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Preserving Single Cells in Space and Time for Analytical Assays

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Abstract

Analytical assays performed within clinical laboratories influence roughly 70% of all medical decisions by facilitating disease detection, diagnosis, and management. Both in clinical and academic research laboratories, single-cell assays permit measurement of cell diversity and identification of rare cells, both of which are important in the understanding of disease pathogenesis. For clinical utility, the single-cell assays must be compatible with the clinical workflow steps of sample collection, sample transportation, pre-analysis processing, and single-cell assay; therefore, it is paramount to preserve cells in a state that resembles that *in vivo* rather than measuring signaling behaviors initiated in response to stressors such as sample collection and processing. To address these challenges, novel cell fixation (and more broadly, cell preservation) techniques incorporate programmable fixation times, reversible bond formation and cleavage, chemoselective reactions, and improved analyte recovery. These technologies will further the development of individualized, precision therapies for patients to yield improved clinical outcomes.

Keywords

laboratory medicine; single-cell analysis; sample preparation; fixation; preservation; signaling; analytical chemistry; bioanalytical chemistry; clinical assays

1. Introduction

1.1. Lab Medicine and Single-Cell Assays

The clinical laboratory provides healthcare professionals with myriad information used in disease detection, diagnosis, and management. The outcome of laboratory assays influences

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Declaration of interests

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upwards of 70% of medical decisions, highlighting the importance of obtaining reliably accurate results [1]; Through the Laboratory Medicine Best Practices (LMBP) Initiative, the Centers for Disease Control and Prevention's (CDC) Division of Laboratory Systems (DLS) has sponsored the development of a wide range of best practices guidelines for various aspects of the clinical sample pipeline in an effort to identify "effective laboratory medicine practices associated with improved healthcare quality outcomes [2]." The process of testing a primary patient sample consists of several steps that can be sorted into three main phases: pre-analysis, analysis, and post-analysis (Fig. 1A) [3]. Each step may be performed by different personnel in different locations and at different times, creating multiple points during which unintended sample alterations might occur which could yield errors in sample assay outcomes [3]. Primary tissue collection is performed by on-site clinical personnel with initial sample processing beginning in the collection tube (Fig. 1A). For example, ethylenediaminetetraacetic acid (EDTA) is a common additive to hematology collection tubes and acts to chelate divalent ions for preventing blood coagulation. Other sample modifiers include oxalate, citrate, heparin, fluoride, and others to initiate separation or preservation [4]. These early sample processing steps can alter assay outcomes if their impact is not considered [4]; for instance, EDTA reduces the concentration of free calcium within serum and thus is not an appropriate modifier for calcium assays. After collection, the sample must be transported from the clinic to a laboratory, with delivery times ranging from tens of seconds when using an automated pneumatic tube system to several minutes for manual deliveries (Fig. 1A) [5]. Once the sample has arrived at the clinical laboratory it must undergo any number of appropriate subsequent processing steps prior to analysis, including aliquoting, enrichment and purification processes, centrifugation, fixation, and incubation (Fig. 1A) [3]. Following analysis, data must be processed and results reported to the physician where they are interpreted and integrated with additional information to decide on a clinical treatment or prevention plan (Fig. 1A). Although the timing of each process within the clinical workflow varies from assay to assay and can be difficult to monitor [6], total turnaround times (TAT) typically vary from 30 to 90 minutes [6–9]. As an example, transportation times can range from tens of seconds when using an automated pneumatic tube system to tens of minutes for manual deliveries [5].

Various instruments and analytical methodologies are used to quantify analytes in the clinical laboratory, and an extensive list has been described previously in detail [10,11]. A large fraction of the assays within the clinical laboratory are serologically based. The commonly used comprehensive metabolic panel consists of a series of assays (including those that use ion-selective electrodes and spectrophotometric methods) to quantify serum analytes, thus providing information on a range of organ systems such as the heart, kidney, and liver [12]. These assays are ubiquitous since the analytes remain stable for times on the scale of hours and the assays themselves are remarkably robust and reliable and provide high-value data on organ function [4]. However, serologically based assays provide indirect, population-averaged measures of cell health and well-being within the organs. Other clinical assays focus on direct quantification of intracellular constituents but are still averaged over many cells. For example, red blood cells are used as a source of hemoglobin in an assay that electrophoretically separates and measures defective hemoglobin proteins for diagnosis of thalassemias or sickle cell [10]. Liquid and gas chromatography coupled with mass

