True One Cell Chemical Analysis in Cancer Research: A Review

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Abstract: True One Cell (TOC) analysis Is becoming highly critical for functional studies of cancer cells. This is partially because it is the only form of analysis that provides an avenue for studying the heterogeneity and cell-to-cell variations of individual cancer cells, thus providing unique insight into complex regulatory processes that govern TOC functions within a tumor. Additionally, true one cell techniques are playing an increasingly important role in current attempts to implement TOC metabolomic and proteomic studies, as well as emerging attempts to spatially resolve TOC information. In this review we provide a brief overview of the basis of the field and discuss its applications in TOC metabolomics and proteomics.

Keywords: True One Cell (TOC), metabolome, proteome, genome, fluorescence, mass spectrometry, Raman spectroscopy, microscopy.

INTRODUCTION

The human body is a complex and highly organized structure that contains hundreds of times more cells than there are stars in the Milky Way [1]. Such a perspective is a reminder of how intricate our bodies are. It is also true that there are more viruses than stars in the universe, and while several of these existing viruses do not pose a threat to humans, it serves as a small reminder of the viruses and diseases that do, of which several remain uncurable and merely treatable, such as Cancer [2, 3]. Recent advancement in cancer detection, diagnosis, and prognosis, is mainly owed to True One Cell (TOC) analysis [4-8]. This TOC cell approach has gained immense attention due to its ability to provide a unique insight into the inner workings of cancer cells [9-12]. A living organism is composed of numerous types of cells, each containing a specialized structure, function, and unique gene expression [13, 14]. Therefore, two cells with an identical chemical composition will never exist; even cells of the same type/sub-type with identical or near identical genomic DNA sequences will contain unique gene expressions and thus unique metabolome profiles [13]. Such heterogeneous nature is a result of complex mechanisms that arise from several biochemical interactions occurring between the genome, epigenome, transcriptome, proteome, and metabolome of a system. Such complexity is unfortunately translated into cancer cells [15, 16]. Several types of cancers exist, each with their own characteristics and susceptibilities to anti-cancer drugs, radiation, and other cancer treatments [17, 18]. One of the prominent hallmarks of cancer is malignant tumors. These tumor growths can be slow and, in some cases, non-existent, thus presenting a challenge for early diagnosis [17]. Currently, the “gold standard” for cancer diagnosis is histological and pathological analysis of tumor tissue but liquid biopsy Is quickly gaining traction in cancer research and clinical application. A liquid biopsy is a blood test that detects circulating tumor cells (CTCs) that cause cancer metastasis [17, 19]. Cancers become metastatic when cells from primary tumors begin to spread locally, attacking neighboring sites [19, 20]. These cells then continue to spread into nearby blood vessels and once in the bloodstream they are known as CTCs [19, 20]. In the bloodstream they now have access to the entire body and are capable of infecting other organs, at which point the cancer is targeting more than one area. Therefore, it can be said that these CTCs are precursors that give rise to metastatic populations in other areas of the body. And even though these cells come from a primary solid tumor, tumor analysis is not entirely representative of their molecular nature. Therefore, emerging technological advances for TOC analysis of CTCs are urgently needed, yet their scarcity in blood presents several challenges [20]. Herein we will discuss TOCs’ role in improving our understanding of CTCs and solid tumors.

Currently, research efforts in TOC analysis aim to examine the heterogeneity of individual cells, for such information can be used to improve our understanding of chemical mechanisms dictating cell biology health and disease [21-23]. Consequently, the unique chemical composition of individuals cells cannot be analyzed using traditional bulk-cell techniques since such an approach greatly hinders any attempt to decipher and understand intercellular heterogeneity at the TOC level. This is because bulk-cell techniques provide average values of proteins, genes, and

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ISSN: 1927-7210 / E-ISSN: 1927-7229/23

metabolites by averaging ensemble measurements of millions of cells [21]. Therefore, only one-cell methods such as TOC analysis can provide unique insight into the chemical composition of an individual cell. True One Cell (TOC) studies can incorporate a broad range of techniques such as micro- and nanomanipulation, Raman spectroscopy, microscopy, mass spectrometry (MS), fluorescence, microfluidics, and several other techniques [4-8]. The techniques used for TOC analysis greatly depend on the physical and chemical state of the sample to be analyzed. Often, TOC analysis incorporates technology capable of detecting, isolating, and analyzing individual cells and their intracellular content with increased specificity, thus permitting an enhanced tailored approach for studying cell-intrinsic factors that dictate a cell's functional state. Such capabilities provide scientists with the opportunity to study unrevealed epigenetic differences between individual cancer cells of the same specialization for drug discovery, disease diagnostics, and prognostics. In cancer research, TOC analysis for several omics has been successfully implemented [22-24]. These studies have greatly improved the scientific community's understanding of regulatory processes within cancer cells. Yet, although cancer research has greatly benefited and advanced due to TOC techniques, scientists have yet to identify and fully understand key chemical reactions that govern cancer. As a result, several cancer types remain merely manageable and incurable [25]. One of the current challenges in TOC studies is the volume of chemistry available in individual cells. Cells are small, and their organelles are even smaller, thus making the volume of chemistry a challenging aspect for certain areas within the field of TOC analysis. For example, TOC DNA and RNA can be amplified in genomic and transcriptomic investigations, while proteins and metabolites in proteome and metabolome studies cannot. It is therefore no secret why there has been more success in genomics and transcriptomics. Yet, these omics layers do not provide the best description of what chemical processes take place in a cell [23, 24].

Genomics is known as the most mature of omics [26]. The genome is responsible for associating genomic alterations with genes expressed by analyzing structural and single nucleotide variations at the TOC level. And from such information, scientists can infer what a cell is capable of. In a manner that greatly complements genomic studies, transcriptomics both qualitatively and quantitively examines RNA levels genome-wide [26, 27]. These levels can also be linked to specific physiological processes and cell-diseased states, providing insight into what processes a cell is intending to execute. While TOC genomic and transcriptomics studies continue to improve our understanding of cell biology in health and disease, these omics layers merely give a snapshot view of a cell's phenotype; metabolomics and proteomics are the realms that provide direct detailed insight into an individual cell's phenotype. Subsequently, it is a cell's metabolome and proteome profile that provides the best view of intracellular chemical processes and their relationship with the surrounding environment, thus providing the ultimate census of what is actually taking place within a given cell. Such knowledge has led to increased interest in TOC metabolomics and proteomics in cancer research. And so, cancer continues to be propelled forward by TOC analysis. These accomplishments need to be documented in review papers such as this one, to keep readers updated on the subject. In this paper, we will discuss past and current contributions of TOC analysis in the realm of cancer research and since the field of TOC analysis is very broad, comprising of several bioanalytical techniques, the discussion will be limited to techniques of Raman spectroscopy and mass spectrometry.

The metabolome and proteome of TOC often entails thousands of molecules, and such profiles are constantly changing due to several intrinsic and extrinsic factors [28, 29]. Adding to the complex dynamic, metabolites and proteins consist of a wide range of precursors, intermediates, and end products of cellular processes, all of which greatly vary in structure, concentration, and properties. Analyzing these molecular entities provides extremely valuable information into a cell's phenotype [24, 30]. Yet, such rich chemical diversity only increases the number of challenges to be encountered during analytical processing. The abundance of metabolites and proteins in a TOC varies wildly, while some are present in millimolar concentrations, others are only present in nanomolar concentrations. Ans so, discovery in the realm of metabolomics is greatly driven by analytical and technological techniques that are capable of manipulating TOCs, as well as detecting low concentrations of chemically diverse compounds intracellularly. In general, TOC studies require tools sensitive and selective enough for extremely small volumes, which for cells is typically around the picoliter-microliter range. Such a feat has been a major challenge in the field of TOC analysis, yet continual

development of analytical and instrumental techniques makes TOC analysis possible.

