Review of Methods to Probe Single Cell Metabolism and Bioenergetics

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Abstract

Single cell investigations have enabled unexpected discoveries, such as the existence of biological noise and phenotypic switching in infection, metabolism and treatment. Herein, we review methods that enable such single cell investigations specific to metabolism and bioenergetics. Firstly, we discuss how to isolate and immobilize individuals from a cell suspension, including both permanent and reversible approaches. We also highlight specific advances in microbiology for its implications in metabolic engineering. Methods for probing single cell physiology and metabolism are subsequently reviewed. The primary focus therein is on dynamic and high-content profiling strategies based on label-free and fluorescence microspectroscopy and microscopy. Non-dynamic approaches, such as mass spectrometry and nuclear magnetic resonance, are also briefly discussed.

1. Introduction

Understanding metabolism and energy flow through cells has recently generated considerable interest. This is due to implications in metabolic engineering and the use of microbes as factories for the production of chemical compounds. Exploratory or optimization investigations to this end commonly take place within the widely available flasks and petri dishes, as well as dedicated bioreactors (**Fig. 1a-b**). In these, many cells ($> 10^{11}$) are simultaneously stimulated and their output is collectively analyzed.

In population level studies, however, the performance of individuals is masked and stimuli gradients across the population are enforced due to the geometric boundary conditions of the growth microenvironment. Single cell methods need to be implemented to address these, as recently evidenced by the wide success of single cell genomic and proteomic approaches. Herein, we review methods for studying single cell physiology – specifically metabolism and bioenergetics – where cells are now treated as isolated biochemical factories, thus enabling the precise measurement of (a) their metabolic profile and (b) culture heterogeneity, as well as (c) the effect of the microenvironment.

Despite their potential in gaining a unique insight in cell metabolism, it is important to note that care has to be taken when designing single cell experiments and interpreting results. In addition to the need for statistical significance, the differences between experimental conditions at the population-level and the micro-volume that contains an individual cell need to be considered. Such differences include population-level characteristics that are challenged by isolating an individual, such as cell-to-cell communication (e.g. via quorum sensing), spatiotemporal gradients of stimuli and the co-existence of cells in various growth stages.

The review is divided in two parts. In the first, we discuss methods for biomass manipulation down to the single cell level. This is a critical step, as the volume of a typical microbial cell (bacteria or yeast) is approximately 12 orders of magnitudes smaller than common reactor volumes ($\sim$ L). *This is highlighted in Fig. 1a-c in the scale comparison between typical industrial and laboratory scale reactors with that of an individual Yarrowia Lipolytica yeast cell.* Thus, the study of single cells necessitates appropriate sampling and manipulation to address this considerable volume mismatch. This can be achieved via multiple strategies, both stochastic (e.g. serial dilutions) and deterministic (e.g. micromanipulators).

In the second part of the review, we discuss methods for probing the metabolism and bioenergetics of single cells. Areas such as single cell mass-spectrometry, bioimaging, optical sensing and spectroscopy are covered. The applications of such methods, such as strain selection, profiling intracellular metabolites, and dynamic metabolic mapping (e.g. respiration monitoring), will also be discussed for each individual technique.

2. Single Cell Manipulation

The sampling and manipulation of cells down to the individual has been of substantial interest since the very beginning of Life Sciences. The first recorded single cell study involved the use of a micropipette to place a single *Erwinia amylovora* cell on an apple blossom and study its infectivity [1]. This technique - with minor modifications - served with great success the early physiology, [2] genetics [3] and microbiology [4] studies. Since then however, a significantly wider variety of methods has emerged. These are primarily based on modern micro- and nanotechnologies involving advanced fabrication, materials and chemical functionalization methods.