spectrometry are used to separate, identify, and quantify analytes of interest from populations of cells from various biologic fluids [10]; additionally, mass spectrometry imaging techniques have been used clinically to probe tumor heterogeneity and diagnose disease [13]. Bulk assays that analyze cellular components are of high clinical value in that the pooled analytes readily exceed the limits of detection, provide large sample sizes which increase the ease of assay, and can be automated [3,7]. While these clinical assays provide direct information about cellular components, the measurements are population-averaged and may not accurately describe the diversity of cells within the population; however, this diversity is increasingly recognized as impacting critical attributes of many diseases, including their rate of progression, and as such single-cell assays have been developed to probe the properties of individual cells.

Single-cell analyses enable researchers to identify rare cells and cell subpopulations, trace cell lineages, and probe mechanisms that are obscured by bulk analysis [14–17]. Disease may arise from the properties of individual or founder cells; further, disease treatment failures may arise from the diversity of cell behaviors and responses. For example, single-cell analyses performed in academic laboratories have revealed that intratumor heterogeneity is largely responsible for supporting cancer evolution, impacting the efficacy of treatments, and fostering drug resistance [15–28]. Assays of single cells can be used within the clinical laboratory to provide significant disease detail, promoting improved therapeutic decisions and providing more accurate prognoses (Fig. 1B). Microscopy-based single-cell assays have commonly been employed in the clinic, including peripheral blood smear analysis to examine cell phenotype. In this analysis, microscopy is used to investigate the size, shape, and morphology of individual circulating blood cells [10]. High throughput laser-based single-cell analyses, including flow cytometry, are also common in the clinical laboratory [29–31]. Flow cytometry has been used clinically to characterize leukemic cells and immune deficiencies, to predict response to monoclonal antibody therapy, and to screen for genetic diseases [32]. Other single-cell assays developed in academic and industry laboratories are on the horizon and are expected to be incorporated into the clinical workflow in the near term. Chief among these is the Quanterix digital enzyme-linked immunosorbent assay (D-ELISA) capable of detection of sub-femtomolar protein concentrations and hence many contents within a single cell [33]. More recently, CellSearch was cleared by the Food and Drug Administration for clinical isolation and enumeration of rare circulating tumor cells of epithelial origin in whole blood [34,35]. These single-cell assays and others show promise to be implemented in the clinic with potential to revolutionize disease detection, diagnosis, and monitoring.

Since single-cell analyses have enormous potential to improve personalized and precision diagnostics [36–40], there is growing commercial interest in the development and clinical trials of these methods. A recent industry report on the global single-cell analysis market (for all technologies and applications) valued it at 1.4 billion dollars in 2016 and forecasts a compound annual growth rate of 17.3% through 2025; further, hundreds of clinical trials are currently evaluating single-cell analyses for diagnosis and therapeutic decision-making for diseases such as cancer and HIV [41]. However, many of these assays focus on genome sequences, RNA expression, and protein presence while seemingly few investigate protein activity or signaling metabolites. Growing evidence suggests that direct assay of signaling

activity within cells may provide a more information-rich picture of cellular physiology and most closely predictive of cell behavior relative to assays that measure gene sequence or mRNA expression [42]. For example, the correlation between DNA copy number alterations (CNA) and mRNA abundance in tumors is weak and the correlation between CNA and protein presence is even poorer [43]. Further, correlation coefficients between mRNA and protein abundance are <0.7 - 0.8 for steady state measurements and significantly worse for dynamic measurements [44]. Several studies have demonstrated that only a fraction (between 40 and 84%, depending on the sampling method used) of protein variance can be explained by mRNA variance - with the remaining protein variance being due to translation rates, translation rate modulation, modulation of a protein's half-life, protein synthesis delay, and protein transport - suggesting that the abundance or presence of these molecules may not be the best predictor of cell behavior [44]. Never-the-less, due to wide availability, genomic sequencing and measuring RNA expression is increasingly common in clinical medicine with the rise of next-generation sequencing and other methodologies [45,46]. A multitude of single-cell protein assays are now under development; however, these assays remain difficult and not widely adopted clinically due to technical challenges and their significant costs [47]. Further, cellular protein concentrations alone may not be sufficient to predict disease states since proteins can actively transition between inactive and active states. Perhaps the most direct predictor of cell behavior then is protein activity and signaling drivers such as second messengers and phosphorylated proteins since these attributes act directly to alter cell actions.