Commonly implemented analytical techniques in TOC studies pertaining to the metabolome and proteome are micro-and nanomanipulation, Raman spectroscopy, microscopy, and mass spectrometry. Fluorescence and microfluidics techniques are also greatly used in TOC analysis but will only be briefly discussed in this review. Mass spectrometry (MS) is one of the commonly implemented techniques in TOC studies of cancer tumors due to its ability to provide superior sensitivity, resolution, specificity, and both quantitative and qualitative data. [31-33] A key advantage of using MS for TOC analysis is its ability to work with extremely small volumes of sample which is a requirement in one cell measurements where volumes lie in the picoliter range [34]. Another key benefit MS provides is the tunable nature of its front end to fit specific sample introduction and ionization needs [32, 33]. Other analytical instruments used in TOC studies, such as Raman spectroscopy, fluorescence microscopy and flow cytometry are not capable of providing the resolution nor sensitivity that comes with using MS [34]. Despite such powerful capabilities, several challenges while using MS exist due to a cell's inherent physical and chemical properties. And while MS is not often used in genomic and transcriptomic studies, where fluorescence detectors are the popular choice, it is the instrument of choice for metabolomics and proteomics [31, 33].

Genome and transcriptome studies come with their own set of challenges; they are easier to study mainly because gene information can be amplified thus eliminating challenges that arise with not having enough samples [34]. Such gene amplification capabilities explain the popularity of these 'omics' in cell research. Before the development of TOC technology, such information could only be extracted in a "bulk" manner which involves the simultaneous extraction of genetic material from thousands of cells which is then averaged [21]. The evolution of TOC technology greatly propelled progress in genomics and transcriptomics on its own, yet such progress was further enhanced when high-throughput systems were introduced for rapid analysis of TOC genetic material. These high-capacity systems allow the genetic information of hundreds of individual cells to be processed rapidly and with ease [35]. Now it is common practice to use single-cell sequencing (SCS), such as: single-cell DNA sequencing (scDNA-seq), RNA-sequencing (scRNA-seq), DNA methylome sequencing, Transposase-accessible chromatin sequencing (scATAC-seq), and Transcriptome sequencing (scRNA-seq) methods in cancer research and clinical practices. Several reviews on TOC analysis on the genome and transcriptome of cancer cells exist therefore such omics will not be further discussed in this paper [36-38].

DISCUSSION

Spectroscopy and Spectrometry

Fluorescence and Microfluidics

Cells contain intracellular content that is naturally fluorescent, such characteristics are exploited in fluorescence lifetime imaging (FLIM) [39, 40]. FLIM is an imaging technique that can capture and monitor the lifetime of a fluorophore regardless of concentration making it very useful for cell molecular function, interactions, and environment analysis. [39, 40] FILM is sensitive enough to detect and quantify the cellular metabolism of intrinsic fluorescent metabolites such as intracellular proteins and other small molecules. For this technique there are several manners in which data can be collected and analyzed. Shirshin *et al.* introduced an improved method for processing FILM data to differentiate between metabolically heterogeneous cellular subpopulations with high accuracy. [41] By implementing non-parametric analysis, they were able to increase the discrimination between metabolically distinct subpopulations. To validate their method of data analysis they monitored the fluorescent lifetime of NAD(P)H in cultured cancer cells via FILM images as pictures in Figure **1** [41].

After the FILM imaging the cancer cells underwent chemotherapy and FILM images were taken once more. The FILM data was deconvoluted using both traditional non-parametric and parametric data as well as their improved method. Their new non-parametric analysis method, based on BI calculations, clustered fluorescence decay curves using K-means algorithm. Their improved approach showed to be the best for detecting metabolic heterogeneity for cell clusters at an efficient pace, yet it is limited by its ability to only screen two groups of cells, which is not ideal in multimodal cells studies. Nevertheless, this label-free sensing technique paves the way for studies which aim to investigate the heterogeneous nature of cellular subpopulations with high accuracy and sensitivity. Furthermore, this approac is very important since cance cells are known to be capable of switching between metabolic pathways. Monitoring such cellular

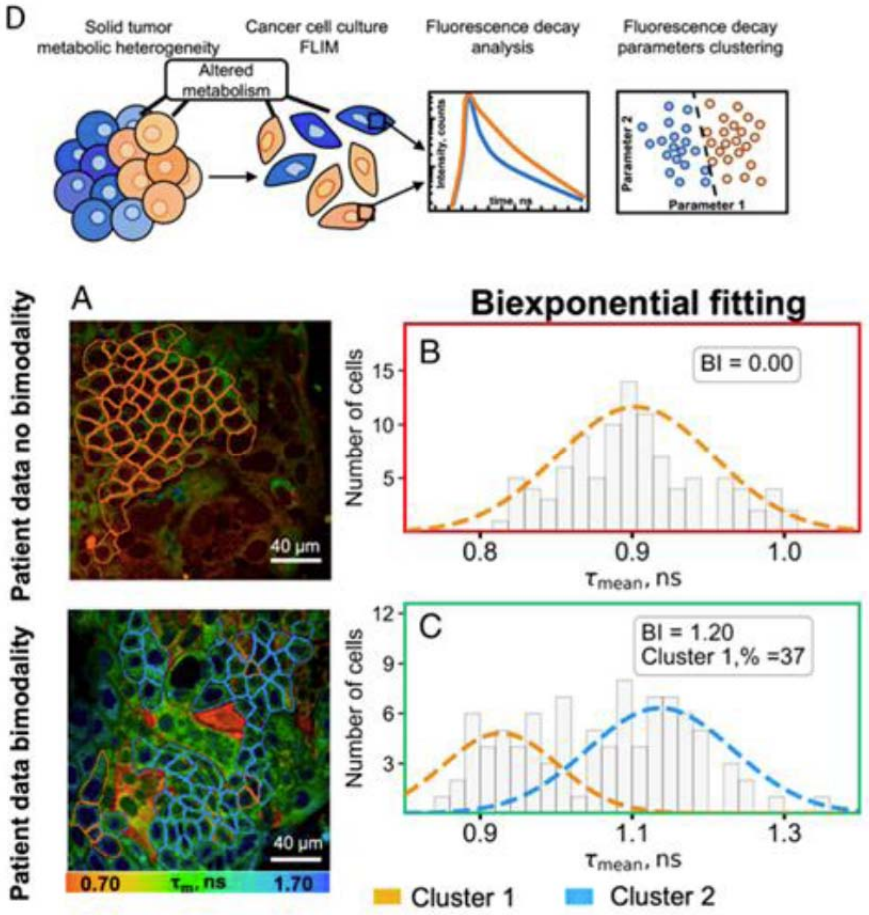


Figure 1: Illustration of tumor metabolic heterogeneity evaluation using FLIM **A**) (**A**) Representative FLIM images of NAD(P)H fluorescence of untreated (Top) and treated cells (4 μM for 24 h, Bottom). Orange and blue contours correspond to two clusters as determined by the K-means algorithm applied for the fluorescence decay curves of the contoured cell. (**B** and **C**) The distributions of the mean fluorescence lifetime t mean for the untreared (**B**) and treated (**C**) cells and their fits to two Gaussians. (**D**) Cancer cells are examined using metabolic FLIM, which provides the kinetics of fluorescence decay from each pixel of the image. The obtained fluorescence decay signal depends on the metabolic state of the cell and can further be analyzed using various parametric and nonparametic methods, which can accurately predict metabolically distinct subpopulations. Reproduced (adapted) with permission from ref. [33] Copyright (2022) by proceedings of the National Academy of Sciences of the Unites States of America (PNAS).