Herein, our objective is to highlight the most recent developments in single cell manipulation, as well as pioneering ones; it is also worth noting that excellent reviews exist on similar topics [5-15]. First, *flow-through* methods will be discussed, namely methods in which single cells flow continuously in an ordered manner during their analysis. This section will be followed by *confinement* techniques that enable cell isolation and confinement in zero-, one, or two-dimensions (**Fig. 1d**) [12]. Such confinement techniques can be further divided into permanent and reversible or dynamic ones and can be based on both chemical and physical approaches. The section will conclude with a comparison between these approaches.

A critical aspect of both *flow-through* and *confinement* strategies is the enabling fabrication method, which in the majority of examples is based on Soft Lithography. The latter will not be discussed in detail in the present review and the potentially interested reader is referred to recent excellent recent reviews on the topic [16-18].

2.1 Flow-Through Methods

Flow cytometry and droplet microfluidics are the two most common methods of flow-through analysis. While both are high-throughput techniques [19], their difference is that the droplet encapsulated cells experience a restricted and personal microenvironment, while in flow cytometry cells experience similar nutrient and stimuli initial concentrations. These methods are rather well established; however, they recently enabled immense strides in single cell phenotypic analysis, namely the identification and analysis of metabolically distinct individuals from an isogenic population using both droplet microfluidics [20] and flow cytometry [21].

2.1.1 Flow Cytometry

Similar to its first ever designs [22, 23], most modern flow cytometers contain a nozzle and a flow chamber. These, through the principle of hydrodynamic focusing, guide individual cells to flow sequentially through a microanalysis location, mostly optical (i.e. fluorescence or light scattering) or mass spectrometry based. In this way, high content screening at the single cell level can be achieved at rates as high as 10^4 cells/s (Fig. 2a) [24, 25]. In minor modifications, the cells can also be sorted depending on their fluorescent (or other) properties, thus forming the so-called Fluorescence Activated Cell Sorters (FACS).

Flow cytometers are widespread and accessible instruments, with applications ranging from the most early studies on biological noise (stochasticity) [26-28], to more recent investigations in metabolic engineering [29]. Additionally, research and development on cytometers is still an active research field, with recent advances in instrument miniaturization and the introduction of novel types of cell interrogation. Microfabricated cytometers (*microcytometers*) emerged with the advent of microfluidics [30], aiming primarily at reducing capital investment requirements [31]. Another advantage of scaling down cytometers is the ability to integrate more complex functionality, such as more efficient cell transport and sorting [32], as well as more efficient single cell analysis [33].

Regarding the available types of interrogation, most conventional cytometers analyze and sort cells based on their fluorescent and light-scattering properties. More recently, especially in microcytometers, the use of dielectric [34] or Raman [35] spectroscopies, as well as the measurement of cell capacitance [36], impedance [37] and mechanical properties [38] have emerged. Highly-multiplexed single cell analysis using flow cytometers recently became

available through the ‘mass cytometry’ based on inductively coupled plasma (ICP) mass spectrometry [39].

2.1.2 Droplet Microfluidics

Droplet microfluidics (including *digital microfluidics*) is essentially a two-phase fluidic microsystem formed *via* the interaction of two immiscible fluids, specifically by shearing one into the other [40-42]. Droplets in microfluidics offer several distinct advantages, forming thus an ideal assay platform for screening or other purposes [20]. These advantages include the generation of highly mono-disperse [43] and size-variable microreactors [44], which can additionally be transported in a dilution free and zero cross-contamination manner [45]. More recently, techniques for accurate mixing of pL volumes using droplets have been reported [46-48].

A wide variety of methods to encapsulate single-cells into individual droplets also exist ([Fig. 2a, b](#)) [49-52], including droplets made of hydrogels such as agarose [53]. Thus, the aforementioned compartmentalization-driven advantages of droplets can translate into unique features in investigating single cell physiology [54, 55], and genomic content [56-58]. The first is that cell-secreted molecules remain confined within the volume of the droplet, and thus can rapidly reach detectable concentrations [20, 59, 60]. The second advantage is the ability to rapidly mix minute (pL) volumes of reagents, hence enabling reduced reagent consumption [61], as well as the high-precision timing of reaction kinetics. The latter was recently demonstrated by identifying mutants producing enzymes with improved productivity and activity [62].