1.2. Challenges and Strategies for Analyzing Direct Drivers of Signaling Behavior in Single Cells

Analyzing protein activity or signaling behavior within single cells, while extremely beneficial in understanding cellular heterogeneity, does not come without challenges. The volume of single mammalian cells is small: red blood cells are approximately 0.1 pL in volume [48,49] and typical mammalian cells approximately 1 pL [50]. Detection limits for single-cell analyses may need to be as little as 1 zeptomole (10^{-21} moles). For example, MAPK phosphatase regulates growth and development and is present at concentrations as low as 2 nM (2×10^{-21} mol) [51]. Some cellular analytes are relatively stable (DNA $\tau_{1/2} \sim$ hundreds of years [52]) mRNA $\tau_{1/2} \sim$ tens of hours [53], and proteins $\tau_{1/2} \sim$ hours to years [53]) and thus allow a more flexible measurement time. However, key drivers of cell physiology such as metabolites possess concentrations that can change on timescales of seconds or less ($k_{cat} \sim$ tens of thousands per second [54]), and as such accurately measuring these metabolites in single cells remains difficult [55,56]. Furthermore, an added obstacle is the rapid temporal change in signaling drivers such as protein activation in response to environmental cues. Two of the most common errors in the pre-analytical clinical steps create further obstacles for measurement of rapidly changing, environment-sensitive analytes: (1) improper sample storage and (2) delayed sample transport [4]. Notably, specimen transport accounts for approximately one third of TAT although transport can take tens of minutes at large hospitals [4,5]. All of these attributes require development of novel strategies to preserve cellular signaling molecules in their native state up to the time of analysis (Fig. 2).

A number of sample preparation techniques are employed to maintain cellular constituents in a stable form and improve analytical assay performance. These strategies often use fixatives, of which a wide range of methodologies are available, with the most common use being for maintenance of the cellular architecture in a near-native state for histological assays [57–64]. Specialized versions of these preservation techniques applied near the time of sample collection offer the potential to preserve clinical samples in a stable state during sample transport and preparation for non-histologic, specialized assays that measure protein function and/or catalytic activity at the single-cell level. These novel strategies may enable the introduction of a much broader and information-rich set of cellular assays into the clinical laboratory. This review will introduce these emerging cellular preservation techniques with particular focus on those that may have the potential for incorporation of single-cell assays into clinical workflows.

2. Traditional Cell Preservation Techniques

2.1. Considerations for Choosing a Cell Preservation Method

Sample preservation methods act to keep the cellular analyte or attribute of interest in a state as close to that *in vivo* as possible by maintaining cell and/or tissue architecture and/or halting cellular processes to protect cellular analytes, including terminating enzymatic reactions. Selected preservation strategies should have the following characteristics [59]:

- Acts on a timescale suitable to maintain the targeted analyte in its desired state.
- Does not cause degradation of intracellular analytes of interest.
- Maintains the analyte at a concentration near or at its *in vivo* concentration.
- Supports high efficiency analyte recovery if the analyte must be removed for assay.
- Avoids introducing assay interferents.
- Provides the necessary cellular/tissue spatial resolution (if any) for the assay.
- Prevents cross-contamination between individual cells for single-cell assays.
- Remains compatible with reagents to be used in subsequent assays.

2.2. Physical Fixation Techniques for Single Cells

Physical fixation techniques include dehydration, precipitation, cryopreservation, and freeze-drying, all of which inactivate cellular enzymes and preserve many cell constituents for later analysis. A common clinical application of a physical fixation technique is the drying and alcohol fixation of peripheral blood smears on a substrate prior to staining for evaluation of hematological disease [65]. Fine-needle and other aspirate smears are commonly preserved with alcohol-based fixatives prior to histologic analysis [66]. Fixation solely by alcohols (methanol or ethanol) or acetone yields dehydrated samples with precipitated and inactivated proteins but may also lead to collapse of cellular features and cell shrinkage [60]. To counteract this, alcohol-based fixatives often contain chloroform and acetic acid, which form hydrogen bonds with the tissue to prevent alteration of structural features [67]. Benefits of

alcohol fixation include maintenance of antigenicity for downstream immunohistochemistry and preservation of genetic material for downstream amplification and sequencing of DNA or cDNA prepared from RNA [60]. For this reason, single-cell sequencing for clinical applications will likely employ alcohol fixation [68].