plasticity can greatly aid in several cancer research areas. In a study conducted by Marcus *et al*. phase-contrast with fluorescent time-lapse microscopy was used to investigate the efficacy of an anti-cancer drug known as Selinexor [42]. Here cancer cells were first immunofluorescent stained with anti-RanBP1 and anti-Ki67 for the detection of RanBP1 and Ki67 enzymes. Once stained, the cells were treated with varying concentrations of the anti-cancer drug, and the cells, and intracellular regions of interest (ROI) were monitored using time-lapse microscopy imaging. Both phase-contrast and fluorescence images were taken every 10 minutes. In Figure **2** the concentration of RanBP1 is seen to correlate with the concentration of the drug, and fluorescent cell cycle indicators (FUCCI) signals are seen to be representative of the cell's fates after Selinexor treatment [42]. Red fluorescence in cancer cells increased during mitotic arrest due to a decrease in SCF2 E3-ubiquitin ligase activity; such findings are consistent with the 'competing pathways' hypothesis for cells under mitotic arrest. Through the fluorescence cell cycle inhibitors Marcus and his colleagues were able to conclude that Selinexor inhibits a single protein's function which leads to the blockage of nuclear exports, many of which are key players in cell growth and death regulatory pathways in G1- and early S-phases of a cell. Thus, this study proves that Selinexor has a strong effect on cells with G1/S-phases, such as the cells that make up slow-growing tumors.

Microfluidic devices have also shown great use in TOC isolation and analysis. Nagrath *et al*. constructed a microfluidic chip platform to efficiently isolate CTCs

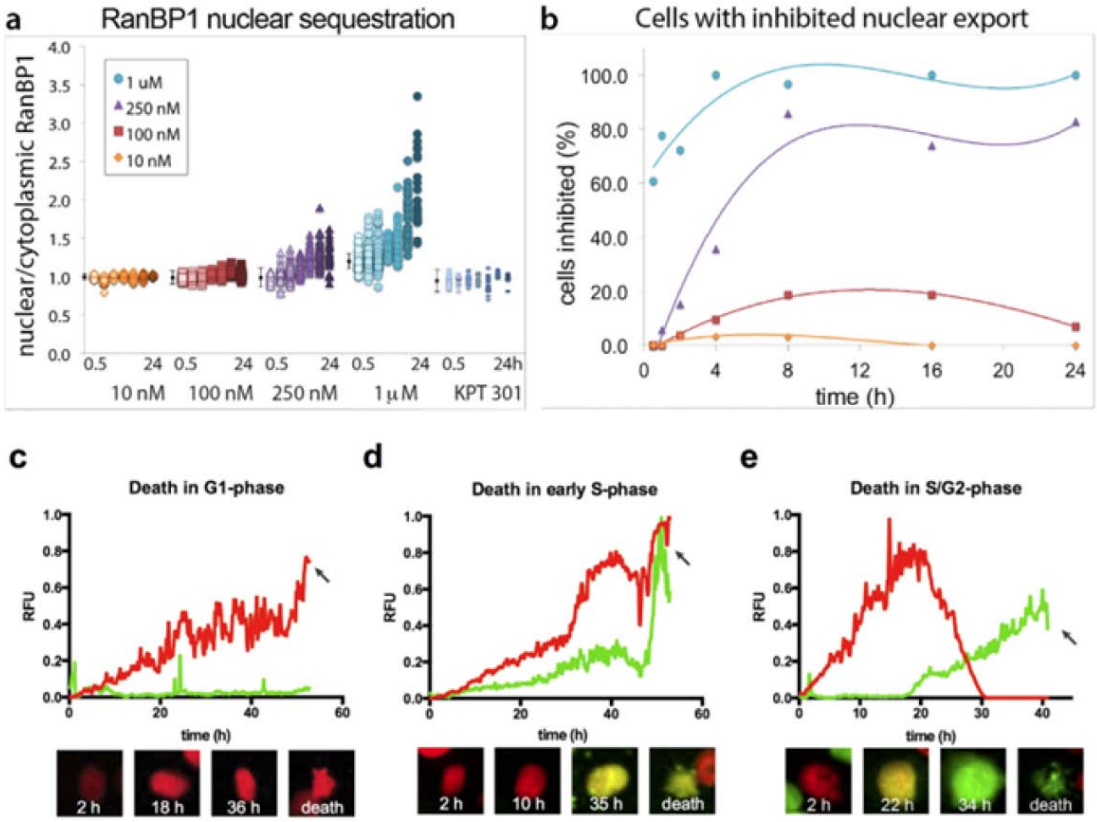
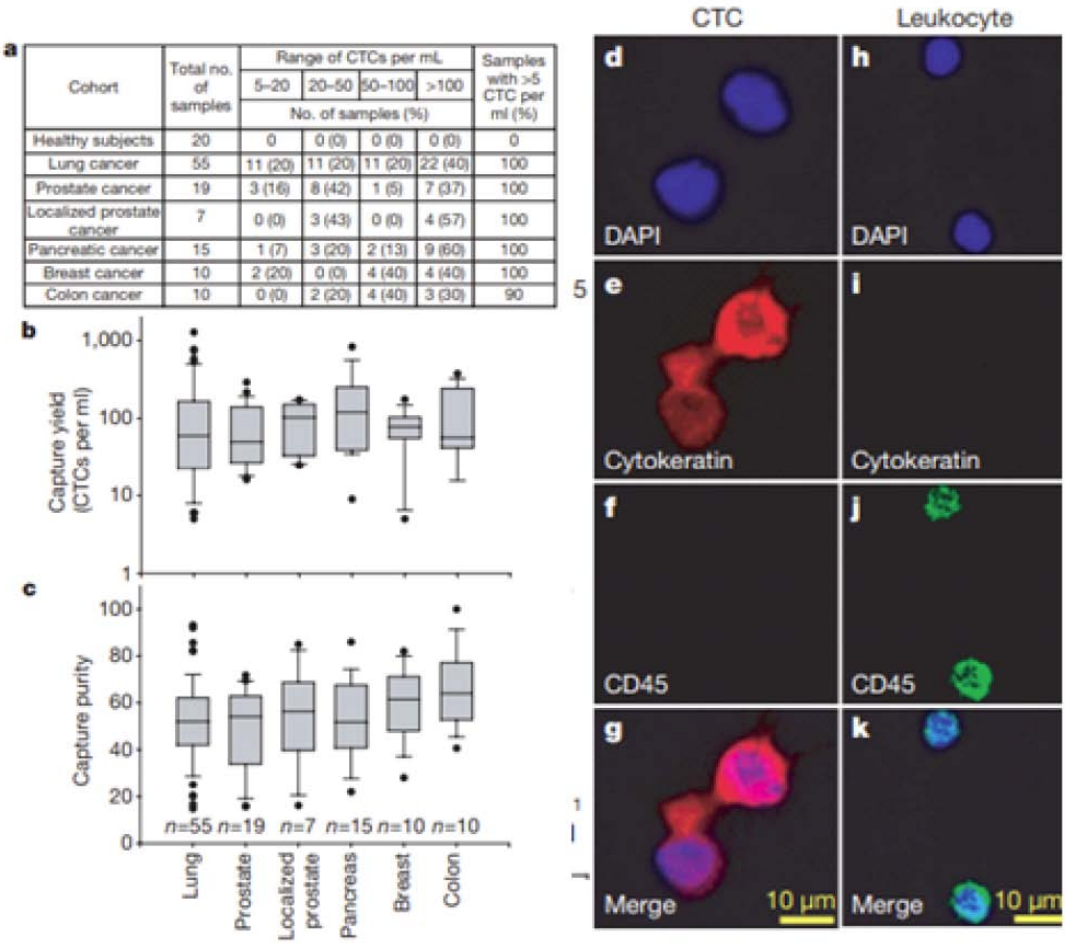