One disadvantage of droplet based single cell analysis compared to conventional flow cytometry is the stochastic nature of encapsulating cells in droplets, which may reduce cell throughput [63]. On the other hand, single cell confinement in microdroplets allows for identification of cells with

superior properties of secreting a product metabolite and identifying genetic elements that are responsible for an elevated production phenotype of secreted products [20]. No other methods are available to identify such super-producing cells.

2.2 Permanent Confinement

Permanent confinement methods enable the irreversible and non-dynamic positioning of an individual cell or of a population of single cells. This, generally, can be achieved *via* chemical or physical potentials (or barriers) that forbid cell motion in all, or in specific directions. Depending on the type of confinement, the cell position may fluctuate within controlled *multicellular* or *subcellular* distances (i.e. a few μm 's down to a few nm) in one-, two- or even three dimensions (Fig. 1). Such methods will be reviewed in this section and classified depending on their confinement size and dimensionality.

2.2.1 3D Microcavities

In this embodiment, a micron-scale topographic cavity – or an array of cavities – is engraved on a surface, thus confining isolated cells in that microenvironment, however, without generally restricting their motion. *Microwells* and *microchemostats* belong to this category, offering confinement levels of generally cellular resolution.

Microwells: A type of microcavity is a lithographically defined microwell (Fig. 3a) [64]. In these, a cell suspension is introduced over the microwell array, and cell loading in the microwells commonly occurs by sedimentation (gravity) [65]. Single cell immobilization is ensured by employing appropriate seeding cell concentration, seeding time and microwell dimensions [66, 67] (Fig. 3b). It is worth noting that this type of cell confinement is very common, as it is

compatible with a wide range of microfabrication and functionalization techniques, as well as types of materials, including SiO₂ [68], poly(ethylene glycol) [69], PDMS [70], (including functionalized PDMS [71]), functionalized hydrogels [72], the photoresist SU8 [73], and solid agarose [74]. Up to 3600 cell traps have been reported using microwells [75].

Microchemostats: Microfabricated chemostats enable continuous and dynamic stimuli delivery and product removal from the microenvironment surrounding the confined cells [76, 77]. In one of the first of such demonstrations, *Khademhosseini et al.* reported an array of microwells impregnated at the bottom surface of a microfluidic channel. The array was able to trap populations of single mammalian cells simply by controlling the flow conditions [78] (**Fig. 3c**). This architecture was recently evolved into microwell arrays separated by the microfluidic channels *via* hydrogel layers [79]. To enable handling of smaller and more motile cells, *Groisman et al.* developed an array of microchambers ($\sim 100 \times 100 \mu\text{m}^2$) connected to apertures whose diameter was flow dependent [80]. This enabled bacteria loading and at high flow-rates (large aperture), and subsequently trapping them at lower flow rates (smaller aperture). Similarly, ‘*Lobster Traps*’ were demonstrated in a crosslinkable bovine serum albumin (BSA) matrix; in these, - following cell seeding - the loading apertures permanently seal by raising the temperature and forcing the BSA matrix to expand [81].

2.2.2 Confinement in 2D

Single cell populations are most commonly studied in microsystems that enforce 2D confinement, namely allowing cell motion within a 2D plane. This is typically achieved by volume exclusion by geometrically minimizing the available space in the third dimension, thus ensuring the formation of a 2D cell monolayer. In one of the earliest reports of this type,

Cookson et al. reported the Tesla microchemostat ($T\mu C$) by integrating large seeding microchannels with a 4 μm thick trapping region (**Fig 4a**) [82]. This size mismatch enabled the stable trapping of many single cells of *Saccharomyces cerevisiae* within a single focal plane for more than 24h (**Fig 4b**). This basic architecture has found many applications in long-term bioimaging of both eukaryotic cells [83, 84], and bacteria [85, 86]. *More recently, Dénervaud et al. reported a massively parallel array of microchemostats capable of confining 1,152 individual cells [87]. In this embodiment, a DNA spotter was used to array single cells on a surface and subsequently align them with individual chambers interconnected to media delivery microchannels.*