Freezing is commonly employed for clinical samples when the cells need to be retrieved for later assay [69,70] such as recovery of genetic material for whole genome sequencing. DNA recovery from frozen tissue is typically excellent [71]. Though not yet common in the clinic, freezing samples prior to single-cell genetic sequencing is often reported in laboratory use, with transcriptome library preparation and sequencing exhibiting minimal to no differences when compared to fresh samples [72]. Frozen cells are sometimes subjected to freeze-drying with retention of some cellular biomarkers but deficits in others *e.g.* protein secondary structure loss and DNA damage after 1 week [73]. Cryopreservation with an additive (DMSO or trehalose) to prevent ice crystal formation, which can disrupt cell membranes, is commonly employed for clinical samples to be retained in a viable state such as human gametes, early stage embryos, amniotic fluid, umbilical cord blood, and bone marrow [69,70]. While freezing and cryopreservation offer the benefit of long term storage, a drawback to these techniques is the cost and maintenance of freezers at ultra-low temperatures (-80°C).

2.3. Chemical Crosslinking for Single-Cell Preservation

Chemical fixatives generally act by crosslinking chemical groups through either inter- or intra-molecular reactions to halt cellular functions, including inactivating enzymatic processes. Aldehyde crosslinkers such as formaldehyde, glutaraldehyde, and glyoxal crosslink free-amine groups of proteins and other amino-bearing molecules and have a long history of use (decades). These preservatives are currently the gold standard in clinical pathology for histopathological and immunohistochemistry of all tissues [61,62,74,75]. Aldehyde fixation of single cells has been demonstrated as a preservation method for multiple research-focused assays: mass spectrometry imaging of single cells after formalin fixation/freeze drying has been used to examine lipids, amino acid residues, and nucleic acid bases [76] and capillary electrophoresis (CE) has been used to analyze glycosphingolipid, phosphatidylinositol, or sphingosine metabolites from individual aldehyde-fixed cells [77–81]. However, these aldehyde fixatives are inherently aggressive and promiscuous for numerous functional groups and are thus unsuitable for many applications, including analysis of DNA since it is damaged by aldehyde fixatives [60,71,82].

Newer technologies have evolved over the past several decades that preserve RNA, DNA, small molecules, and proteins for high quality downstream analysis and as such are complementary to aldehyde fixatives [60]. These “molecular fixatives” protect macromolecules by utilizing mixtures of alcohols or acetone with stabilizing reagents to counteract shrinking during the dehydrating fixation process. Not yet widely utilized in the clinical setting, these fixatives are increasingly used in the research laboratory for proteome and genome preservation for downstream assay and may find utility for single-cell analyses in future clinical applications. Examples of analytical assays successfully paired with the molecular fixatives include extraction of intact, immunoreactive proteins for Western blot

and reversed-phase protein assay using a mixture of alcohols, acetic acid, and a proprietary soluble organic compound [83]. Other strategies employed a mixture of ethanol, acetic acid, and trehalose for preservation of morphology, proteins, and nucleic acids in tissue slices [84].

3. Novel Single Cell Preservation Techniques

3.1. Novel Chemical Chemoselective & Reversible Single-Cell Preservation Strategies

Recently developed preservation strategies focus on chemoselective reagents with a goal of providing increased flexibility in the timing between the analytical assay steps within the clinical laboratory environment. These “programmable” preservation strategies often employ on-demand initiation or reversal of crosslinks between cellular constituents and are readily compatible with single-cell assays (Fig. 3). Rapid halting of targeted cellular reactions assists in minimizing cell-to-cell variability resulting from the analyte collection process. Reagents to support stable entrapment or covalent crosslinking of analytes within cells with on-demand release also prolong potential sample storage times prior to assay and increase retrieval or extraction efficiency. In recent years, this nascent field has experienced an explosion of novel protocols for performing a wide array of single-cell bioanalytical assays including immunohistochemistry, proteomic measurements, and DNA/RNA-sequencing [68,85–89]. The following sections will serve to characterize the most significant recent developments of innovative single-cell preservation protocols.