Figure 2: (**a**) Scatter plot showing normalized (control) nuclear/cytoplasmic RanBP1 rations in HT1080 cells for different selinexor concentrations. Cells were treated for: 0.5, 1, 2, 4, 8, 16 and 24h. Error bars show the control average +/- two standard deviations for each experiment. Increased nuclear/cytoplasmic ratios are concentration and time dependent. (**b**) percent of HT1080 cells showing nuclear export inhibition after selinexor is concentration dependent. (**c**) Death in G1-phase. (**d**) Death in early S-phase. (**e**) Death in late S/G2-phase. Reproduced (adapted) with permission from ref. [34]. Copyright (2015) by Springer Nature Limited.

from peripheral whole blood samples [43]. To achieve such selective separation of CTCs, the microfluidic platform was made to consist of antibody EpCAM-coated microposts which can selectively connect with CTCs that overexpress EpCAM. With high sensitivity and specificity, this platform has great use in clinical cancer procedures. Furthermore, this method is very appealing since no pre-labelling of the whole blood is required before microfluidic separation. The platform showed a high success rate (99%) in selectively separating CTCs in whole blood samples from patients with metastatic lung, breast, pancreatic, and colon cancer, as seen in Figure **3** [43]. Once captured, cell types are identified by staining with 4,6-diamidino-2-ohenylindole (DAPI) and adding the antibodies: rhodamine-conjugated anti-cytokeratin (KERSMCR) antibodies, and fluorescein-conjugated anti-CD45 antibodies. As observed in Figure **3**, the different fluorescent tagging of captured cells identifies CTCS which are observably larger in size than normal cells [43]. This method as well as other microfluidic devices are revolutionizing the rate at which CTCs are being isolated and identified for further TOC cell intracellular investigations.

Raman Spectroscopy

Raman spectroscopy is an analytical technique whose use in TOC analysis is rapidly increasing. This vibrational spectroscopic technique provides detailed information about a chemical's structure and composition [34, 44]. Since Raman scatter from water is negligible, this technique is great for cells, which mainly consist of water (70% or more of total cell mass), yet on its own Raman spectroscopy suffers from low sensitivity and spatial resolution for single-molecule detection [44]. Yet, its integration with other advanced analytical techniques and modern data analytics has led to increased resolution, sensitivity, and specificity in in situ, *in vitro*, and *in vivo* TOC studies [44]. Surface-enhanced Raman spectroscopy (SERS) enhances Raman scattering of molecules by taking advantage of the surface plasmon resonance of metal nanoparticles [45]. By coupling such technology, signals from low-concentrated analytes are amplified,



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Cohort	Total no. of samples	Range of CTCs per mL				Samples with ≥5 CTC per ml (%)
		5–20	20–50	50–100	>100	
		No. of samples (%)				
Healthy subjects	20	0	0 (0)	0 (0)	0 (0)	0
Lung cancer	55	11 (20)	11 (20)	11 (20)	22 (40)	100
Prostate cancer	19	3 (16)	8 (42)	1 (5)	7 (37)	100
Localized prostate cancer	7	0 (0)	3 (43)	0 (0)	4 (57)	100
Pancreatic cancer	15	1 (7)	3 (20)	2 (13)	9 (60)	100
Breast cancer	10	2 (20)	0 (0)	4 (40)	4 (40)	100
Colon cancer	10	0 (0)	2 (20)	4 (40)	3 (30)	90

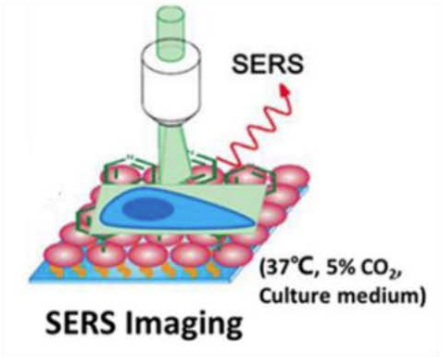
Figure 3: a) Enumeration of CTCs from cancer patients. A, Summary of samples and CTC counts per 1 ml of blood in patients with various advanced cancers and localized prostate cancer. **b**) Frequency of CTCs per 1 ml of blood, by diagnosis. The box plot presents the median, lower and upper quartiles (25^{th}, 75^{th} percentiles). Data point that lie outside the 10^{th} and 90^{th} percentiles are shown as outliers). **c**) Purity of captured CTCs (ratio of CTCs to total nucleated cells), by diagnosis. **d-k**, Higher magnification (20x) images of captured CTCs and haematologic cells from NSCLC patients, stained with DAPI, and for cytokeratin and CD45. Merged images identify CTCs in panels **d-g** and heamatologic cells in panels **h-k**. Reproduced (adapted) with permission from ref. [35]. Copyright (2007) by Nature Publishing Group.

providing ultra-high sensitivity and selectivity [45]. Wilson *et al*. used SERS and nanotags to isolate circulating tumor cells (CTCs) from human whole blood on a microfluidic device. [46] As previously stated, CTCs are malignant cells that are discarded by primary tumors in the bloodstream [47, 48]. Once in the bloodstream they can travel to the entire body and infiltrate distant organs [47]. They are scarce and thus challenging to detect in blood samples [47]. It is therefore not a common clinical practice to screen for CTCs, yet early detection of these cells allows early prognosis and treatment of cancer metastasis [47, 48]. Which is why technologies capable of rapidly detecting and isolating cells with high precision is a key aspect in current attempts of implementing TOC techniques in the clinical field. Wilson and colleagues present an efficient and simple platform that can be potentially used to detect and monitor cancer metastasis. In their study, healthy human blood was spiked with CTCs from cell lines SK-BR-3, MDA-MB-231, and MCF-7 and then underwent tagging with four-color SERS nanotags (iron oxide-gold (IO-Au) core-shell) [46]. The four-color nanotags are capable of binding to four unique surface proteins of CTCs. The CTCs were then captured and immunomagnetically isolated on a microfluidic chip. Isolated cells then underwent Raman spectroscopy analysis to simultaneously detect the nanotags. This study demonstrates that SERS can be successfully used to detect and isolate CTCs from human whole blood for downstream TOC analysis.

SERS can also be used to monitor extracellular content from a TOC. In normal tissue the extracellular pH (pHe) is higher than the intracellular pH, while cancer tissue is observed to have lower extracellular pH (pHe) than normal tissue [49]. This difference in

gradient between the tissue and intracellular content leads to the activation of glycolysis which ultimately leads to cell wall thinning and degradation, as explained by Xu *et al.* [49]. Xu and his collaborators used SERS to monitor the extracellular pH (pHe) of individual cells in-situ during apoptosis [49]. This was done by exposing squamous carcinoma cells to transforming growth factor-b (TGF-B) which is a protein known to suppress or enhance cancer under varying conditions. Once cells underwent TGF-B stimulation, the pHe of temporal and spatial locations was monitored and imaged. This study proved that SERS can be used to investigate the extracellular content of TOCs. This is the sensitivity of varying cells to a drug [49]. In TOC studies, Raman is commonly coupled with optical microscopy. Integration of optical microscopy allows for advanced visualization and analysis of cellular biomolecules such as lipids, proteins, carotenoids, carbohydrates, etc. Such metabolome data reflects the physiological states, phenotypic traits, and metabolic activities of cells. Tip-enhanced Raman spectroscopy (TERS) is a nano-spectroscopic technique that emerged from this coupling. The technique has gained remarkable attention due to its ability to provide site-specific information of material surface regardless of morphology and requires little to no sample preparation. This tool is especially popular in TOC special resolution where both the chemical and morphological information is essential [50]. A study conducted by Lifu *et al.* used both SERS and TERS to investigate the ligand−receptor binding chemistry and specificity of integrin αvβ3 in a cancer cell's membrane[51]. The implemented technique demonstrated high resolution and sensitivity capabilities for molecular ligand studies on TOCs. These capabilities can aid drug discovery and TOC spatial studies where it is necessary to study the molecular and interactive nature of ligands and receptors. Other analytic techniques, such as atomic force microscopy (AFM) and fluorescence-based superresolution microscopy provide kinetic and binding affinity information but are unable to provide molecular information for the binding structure [51]. X-ray crystallography and nuclear magnetic resonance can provide molecular information but require sample extraction methods that membrane destruction and are therefore not useful in spatial studies [51-54].