Despite their simplicity, most 2D confinement architectures may suffer from significant build-up of chemical gradients of nutrients and waste throughout the cell monolayer which may lead to significant phenotypic heterogeneity within the colony [88]. More recent 2D confinement architectures have partially addressed this. In one approach, some level of environmental control was possible by including multiple inlet and outlet microchannels for the supply of nutrients and waste removal [89] (**Fig 4c**). It is worth noting that the effect of nutrient gradients on bacterial metabolism and chemotaxis has also been investigated in pore-scale microfluidic models, which elegantly simulate subsurface and bioremediation conditions [90-92].

2.2.3 Confinement in 1D

In this embodiment, the cells are contained within a linear track, thereby placing strict boundary conditions for motion and proliferation in directions perpendicular to the track itself [93] (**Fig 1d**). This enables more straightforward tracking of both individual cells and their lineages in comparison to 2D or 3D growth, as illustrated in **Fig 4d**. In the earliest report of this type, Balaban et al. grew *E. coli* cells within grooves of dimensions similar to the width of the bacteria

[94]. The grooves were molded in PDMS and bonded to a membrane through which nutrients and stimuli were delivered to the cells.

Subsequent attempts aimed at facilitating cell loading and nutrient supply. Regarding the former, in one approach, the *Weitz group* integrated the tracks with indentations of sub-cellular dimensions [95]. This prevented the cells from flowing through, thus enforcing their trapping within the track. Upon loading, the hydrodynamic resistance of the apertures increased ensuring single cell occupancy. In a more multiplexed assay – the ‘*mother machine*’ – cell seeding took place by diffusion from much larger microchannels, thus infilling approximately 4000 growth tracks [96]. Regarding nutrient supply, this has been optimized from the initial designs by employing agarose-made linear tracks instead of the more conventional PDMS [97]. The inclusion of media carrying trenches next to the tracks [98], as well as the use of channels of an orthogonal – asymmetric – geometry have also been reported [99]. Advanced microfabrication techniques based on electron beam lithography allowing the fabrication of much narrower tracks have been recently reported, primarily for the 1D confinement of much smaller bacteria [100].

2.2.4 Confinement in 0D

Under 0-D confinement, the cells are strictly immobilized at a fixed location within only minor sub-cellular position fluctuations. Generally there are two ways of achieving this: the more traditional approach involving gels and surface functionalization strategies, as well as more recent ones based on micro- or nanofabricated filter beds, through which the cell suspension is flown. We will employ early microfluidic nomenclature of the latter strategy, namely *percolation filtration*, [101, 102] in which the filter exhibits dimensions smaller than the cell in order to trap the targets under continuous flow-driven, shear pressure.

Axial percolation filtration (APF): The cell suspension flows perpendicular to the filter bed in this embodiment. In one of the earliest reports of this type, *Wheeler et al.* integrated a microfluidic ‘T-junction’ with a micron-scale trap: a stagnation point develops at the center of the T-junction (**Fig. 5a**), which locally stabilizes the flow stream and cells gently land and stay in the dock [103]. In a subsequent, pioneering report, *Di Carlo et al.* developed an array of hydrodynamic trapping structures with a U-shaped geometry (**Fig. 5b**) [104]. The U-shaped trapping site contains a narrow aperture smaller than the cell size, preventing it from flowing through and enforcing to reside within trap. *This basic design later evolved to enable the integration of the trap with a pneumatically isolated microchamber [105], as well as trapping of individual bacteria [106, 107], including architectures capable of removing the daughter cells [108]. Similarly,* instead of the U-shaped geometry, monolithic PDMS pads have been recently reported (**Fig. 5c**). These also enable selective trapping of mother and release of daughter cells, respectively [109].