3.2. Chemoselective Single-Cell Preservation Strategies

The search for methods to perform controlled chemical reactions to preserve analyte structure and concentration in cells has led to a panoply of bio-conjugation reagents with high specificity towards specific functional groups commonly found in intracellular proteins [91]. The goal of these reagents is to halt biological reactions via the formation of chemoselective protein-protein crosslinks, which result in protein inactivation and immobilization of protein within the cell structure for later assay. Common functional groups found *in cellulo* that are often targeted in selective reactions include primary amines; thiols (sulfhydryls); and aromatic moieties such as indole, imidazole, and phenol [91]. These classes of functional groups have historically been targeted due to their intrinsic reactivity derived from their acidity/basicity, electrophilicity/nucleophilicity, oxido-reductive characteristics, and the bio-chemical environments in which they occur [23,91–93].

Primary amines are the most prevalently targeted functional group when preserving biological tissues and single cells [94]. This is due to the high abundance of lysine (Lys, K) residues in proteins, which comprise 4–6% of all amino acid residues in the human proteome and bear an ϵ -amino group that is protonated at physiological pH [91,95]. Due to their high degree of hydrophilicity, Lys residues predominantly occur on the solvent-exposed outer surfaces of cellular proteins, making them accessible to a wide array of aqueous chemical transformations. Notable functionalities which selectively target primary amines include aldehydes, ortho-phthalaldehydes, NHS-esters, phosgene/thiophosgene, sulfonyl chlorides, fluorobenzenes, and imines (Fig. 4A) [91]. Reagents such as homobifunctional NHS-ester crosslinking reagents are more selective and less reactive than aldehydes; these reagents

have been used to successfully preserve single cells while maintaining cellular structure and surrounding membranes, facilitating RNA-seq on single cells stored at 4°C up to 3 days post-fixation [89,96].

The second most targeted functional group is the thiol moiety (sulfhydryl) on cysteine (Cys, C). Sulfhydryls are among the most reactive of all nucleophilic moieties in proteins and are significantly less abundant than primary amines. Cys only accounts for 2% of all residues in eukaryotes, and approximately 10% of Cys residues are located within a protein's hydrophobic interior as oxidized disulfide bonds [101]. Another source of sulfhydryls *in cellulo* is cytosolic glutathione (GSH), which is typically found at concentrations ranging between 0.1-15 mM in human cells [102]. Thus, thiol-reactive preservation reagents must be present at sufficient concentrations to quench the native glutathione and therefore crosslink intracellular proteins. Notable functionalities that selectively target sulfhydryls include α -halocarbonyls, maleimides, vinyl sulfones, alkene/alkyne coupling, thiosulfate/-sulfonate, pyridyl disulfides, and others (Fig. 4A). To date, preservation protocols targeting sulfhydryls have been used in single-cell DNA-seq and show promise for single-cell proteomics protocols [103,104].

Aromatic functional groups such as imidazole on histidine (His, H), indole on tryptophan (Trp, W), and phenol on tyrosine (Tyr, Y) comprise most remaining functional groups in cells that have been chemoselectively targeted. Due to the decreased prevalence of aromatic residues in proteins and the fewer number of available chemoselective transformations in aqueous media, aromatic residues have traditionally been limited to site selective modifications of proteins for structure elucidation, purification, and biolabeling [91,97]. However, this does not exclude them as targets for future preservation reagents that focus on minimizing the extent of crosslinking within cells or tethering desired analytes to proteins to mitigate analyte losses from preserved cells. Notable functionalities that target aromatic residues include epoxides, iridium complexes, and Michael addition for imidazole; malondialdehydes, metallocarbenoids, and aryl diazoniums for indole; and o-acylation, o-oxidative coupling, diazoniums, and dicarboxylates/dicarboxylamides for phenol (Fig. 4A) [91]. While the chemical reactivity of the aforementioned functionalities has been well established, their ability to terminate chemical reactions and preserve cellular analytes in whole cells has yet to be demonstrated; we expect novel aromatic targeting reagents will provide unique solutions to single-cell fixation and preservation applications in the future.