Higher sensitivity and resolution of SERS can be achieved by incorporating quantum sensing probes like Dharmalingam *et al*. effectively demonstrated. In their study, self-functionalized anionic quantum probes in conjunction with SERS were used to study TOC metastatic cancer cells *in vitro* [55] Qi *et al*. used SERS to shed light on the greatly disputed molecular mechanism associated with plasmonic photothermal therapy (PPT)- induced apoptosis between cancerous and normal cells [56]. They designed a SERS nanoprobe that targets the nucleus and mitochondria of cells to subjugate HeLA cancer cells and L929 to thermal stress thus inducing hyperthermia cell death. During apoptosis they monitored protein and genetic data from individual cells and found that cancer cell apoptosis was mainly regulated by cellular proteins, while normal cells were mainly controlled by cellular DNA [56]. Oliveira *et al*. are the first to image and characterize TOC cancer cells using both multiplex SERS and microdroplets for rapid cancer cell phenotyping [57]. Using a seed-mediate method they synthesized gold nanostars (GNSs) that were then coded with Raman tags (RaR) 1-NAT, toluidine blue (TB) and Nile blue (NB). Once the quality of synthesized SERS tags was verified, they exposed different cancer cells to these tags and conducted SERS analysis on them, and from the analysis the SERS signals were deconvoluted and cell type was determined [57]. Mrđenović *et al*. used TERS to visualize unlabeled cell membrane molecules within one pancreatic cancer cell [58]. The resolution provided by this technique revealed that BxPC-3 cell membranes contained unexpected biomolecules. Du *et al*. used Raman spectroscopy and Raman scattering (SRS) microscopy to study intracellular lipid droplets from individual metastatic melanoma cells [59]. By taking hyperspectral-SRS images of these droplets they were able to identify fatty acid synthesis pathway as a druggable susceptibility for differentiated melanocyte cells. Their proof on concept study proved that the coupling these two very powerful techniques allow the identification of phenotype-dependent metabolic susceptibilities of TOCs within the Raman spectral fingerprint region, thus providing a general approach for spatially mapping metabolites. Laser tweezers have also been integrated into Raman Spectroscopic techniques. Casabella *et al*. improved the throughput capabilities of TOC laser tweezers Raman spectroscopy (LTRS) by automating the process using low-cost microfluidic chambers. [60] Such an approach allowed them to differentiate and analyze subcellular content of cancer epithelial prostate cells and heathy lymphocytes in a TOC manner. Qiu *et al*. used LTRS for monitoring internal chemical and structural changes of human nasopharyngeal cancer cells exposed to X-ray irradiation at a TOC level [61].



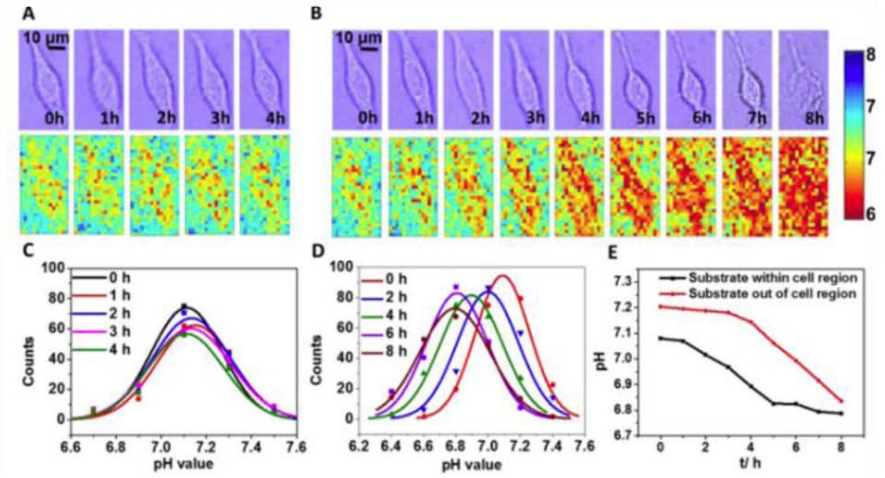


Figure 4: Real-time in situ pHe monitoring of a single living TCA-8113 cell. Bright-field microscopic images (upper panel) and pH images (lower panel) were obtained respectively during (**A**) the first 4 h under normal growth state and (**B**) the following 8 h after the addition of DMEM with 20 ng/mL TGF-b. pH distribution images were produced by converting the experimentally obtained peak intensity ration of 1610-1575 cm^{-1} to pH values using the work curve in Figure **3C**. (**C**, **D**) Statistical profiles constructed using the pH distribution data obtained within the cell region at specific time points in parts **A** and **B**, respectively. (**E**) Comparison of pH variation tendency of the substrate within (black line) and out of (red line) the cell region. Reproduced (adapted) with permission from ref. [49]. Copyright (2018) American Chemical Society.

These studies presented a novel tool for effectively evaluating radiotherapeutic effect on tumor cells at the molecular level. Fang *et al*. further validates the use of LTRS in TOC studies by accurately isolating cells using a polydimethylsiloxane chip [62]. They were able to separate gastric cancer cells from 3 other noncancerous cell types with a ~90% success rate. Raman spectroscopy is also a great technique to implement during anti-cancer drug therapeutics. Wanxue *et al*. established a simple, sensitive, and economical method for measuring the IC50 values of anti-cancer drugs on TOCs [63]. This was achieved by doping cancer cells with varying levels of D2O and monitoring D2O metabolism (done by monitoring intensity ratios of C-D/ (C-D + C-H) peaks) using Raman spectroscopy. This method has the potential of replacing traditional cell viability methods such as CCK-8 assay and ATP measuring kits. Raman spectroscopy and its modified forms continue to demonstrate its vital role in TOC studies where the heterogeneous nature of these cancer cells is extremely important to profile. It has also subsequently shed light on the heterogeneity in the complex microenvironment of intertumoral tumors [64-70].