Lateral percolation filtration (LPF): In this approach, in essence an evolution from APF, the cell suspension flows parallel to the filter plane. LPF is generally based on the ‘basin-style’ design, where the trapping microstructures are aligned perpendicular to the flow direction (**Fig. 5d**). Sophisticated hydrodynamics enable a pressure gradient to build up between loading and trapping sites, forcing the cells to move into the traps. This pressure gradient is reversed once a cell is trapped, thus preventing secondary cells from entering the same trapping site. This was initially demonstrated by *Tan et al.* for microparticles [110], and later evolved into a high-throughput microarray of more than 2000 traps [111, 112], as well microsystems for co-culturing heterotypic cells [113]. It is worth noting that both the APF and LPF confinement can be applied to immobilizing cells encapsulated within droplets, instead of cells by themselves [114, 115].

Hydrogel encapsulation: The encapsulation of living cells in hydrogels is a very traditional approach, with a wide range of available materials and gelation stimuli (agarose, gelatin, poly(ethylene glycol), alginate etc.) [3]. In one embodiment, the cells are deposited on a slide and the gel is placed on top and subsequently solidified [116]. Image analysis enables cell tracking over multiple generations, including the dynamic mapping of their gene regulatory networks and phenotype by imaging [117, 118]. Alternatively, prior to gelation, the cell suspension may be mixed with a hydrogel solution—a very common immobilization technique for maintaining a 3D culture architecture in immune and regenerative medicine [119, 120].

While practical, hydrogel encapsulation suffers from two shortcomings, namely the uncontrolled cell growth and resulting challenge of single cell tracking ([Fig. 4d](#)), as well as limited nutrient and stimuli exchange over time. The latter has been recently addressed *via* the microfluidic integration of hydrogel encapsulated cells; in this way, small molecules present in the microfluidic solution surround and subsequently diffuse into the gel, thereby enabling a dynamic microenvironment chemical control. In such approaches, hydrogel gelation by UV light for integration inside microfluidic networks [121-123], as well as solidified gels integrated vertically with microchannels, have been reported [124]. Gelation in these methods may involve exposure to high temperatures or UV light [125], or the potentially safer ion-based crosslinking [125].

Surface – cell interactions: Cell-surface interactions still receive substantial attention since they are involved in areas such as biofilm formation, antifouling of implants and tumor architectures [126-128]. Such interactions may arise from physical (e.g. Van der Waals), or biochemical forces (e.g. protein, lipopolysaccharides and DNA mediated), and their combinations. Despite ongoing investigations, surface tethering of single cells has been employed for many years and such key examples in single cell physiology technologies will be highlighted.

In an early example, polylysine coated nitrocellulose membranes were employed for immobilizing *S. cerevisiae* and *Escherichia coli* for up to 4 hours, while continuously releasing their newborn cells [129, 130]. The technique, termed ‘*baby machine*’, is the chemical equivalent of the recently reported ‘*dissection platform*’ [131]. Polylysine is water soluble and thus compatible with PDMS, which has enabled many microfluidic investigations on single bacteria [132, 133]. Cholesterol containing co-polymers, and APTES (aminopropyltriethoxysilane), are also water soluble and have been shown to be straightforward methods for tethering yeast in microfluidics [134, 135].

Chemical patterns on surfaces will in turn enable patterned immobilized cells even down to the single cell level [136, 137]. Many techniques exist for generating such surface chemical patterns, such as microcontact printing of self-assembled monolayers (SAMs) [138], which can also be reversed electrochemically [139]. Photolithographically defined SAM patterns using UV [140] or visible radiation [141], as well as electrochemical [142] and photocatalytic methods, have been reported more recently [143]. Again, the microfluidic integration of chemical patterns is an important aspect. To this end, chemical patterning *in-situ* has been recently demonstrated [144], including the integration of metabolic monitoring capabilities using electrochemical pH sensors [145].

While in most cases, functionalized surfaces have been employed, bare – non functionalized – coverslips have also been shown to provide a strong enough tether both for yeast [146] and bacteria [147]. Cell-specific tethering is also possible, for example, via antibody, protein, or peptide specific adhesion, as recently reviewed elsewhere [148].