3.3. Reversible Single-Cell Preservation Strategies

Reversible preservation protocols have the potential to chemically cleave covalently bound analytes or open-up the cellular network to release targeted molecules on cue to preserve their integrity prior to assay and enhance analyte extraction efficiency [90]. To date, the reversible chemical preservation strategies employ features such as cleavable chemical bonds, encapsulating matrices, cellular nano-shells, and chemoselective and/or promiscuous protein crosslinks [90,96,105–108]. All of these strategies are compatible with single-cell assays and may enjoy clinical usage upon further refinement.

3.3.1. Reversible Chemical Crosslinks—Chemoselective modifications of proteins *in cellulo* have provided reagents with reversible chemical bonds for both biolabeling and cellular preservation. Chemical bond cleavage on demand has been achieved using chemical, physical, and/or light-mediated bond breakage (Fig. 4B). Paraformaldehyde (PFA) crosslinks have been successfully reversed at the tissue and single cell level facilitating MS-based measurements of proteins and metabolites. However, to successfully reverse the PFA crosslinks, the biological samples must be heated to 95 - 100 °C, beyond that which the samples are rendered unusable for other purposes. PFA fixation also results in Schiff-base artifacts on proteins within the sample, including multiple signals in mass spectra with a mass difference of 12 Da that serve to clutter the spectra and decrease signal intensity of the major ion; this must be accounted for using MS-based informatics [109]. While reversing PFA crosslinks remains a pioneering step in the field, additional methods of reversible fixation are required to eliminate the harsh reaction conditions, while increasing the efficiency of the crosslink reversal process and employing highly chemoselective reagents. To date, the most common reversible functionality to be integrated into cell preservation protocols is the disulfide bond. Disulfide bonds are attractive crosslinks for use in cell preservation since they undergo facile cleavage when exposed to reduced thiol reagents such as β -mercaptoethanol (β -ME) and dithiothreitol (DTT) at physiological pH and temperature. This has led to the proliferation of disulfide-containing crosslinking reagents that facilitate bond formation between a thiol-containing reagent and a cellular thiol for formation of disulfides. Examples of this latter group include dithio-bis(succinimidyl-propionate) (DSP) and dithio-bis(male-imidoethane) (DTME) [89,91,96]. DSP is perhaps the most common reagent in this space with applications in single-cell RNA-seq and proteomics workflows [89,90,96]. DSP has been shown to offer an 88% increase in protein extraction efficiency over PFA [90]. Additionally, gene expression profiles in DSP-fixed single-cells have been found to be highly correlated ($r^2 > 0.9$) with unfixed single cells [85]. These results likely stem from several desirable attributes of DSP as a cell preservation reagent including: its cell permeability, the chemoselective nature of its NHS-ester, the mild chemical conditions under which its disulfide crosslinks are reduced, and the relatively fast rate of reductive cleavage [89,90,96,102]. Another class of reversible crosslinks are vicinal diol-containing compounds such as disuccinimidyl tartrate (DST), which undergoes cleavage to form the corresponding aldehydes when exposed to a strong oxidant such as sodium periodate (NaIO_4) [90]. While reversible vicinal diol reagents outperform PFA in tissue-level preservation for MALDI-MS proteomics analysis, the strategy tends to suffer from a lower cleavage efficiency than comparable disulfide-based reagents such as DSP [90]. Reversible preservation reagents are expected to be of increased utility in clinical cell preservation workflows as these reagents are further refined and their utility broadened.

Azo-arene bridges are another class of reversible crosslinking agents that have seen increased usage since they are known to undergo cleavage upon incubation with strong reducing agents such as sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_2$) and/or hydrazine [97,98,100]. Although not yet employed in single-cell preservation workflows, azo-arene crosslinks show promise as dual functionality reagents that exhibit both chemoselective reactivity (e.g. tyrosine selectivity of aryl diazonium salts) and reductive reversibility under relatively mild pH and temperatures [97,98]. Drawbacks to the use of azo-arene crosslinks include the use

of harsh reducing agents, which are known to result in chemical modifications to nucleic acids and select amino acid residues at elevated temperatures [110]. Additionally, the required thermal de-crosslinking step may restrict this type of reversible crosslink to tissue-level analyte extractions since the sample must be submerged in a de-crosslinking solution.