Mass Spectrometry

True one cell biomolecular imaging can also be conducted using mass spectrometry (MS) analysis which is a technique by which volatile and semi-volatile chemical substances are identified based on their unique mass to charge ratio (m/z) [31, 33]. This technique provides unprecedented resolution and sensitivity for measuring and visualizing the spatial distribution of molecules in one cell. MS encompasses a vast number of instrument ionization techniques for profiling region-specific molecules and lipids within a

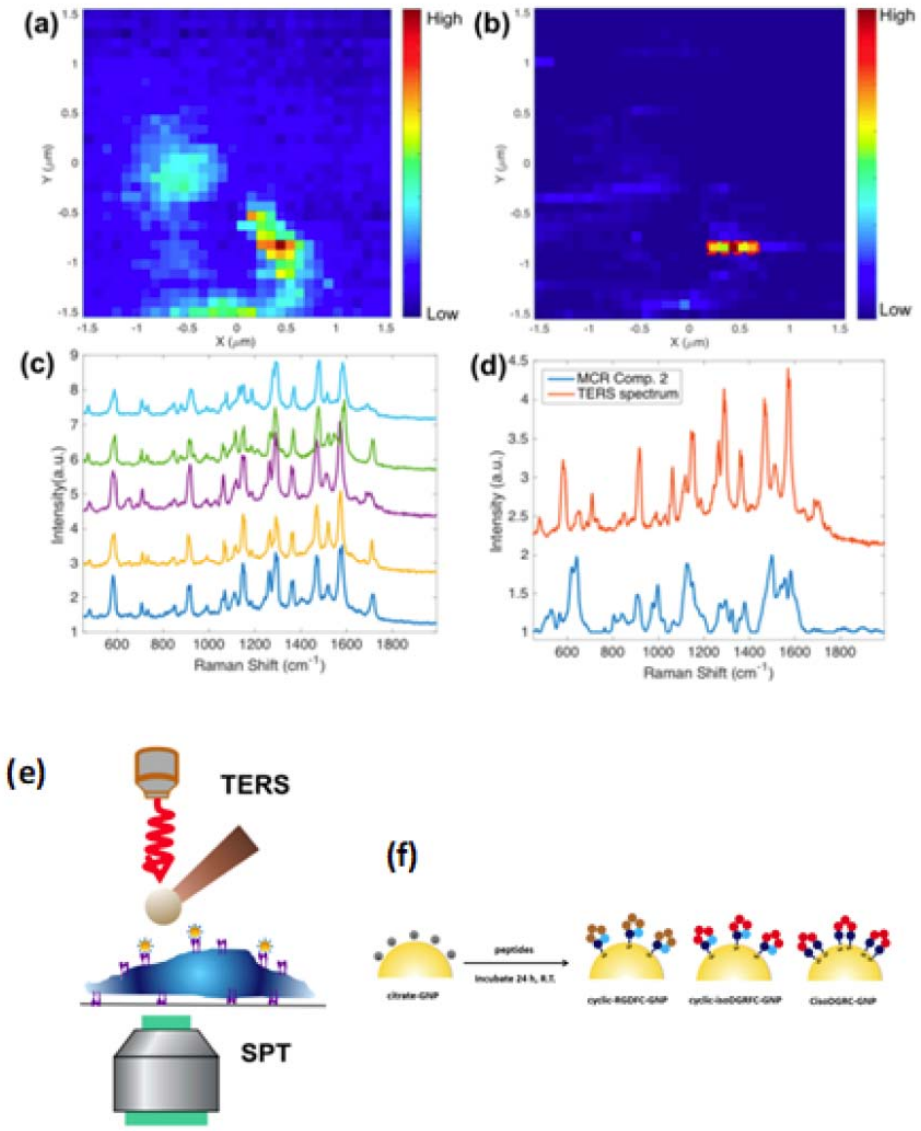


Figure 5: TERS imaging of cyclic-RGDFC-GNRs bound with sw620 cells. (**a**) TERS heat map (3 x 3 μm2) generated using single-peak intensity at 1002 cm^{-1} (step size: 93 nm). (**b**) MCR map generated using scores of each TERS spectra toward the MCR component corresponding to integrin avb3 determined from the SERS experiments. (**c**) TERS spectra selected from high intensity pixels in panel **b**. (**d**) Comparison between TERS spectrum and MCR component. MCR is able to filter TERS data to generate much cleaner maps. (**e**) Illustration of basic technique setup (**f**) Schematic Illustration of Gold Nanoparticles Conjugated with Three Different Peptide Ligands (cyclic-RGDFC, cylic-isoDGRFC, and CisoDGRC). Reproduced (adapted) with permission from ref. [52]. Copyright (2017) American Chemical Society.

cell. The ionization method implemented in TOC studies greatly depends on two main factors: the chemical nature of the analytes to be detected and the end goal of the study. For example, certain MS techniques are more destructive than others, and if studies require the continual use of certain cells, some ionization techniques might not be suitable for such needs. Several ionization techniques for the detection of cellular molecular biomolecules exist in MS. TOC intracellular chemistry can be extracted using laser ablation, ion beam bombardment, and droplet extraction [31, 33]. Thus, MS instrumentation falls into two broad ionization categories, desorption ionization, and atmospheric pressure ionization. There are hundreds of published papers on the use of these laser and ion beam-based MS techniques used for TOC analysis. Therefore, this paper will only update the reader on the newest achievements in TOC-MS technology pertaining to cancer research. A few of the most popular desorption ionization methods used in TOC analysis for metabolomics and proteomics have been matrix-assisted laser desorption ionization (MALDI), secondary ion MS (SIMS), and electrospray (ESI) MS. MALDI is a soft- ionization label-free

technique capable of detecting multiple metabolites from one cell. With pixel resolutions of 5-20um, MALDI is a great tool to implement in spatial resolution studies of cancer cells [71, 72]. Hamilton *et al*. successfully created a method that decreases ion suppression during MALDI lipid imaging [73]. Using direct analyte-probed nanoextraction (DAPNe) they were able to extract lipid content effectively and efficiently from one Snu-5 gastric cancer cell for MALDI MS analysis [73]. By eliminating the need to fix an individual cell onto a MALDI plate they decreased the complexity of the sample thus decreasing ion suppression that often accompanies MALDI imaging of lipids. The use of DAPNe methods in TOC studies present several advantages since such a method provides high specificity and is associated with having little to no cell environment disturbance. In a TOC study conducted by Yang *et al*. 105-106 protein copies were detected per cell using MALDI MS (with a spatial resolution of 50 um) combined with a microfluidic approach for sample preparation, a combination coined "MIMAS" (Microfluidic assay combined with MALDI Mass Spectrometry). This study and another past work allowed Yang *et al*. to develop a method that does not require fluorescent-based method for detecting highly abundant proteins from a cell [74-76]. In this study isotope-labeled peptides were used as internal standards to quantify the presence of protein Bcl-2, known to be a key regulator in cell apoptosis[74]. The combination of these two techniques is MCF-7 human breast cancer cells were isotopically labelled and then underwent freeze-thaw cell lysis inside a microfluidic chip created by the group prior to immunocapture, tryptic digestion, and MS analysis. The design of the chip allowed the chemistry of TOCs to be secluded from other cell chemistry thus keeping the heterogeneity integrity of each individual cell. Once lysed the cells were coated with matrix and underwent MALDI MS. Sun *et al*. recently used MALDI-TOF MS to quantify active metabolites observed in TOCs responding to a widely used chemotherapy drug known as irinotecan (IRI) using a microfluidic platform, with limits of detection and quantitation of 2.2 and 4.5 ng/ml [77]. Wu *et al.* succeeded in increasing MALDI-MS detection sensitivity to cancer cells by 60% [78] by populating cells with nanoparticles using microwave digestion. Conventional MALDI requires cells to be placed in a high vacuum chamber where required chamber pressure and temperature can perturb the spatial distribution of metabolites in a cell, consequently, live cell imaging is impossible with conventional MALDI methods [72, 79]. Such drawback has led scientist to develop atmospheric pressure MALDI MS imaging (AP-MALDI) which allows TOCs to be studied at atmospheric pressure and temperatures which are closer to a cells physiological state [80-82]. TOC analysis has greatly benefited from AP-MALDI since cells no longer need to be put under strenuous high vacuum conditions and the sample preparation required is minimal [80, 83]. Schober *et al.* were the first to develop a TOC technique that provided high spatial resolution (7 um pixel), high mass resolution (R= 100,000 at m/z=200) and high mass accuracy (<3ppm rms) for TOC metabolomics [84]. Such unprecedented capabilities were achieved by combining the powers of (SIMS) and MALDI for TOC analysis. In this study cervical cancer cells (HeLa) were grown on indium tin oxide (ITO) coated glass slides and fluorescently dyed with 3,3'-dihexyloxacarbocyanine iodide which produced emission at 501nm and allowed TOCs to be imaged using an Olympus BX-40 microscope [84]. Once fluorescently analyzed TOCs underwent Atmospheric pressure- MALDI MS imaging with a laser ablation step size of 7 um causing a 5-um ablation spot on a cell, which produced m/z signals for several metabolites of varying size and class.

