in the whole flask extraction, *m/z* 760 matches that of the small lipid droplets, but has new signals at *m/z* 690 and 716 and a loss of peak *m/z* 675. The ESI-MS spectra obtained was analyzed for over

30 minutes with consistent distribution of peaks shown, which did not match any single cell extraction. We determined identity of lipid species using collision-induced dissociation (CID) to observe lipid fragmentation concurrent with fragmentation patterns previously published[6, 21-23] and LIPID MAPS online tools for lipid research (http://www.lipidmaps.org/). For examples see Supplementary Figure 1.

Conclusions:

It has been made clear that vastly different lipid information can be obtained from performing single cell analysis. The single cell approach reported here would be most useful for progressive diseases such as a 3D tumor model as it is a non-invasive technique in which the sample is not destroyed. Obviously nanomanipulation-coupled NSI-MS is not the ultimate method for single cell analysis as it is not high-throughput, but the approach and demonstrative results urge new technology to be created for high-throughput single cell lipidomics analysis. It is evident that in order to understand biological processes as they occur and progress, that we cannot wholly rely on the information obtained from multiple cell analysis. Analyzing diseases on the single cell level, in approaches such as nanomanipulation-coupled NSI-MS, provides the capability to reveal distinct biomarkers for diseases that would otherwise remain absent with current multiple cell technologies.

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