Cell-surface interactions are the simplest method to immobilize single cells with high viability. However, care has to be taken in selecting the appropriate strategy as phenotypic changes may

occur upon surface contact, such as the increased EPS matrix in *E. coli* [149]; for a more detailed discussion on this topic, the reader is referred to the following – bacteria focuses – review article [127].

2.3 Dynamic Confinement

In dynamic confinement, single cells or populations of single cells are immobilized and released upon demand. Applications of this type usually involve the post-trapping analysis of the target, such as lysis mediated proteomics and transcriptomics. External stimuli (optical, electrical, acoustic etc.) and recently passive microstructures that generate a reversible trapping potential are commonly employed to this end. These approaches will be reviewed in this section and classified depending on the potential type they employ.

2.3.1 Mechanical Micromanipulation

Different types of mechanical micromanipulation exist, such as hydrodynamic, acoustic, or the use of pneumatic valves in microfluidics. Each type is reviewed separately below.

Pneumatically Isolated Microcavities: This is a special class of microcavities formed by two valves that pneumatically seal a portion of a microfluidic channel (**Fig. 6a**). The elastomeric nature of PDMS and multi-layer soft lithography are critical in such embodiments [150]. The cells are generally in-between two valves and can be released by opening the valves [151]. Due to their simplicity, and lab-on-a-chip compatibility, this type of dynamic microcavities has made a tremendous impact in elucidating biological noise [152] and in single cell sequencing and transcriptomics [153-155].

Hydrodynamic: Hydrodynamic manipulation is generally achieved in *contact* and *non-contact* methods. In the former, we recently demonstrated that trapped cells in percolation filters can overcome the trap barrier simply by increasing the flow rate [106]. In this way, the drag force increases, eventually leading to cell escape (**Fig. 6b**). Regarding *non-contact* methods, forms of this type include *microvortices* [156] and *stagnation point microflows* [157]. In *microvortices*, microeddies are generated in the proximity of solid boundaries under fluid oscillation conditions (**Fig. 6c**) [158], with reversible trapping occurring at the eddie center [159]. *Stagnation point* trapping occurs at the junction of two streams (**Fig. 6d**) [160]. By combining active flow control and microscopy, a condition of zero force exerted on a cell can be reached, leading to stable trapping at the junction [161]. Non-contact methods are generally simpler; however, they do not enable strict immobilization with the cell position fluctuating with time.

Acoustic: Acoustic manipulation is based on ultrasonic standing waves and was discovered in the early seventies [162]. In these, ultrasonic transducers, which generate acoustic waves and hence trapping forces, are integrated with microfluidics [163]. Acoustic trapping has shown to preserve cell viability, despite the significant temperature increase ($\sim 15^{\circ}\text{C}$) [164, 165]. More recently, the acoustic trapping power requirements were substantially reduced by employing piezoelectric substrate and surface acoustic waves (SSAW) [166].

2.3.2 Optical Micromanipulation

Optical Tweezers: Optical tweezers (OT) is arguably the most common form of single cell dynamic manipulation [167, 168]. In OT's, a laser beam is focused in the cell medium, generating a strong electromagnetic field gradient that pulls individual cells towards the beam center (**Fig. 7a**) [11], where they remain for prolonged durations [169]. By passing the optical beam through a spatial light modulator [170], or a microlens array [171], multiple focused spots

are generated, hence giving rise to multiple trapped cells—recently as up to as many as 200 have been reported. The optical focused spot can be also moved, enabling thus the deterministic transport of the trapped cells. This has been shown to conveniently enable the isolation and transportation of single cells within microfluidic [172] or hydrogel growth chambers [173]. A unique advantage of OTs is that they immobilize single cells away from surfaces, and are thus very popular with Raman microspectroscopy [174].