A key aspect of TOC analysis is the use of extraction and ejection tools and methods for cell and subcellular organelles [85-87]. Tools for cell manipulation emerged at the same time cell and tissue studies began and their implementation has been and continues to be crucial for conducting certain TOC studies [87-89] . In TOC analysis, nanopipettes are some of the most used probing tools [85, 86, 88-90]. Currently there are two commonly used methods for extracting intracellular chemistry and both use specialized micro and nano probes [88, 89]. The first method entails the destruction of the TOCs analyzed. When the physical integrity of the cell to be analyzed is of no importance, biochemical media can be extracted by lysing such cell. This is readily achieved using microcapillary probes with orifices capable of aspirating and ejecting individual cells. Once aspirated cells can either undergo lysis within the probe or be selectively transferred to another location for dissemination.

Past studies conducted by our group perfectly demonstrate these TOC manipulation techniques [91-94]. In these studies, a micro- and nano-manipulate stage, that was designed and patented by Dr. Guido Verbeck, comes equipped with several types of probes that can be used for one-cell manipulation, as seen in Figure **6** [95-97]. The second method extracts cellular chemistry in a less destructive manner. Instead of

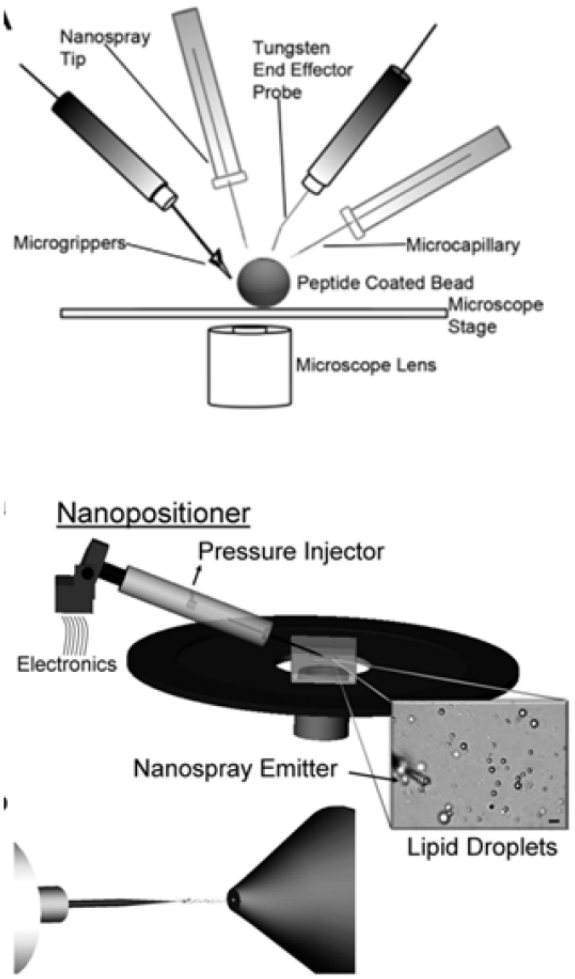


Figure 6: Schematic of direct organelle mass spectrometry and nanoemitters. **A** schematic representation of DOMS using the L200 nanomanipulator coupled to nanospra mass spectrometry. A) Illustration of the types of nanoemitters used for nanonipulation. **B**) The nanopositioner, set on an inverted microscope stage, electronically controls the x, y, and z positioning of a nanospray emitter through a user-operated joystick. The nanospray emitter is prefilled with a microextraxtion solvent and is directly connected to a pressure injector. Approaching purified LDs in buffer, the emitter can selectively load LDs of interest, microextract their lipid contents, and analyze their compositions by nanospray mass spectrometry. Reproduced (adapted) with permission from Ref. [95-97].

breaking apart a cell for its subcellular contents (cell lysis) nanoprobes can carefully pierce a cell at specific locations and aspirate desired content with little disturbance. In a past study conducted by our group, this minimally invasive nanomanipulation technique was used to extract lipid droplets from subcellular organelles [98]. This novel technique is vital in TOC studies since it provides highly targeted cells and subcellular manipulation and extraction thus eliminating the need for lengthy traditional separation procedures. Phelps *et al*. seeded murine fibroblasts 3T3-L1 cells on a glass bottom culture dish where they were introduced to a cocktail of chemistry for lipid growth enhancement [98]. Once lipid droplets were observed to be between 20 - 50 um they underwent nanomanipulation for microextraction. Using a control joystick, the extraction probe tip was positioned within the lipid droplets found inside the cell. The droplet was then aspirated into a nanopipette containing 10 ul of 2:2 chloroform: methanol (v/v). Once contained in the extraction probe, the content was either directly introduced to a nanospray ionization (NSI) source for NS-MS or it was ejected onto a glass slide where the extract was allowed to evaporate to dryness and then spotted with DBH matrix solution using a second emitter for MALDI MS. In Figure **7** you can see the emitters probing a lipid droplet with high precision and the matrix spot that is 57 um [98]. Once spotted heat images of the organelle content can be taken for chemistry density visualization. These spots then undergo MALDI MS and MS/MS analysis. In Figure **8** you can see two spectrums, one for the NSI-DOMS analysis and the other corresponding to the MALDI analysis. From the data obtained the study concluded that NSI-DOMS provided lower resolution than MALDI-MS, thus NSI does not allow for MS/MS studies. These findings have been integral in studies aiming to selectively investigate subcellular chemistry with minimal damage to the cell [98].

Yang and colleagues used single-probe mass spectrometry (MS) to study the metabolites of liver colorectal CSCs at the TOC level. Here they were able to detect abundant levels of tricarboxylic (TCA) metabolites and unsaturated lipids in CSCs compared to normal cells [99]. CSCs were first sorted using fluorescence-activated cell sorting technology, and once sorted these cells were suspended in media allowing them to dissociate from each other. Once isolated these cells were transferred onto a laminin-coated glass slide. Once properly attached the glass slide was placed on a motorized stage and using quartz needles, the chemistry within individual cells was extracted. Chen *et al.* studied the metabolome of drug-resistant cancer cells using single-probe MS [100]. They used HCT-116 cancer cells which were exposed to the drug known as Irinotecan (IRI) which is commonly used in metastatic colorectal cancer chemotherapy [100]. IRI-resistant cells were then isolated and harvested for MS analysis. To extract the subcellular contents of individual cells Chen *et al.* used a dual-bore quartz needle which was connected to a fused silica capillary. Once extracted using the needle