A final promising group of reversible crosslinking reagents includes photoactivatable crosslinkers. While no chemoselective photoactivatable fixation reagents yet exist for single-cell workflows, there exists a rich and diverse chemistry of photolabile moieties that are likely to provide innovative solutions towards the challenges of modern cell-based bioanalytical assays including nitro-benzyl and coumarin analogues [99]. Some of the most promising aspects of photoactivatable crosslinking reagents is the ability to “program” the unmasking of reactive functional groups to initiate crosslink formation and/or de-crosslink the sample with a high degree of spatiotemporal control.

3.3.2. Reversible Encapsulation of Cells—Encapsulation protocols are commonly employed for two distinct applications: retrieval of viable cells or maintenance of intracellular constituent concentrations for later assay. In the first case, living cells are encapsulated within a microenvironment designed to optimize their viability for later culture or transplantation [111]. A selectively or semi-permeable, biocompatible matrix (often an extracellular matrix and/or natural/synthetic hydrogels) surrounds each cell and ideally facilitates exchange of oxygen, nutrients, and waste products to maintain the cell in a viable state with minimal perturbation of intracellular reactions [111]. Newer strategies use non-biologic materials for cell encapsulation and include metal nanoparticles, graphene, and silica shells [112–115]. While typically employed for the preservation of live cells, these shells can also serve to prevent analyte movement between fixed cells.

Recently, a tannic acid-Fe (III) [TA-Fe (III)] nanoshell was used to encase single viable cells while simultaneously displaying cytoprotective effects against UV-C radiation and polyaziridine toxicity *in vitro*. Impressively, the TA-Fe(III) nanoshell could be controllably dissolved, enabling the preserved cells to be expanded in culture or assayed [108]. Such tunable preservation protocols could find use in improving the long-term viability of ultra-small patient samples, enabling prolonged storage, and facilitating cell-based assays [108,112–115].

When viable cells are not desired, cells may be fixed to terminate chemical reactions and then encapsulated in impermeable barriers [111–118]. Since fixation compromises cell membrane integrity, the shell acts to contain the intracellular constituents within the cell body for later assay. A photoactivatable encapsulation matrix (thiol-PEGylated matrix with a photoactivatable nitrobenzyl-maleimide crosslinker) was recently employed by SAMSUNG Electronics Co. to facilitate targeted extraction of primary metabolites from fixed circulating tumor cells (CTCs) [103]. CTCs were released from the encapsulation matrix by UV irradiation followed by sequencing of their DNA. An advantage of these encapsulation matrices is the potential to eliminate analyte movement between cells by preserving the integrity of cell membranes and/or providing an impermeable barrier.

4. Conclusions and Remarks on Future Directions

While preservation strategies such as aldehyde-based chemical crosslinking have been employed in the clinical laboratory for decades, exciting new methods to preserve cellular analytes are under development and hold the potential for integration into clinical settings. Additionally, the utility and importance of analyzing single cells to understand disease pathogenesis and prognosis is increasingly recognized with a consequent growth in single-cell assay methodologies. Emerging technologies hold the potential to provide preservation strategies with applicability to the sample preparation workflow for single-cell assays and their entry into clinical laboratories. Chemoselective preservation methods offer the advantage of being highly targeted, minimizing non-specific reactions with the targeted cellular analytes. Additionally, these reagents provide superb temporal control of intracellular reactions as compared to traditional fixation strategies, and therefore may preserve cells in a more natural state by maintaining analyte structure and concentration within cells. Reversible preservation strategies, including reversible fixation and reversible encapsulation, show potential to permit long sample storage times between collection and assay and enhance analyte extraction efficiency. Both chemoselective and reversible preservation strategies also enable programmable initiation of the fixation process for controllable stoppage of intracellular reactions after sample collection. The development and implementation of novel cell preservation strategies will likely be fundamental for implementation of single-cell assays into the clinical workflow, thus offering considerable benefits in developing individualized diagnostic and prognostic assays for patients with consequent improved clinical outcomes.