Scattering Forces (Radiation Pressure): Focused optical beams may also induce weak repulsive forces along the light propagation axis, [175] a concept that has also found multiple single cell applications [176]. Single cell immobilization is possible at the center of two lightly focused, counter propagating laser beams, an approach frequently referred to as the ‘dual beam trap’ (Fig. 7b) [177]. Alternatively, scattering forces may be employed to force a trapped cell out of position to its release; this approach was demonstrated by *Kovac et al.* for forcing cells outside of their trapping microwells (Fig. 7c) [178]. Recently, single cell trapping and transport was demonstrated using integrated optical waveguides and resonators. This approach reduces the experimental complexity as well as enabling deterministic light propagation and cell transport along a lithographically defined optical waveguide [179, 180].

Optoelectronic Tweezers: In optoelectronic tweezers (OET), the cell suspension is introduced in between two electrodes, one of which is additionally coated with a photoconductive layer. Upon illumination, a non-uniform electric field is generated between the two electrodes, which in turn induces a dipole and a ‘dielectrophoretic’ force on the cells that traps them on the photoconductive surface [181]. Due to their low light intensity requirements and ability to employ spatial light modulators to generate multiple excitation locations, OETs can generate massively high-throughput trap arrays, with up to 15,000 trapping sites demonstrated [182].

Recent progress in OET has enabled their microfluidic integration [183], as well as their further simplification and miniaturization by employing alternative illumination strategies such as micro-LED arrays [184].

Light Stimulated Hydrogels: In [section 2.2.4](#), hydrogels were discussed in the context of 0D confinement. However, hydrogels can be also optically degraded and thus enable dynamic control of their cell confining potential [185]. Alternatively, hydrogels can be also optically polymerized, as recently demonstrated in combination with microwell trap arrays: the trapped cells were covered with a photosensitive hydrogel, which was selectively illuminated and solidified apart from locations where target cells resided; these could then be recovered by simple washing ([Fig. 7c](#)) [186].

Photothermal: Photothermal single cell manipulation is less common, but has been employed to release trapped cells in microfluidic trap arrays, as reported in the seminal work by *Tan et al.* [110]. This involved the integration of an aluminum pad in the cell trap location. Upon illumination with a laser beam, the local temperature around the pad was raised, thus inducing a bubble that forces the cells to overcome the trap potential and be released.

2.3.3 Dielectrophoretic Micromanipulation

Similar to the *Optoelectronic Tweezers* case, in dielectrophoretic (DEP) manipulation, an electric field is generated in the vicinity of microfabricated electrodes; this then induces a dipole in the cells in close proximity [187]. This forces the cells to experience a Coulomb interaction and a resulting trapping force. DEP applications involve cell screening, sorting or transport [188], as well as immobilization, by employing specific quadrupole, octopole or ring electrode geometries ([Fig. 7d](#)) [189-191]. In electrode arrays, the cell may also be transported from electrode to

electrode under the application of specific voltage patterns [192]. Recently, DEP forces were employed to release immobilized cells from a microfluidic trap array [193].

Due to the need of electrode integration, DEP trapping microsystems usually require complex fabrication. Buffer heating effects may also occur due to power dissipation at the electrodes, which was recently measured to reach up to 20°C [194]. Despite such shortcomings, reversible DEP immobilization has found applications in single cell metabolic studies, such as bacterial proliferation in controlled microenvironments [195].

2.3.4 Magnetic Micromanipulation

In magnetic micromanipulation, magnetically labelled cells are placed in the proximity of magnets [196]. The need for magnetically labelled cells was recently successfully eliminated by using paramagnetic buffers [197]. *Gach et al.* demonstrated cell release in magnetic polymer microwells [198], where under the action of a magnetic field, selective removal of an individual microwell was possible (**Fig. 7e**). To eliminate cell, buffer, or microwell labelling, magnetic nanowires or microparticles were recently shown to trap single cells; the microparticles rotate under the action of a rotating magnetic field, thus generating microvortices and trapping single cells within [199, 200].