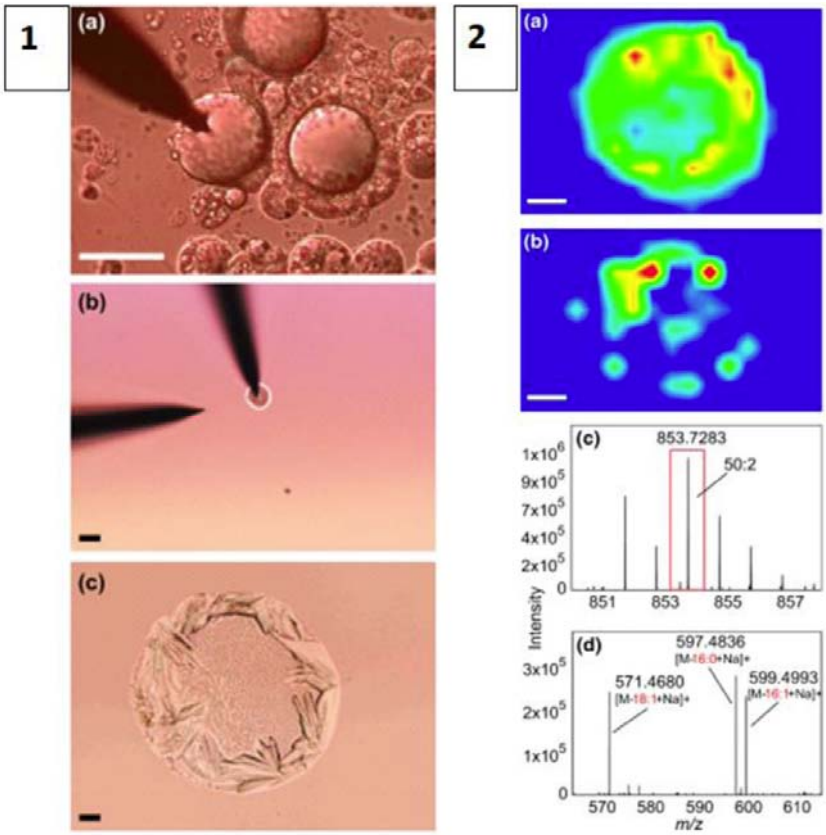


Figure 7: Bright field and heat image of lipid droplet. (**1a**) Nanospray emitter positioned within lipid droplet of a 3T3-L1 differentiated adipocyte in preparation for microextraction. (**1b**) Following microextraction, the nanospray emitter (top) is shown depositing the dissolved lipid droplet contents onto a precleaned glass slide, the second emitter (left) is utilized to spot matrix solution on top of the analyte spot, resulting in an ~450 µm matrix spot (**1c**). Scale bars represent 50 µm. (**2a**) Total ion count of the co-deposited organelle content and matrix spots. (**2b**) Selected TAG [50:2 + Na]+ at m/z 853.72 ± 0.003. (**2c**) Fifty carbon acyl spectrum obtained from total ion count, with red box indicating TAG selected for MS/MS analysis. (**2d**) Resulting spectrum from CID of m/z 853.7283 with fatty acid losses highlighted. One hundred µm scale bars. Reproduced (adapted with permission from ref. [98]. Copyright (2011) by B American Society for Mass Spectrometry.

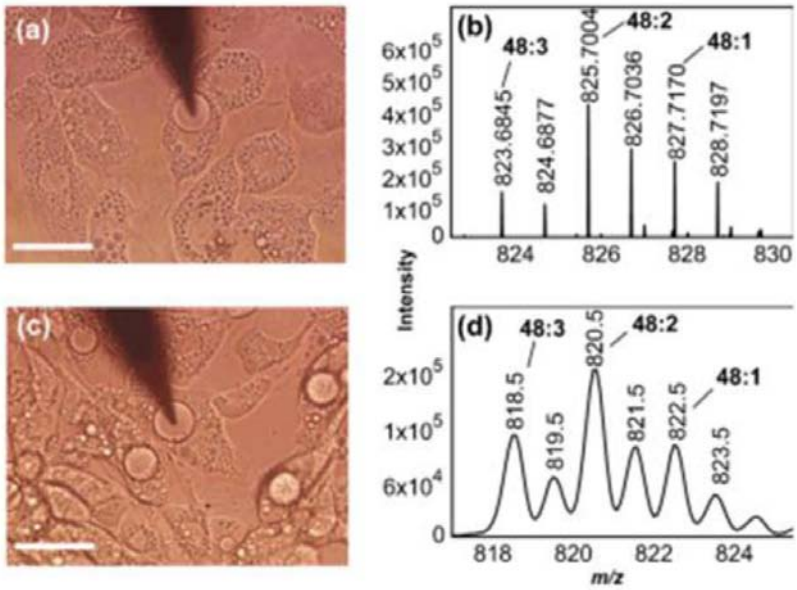


Figure 8: (**a**)-(**b**) Bright field image of lipid droplet microextraction and corresponding MALDI-DOMS mass spectrum of the 48 carbon TAG molecular species. TAG species were detected as [M+Na]+. (**c**)-(**d**) Bright field image of lipid droplet microextraction and corresponding NSI-DOMS mass spectrum of the 48 carbon TAG molecular. TAG species were detected as [M+ NH4] +. Bright field images taken using 40x magnification. Scale bars represent 50 µm. Reproduced (adapted) with permission from ref. [98]. Copyright (2011) by American Society for Mass Spectrometry.

probe, the chemistry was ionized using nano-ESI. Cell-to-cell interaction is extremely crucial in cancer research, especially in tumor microenvironment which is made up of several types of cells communicating with each other for survival. Chen and his colleagues used single-probe mass spectrometry in combination with fluorescence microscopy to study the cell-cell interaction of drug-sensitive cancer cells [101]. Here HCT116 colorectal cancer cells were continuously treated with irinotecan (IRI) for 20 days after which surviving cells were deemed to be drug resistant. These cells were then co-cultured with normal HTC116 that were tagged with a green fluorescent protein. Using fluorescence microscopy these co-cultured cells were located on a glass cover slip and then probed using a dual-bore quartz needle which allowed the extraction of intracellular metabolites for MS analysis using a triple quad mass analyzer. From their study, they were able to detect lipid reprogramming occurring within drug-sensitive cells after they acquired drug resistance from neighbors. Phospholipids (e.g., PC, PE, and LysoPC lipids) were observed to be the main class of lipids altered in cells undergoing drug resistance. The authors speculate that the upregulation of sphingomyelin and the downregulation of the TCA cycle is one of the main driving factors in such lipid alteration [101]. Furthermore, cell wall thickness was observed to increase with the upregulation of SM, thus making them impenetrable to certain drugs. Another desorption MS technique used in TOC analysis is near-field desorption. Yin *et al*. recently showed successful chemical and topographical imaging with resolutions ranging from 350 nm to 250 nm for TOC analysis using near-field desorption post-ionization time-of-flight mass spectrometer (NDPI-TOFMS) [102]. All the studies just mentioned are slowly unearthing cancer cell mechanisms thus proving its immense value in cancer research. The field of TOC analysis is rapidly expanding and has so far experienced great feats, yet in order for this field to continue providing vital and novel information for the purpose of eradicating cancer, some of the limitations present in the field must be removed [103-106]. Many of these restrictions are closely tied to technological advancement in the field. To achieve unprecedented data acquisition, innovative technology modalities need to be developed in the upcoming years.

CONCLUSION

The heterogeneous nature of cancer cells at the TOC level emphasizes the need for a tailored one cell analysis approach. Studying cancer cells in bulk convolutes key molecular metabolite information that is vital for understanding underlying mechanisms that drive cancer development, progression, and disruption. Thus, the emerging field of true one-cell techniques is expected to be the leading realm in cancer cell research. Accomplishments in the field are mainly a result of advancement in instrumentation development, which provides scientists with unprecedented resolution, sensitivity, and selectivity for multi-omics studies at the TOC level. The work summarized in this paper has provided novel insight into cancer cell mechanisms, drug and cancer cell interactions, and cell-cell communication which has led to the scientific communities greater understanding of CTCs and microtumor environment.

CONFLICT OF INTEREST STATEMENT

This review presents no conflicts of interest.

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Received on 28-03-2023 Accepted on 26-04-2023 Published on 10-05-2023

https://doi.org/10.30683/1927-7229.2023.12.06