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Abbreviations:

CE	capillary electrophoresis
CNA	copy number alterations
CTCs	circulating tumor cells
D-ELISA	digital enzyme-linked immunosorbent assay
DSP	dithio-bis(succinimidyl-propionate)
DST	disuccinimidyl tartrate
DTME	dithio-bis(male-imidoethane)
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
TA-Fe (III)	tannic acid-Fe (III)

TAT	turnaround time
β -ME	β -mercaptoethanol

Citations

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Highlights:

- Single-cell analyses will be an important addition to the suite of technologies used for personalized medicine.
- Living cells can individually alter their physiology in response to subtle changes in the microenvironment.
- Analytes within cells must reflect that of the living state for accurate single-cell assays.
- Current cell preservation strategies yield accurate readouts for many single-cell assays but are inadequate for others.
- Novel sample preparation strategies will transform precision medicine enabling improved understanding of the role of single cells in patient disease.

Figure 1.

Overview of the clinical sample analysis pipeline and types of measured analytes. **(A)** Schematic of clinical sample workflow and examples of each step within the workflow. From the left to the right: sample collection (pre-analysis), sample transportation (pre-analysis), pre-analysis processing (pre-analysis), single-cell assay (analysis), and data processing (post-analysis). **(B)** Analyte categories and representative techniques used to measure these analytes within single cells in lab medicine workflows. Techniques in *italics* denote tools that show promise for use in future clinical single-cell workflows.

Figure 2:

Illustration of various preservation techniques. In all panels the red, blue, and green curved lines represent three different possible cellular analytes. The left and center columns are a schematic depiction of the fixation outcome or impact on a single cell (left column) and a magnified version focused on the membrane region (center column). The right column demonstrates an example reaction in each fixation category, and more specific examples and descriptions can be found throughout the text of this manuscript. While this figure depicts a representative preservation technique for each strategy, readers are encouraged to explore each of the primary research articles cited in this review for more specific details. **Chemical crosslinking** results in cell membrane damage and the widespread, nonspecific crosslinking of cellular constituents with amino groups by aldehyde-based reagents such as formaldehyde. **Cell encapsulation** techniques consist of a shell (grey region) which surrounds the cell, does not interact with the cytoplasm, and reduces the loss of cellular molecules. An example is the nanoshell formed the reaction of tannic acid and iron (III). **Chemo-selective crosslinking** incorporates specific crosslinking of targeted reactive groups

between intracellular molecules preventing loss of analytes. Reactants such as ortho-phthaldehydes crosslink primary amine groups with minimal off target reactions. **Reversible crosslinking** may utilize a crosslinked cytoplasm with bonds that can be cleaved on demand to release analytes. Disulfide bonds are a commonly used reversible crosslinking strategy. See Fig. 3 for additional details on reversible crosslinking. **Physical preservation** can often lead to significant loss of cytoplasmic molecules. Lyophilization is an oftenemployed modality for cell dehydration.

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Figure 3.

Representative workflow of reversible cell preservation strategies and comparison of analyte extraction efficiencies between novel strategies and conventional methods. In all panels the red, blue, and green curved lines represent three different possible cellular analytes. **(A)** Reversible cell preservation workflow. From left to right: Cell constituents are exposed to reactants such as NHS-esters that support reversible cell preservation. In their preserved states cells can be transported from the collection to the analytical site. The chemical bonds that support cell preservation can be reversed just prior to analysis through means such as a

photocleavage step. The desired analytes can be liberated from the cell and assayed followed by data analysis. **(B)** Comparison of analyte extraction efficiencies between traditional and reversible cell preservation strategies. All extraction efficiency data is from representative primary sources [89,90], and readers are encouraged to explore each primary article for specific details regarding preservation and extraction protocols. Abbreviations: DSP, dithio-bis(succinimidyl-propionate); MeOH, methanol; EtOH, ethanol; DST, disuccinimidyl tartrate; PFA, paraformaldehyde.

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Figure 4.

A selection of promising chemoselective and reversible reactions for use in the development of novel single-cell preservation reagents [90,91,97–100]. **(A)** Amine-selective conjugation reactions: i) o-phthalaldehyde, ii) NHS-ester, iii) fluoronitrobenzene; Thiol-selective conjugation reactions: iv) maleimide, v) pyridyl coupling; Aromatic and miscellaneous selective reactions, Histidine: vi) epoxides; Tyrosine: vii) aryl-diazoniums; Arginine: viii) glyoxal coupling; **(B)** Notable reactions for use in reversible crosslinking reagents i) disulfide reduction, ii) azo-arene bridge reduction, iii) vicinal diol oxidation, iv) nitro-benzyl photocleavage.