2.3 Summary

As discussed in this section, a wide variety of techniques exist, each with its own resolving abilities and target information (e.g. high-throughput versus dynamic analysis, or high/low content screening, mammalian/microbial cells), as well as access requirements to microfabrication or bioanalytic facilities. **Table 1** summarizes these approaches, highlighting for each technique the capacity, ability to control the microenvironment by supplying nutrients and

removing byproducts, as well as advantages (+) and shortcomings (-) in recovering cells and expertise requirements.

3. Probing Single Cells

Multiple methods to probe single cell physiology exist, operating either in steady state or dynamic fashion. Each method has its distinct resolving capabilities and targets specific cell characteristics, such as its size, mass, and volume, as well as specific organelles or metabolites. Several excellent reviews have been published on the topic [10, 201-203], including recent ones on biomechanics, microanalytical separations and genomics [38, 201, 204], — topics that will not be reviewed herein.

On the contrary, our focus in this section is on methods that directly or indirectly probe the metabolic-state, or energy flow of single cells. Depending on their nature, the techniques are divided into non-destructive ones that permit dynamic analysis, namely *label-free* and *fluorescence mediated microanalysis*. We will also briefly discuss non-dynamic techniques, namely *mass spectrometry* and *nuclear magnetic resonance*. Key applications in single cell physiology and metabolic analysis will be discussed separately for each technique.

3.1 Label Free Microanalysis

Single cell microanalysis techniques will be reviewed in this section, namely techniques that can probe single cell physiology without the need of cell staining. Cell proliferation, mass, volume and chemometric content are the typical pieces of information that can be extracted from single cells with such strategies, and will be reviewed separately.

3.1.1 Proliferation

In proliferation studies, cell growth in terms of number of individuals and their size is investigated, both of which with direct links to cell cycle and metabolic state. The most common instrument used to this end is conventional microscopy; however, more advanced, label-free, holographic approaches are becoming increasingly popular [205]. With the advent of advanced microfluidic architectures, proliferation studies have been revolutionized, and it is now possible to track parameters of multiple individual cells for many generations and under controlled chemostatic conditions [70, 80]. Such recent developments involve the long-term and dynamic analysis of cell size and size homeostasis, as well as the relationship of these characteristics between mother and offspring cells (**Fig. 8a**) [206]. Single cell lifespan analysis was recently reported, linking cell age to specific phenotypic variations [131]; in this study, a microfluidic dissection device enabled single cell tracking with no cell-crowding due to the continuous removal of the newborn cells by flow (**Fig. 5c**). 1D confinement structures have also been extensively employed to track lineages of single cells [95], as well as their response to chemical perturbations, such as antibiotics [94]. It is also worth adding that portable cell-phone compatible microscopy platforms have been recently demonstrated, enabling important single-cell physiology and motility investigations [207, 208].

3.1.2 Single Cell Volume and Mass

The mass and volume are two physical characteristics also directly linked to cell cycle and metabolic state. Their importance has been recently recognized and several strategies for their measurement have been developed to this end.

Volume: Volume measurements can be *imaging* or *impedance* (resistance) based. An advanced *imaging* approach is the Reflection Interference Contrast (RIC) method, where angled white-

light illumination gives rise to interference fringes; the number of which is directly proportional to the height of the cell [209]. In an alternative *imaging* approach, *Bottier et al.* employed ‘negative staining’, namely the addition of a fluorescent, non-permeable dye in the buffer surrounding the cells, followed by substantially simpler image analysis [210]. *Impedance* based methods are also very popular, such as the commercial *Coulter* technique, where cells decrease the electrical conductivity of an aperture as they transit in front of it. The conductivity decrease has an amplitude directly dependent on the cell volume [211].

The *Coulter* instrument was recently integrated with a temperature stabilized chemostat to enable ‘continuous’ population-scale measurements; the authors reported volume measurements of both yeast and mammalian cells for more than 3 hours of continuous growth [212]. Microfluidic approaches have also been reported. In one approach, microfluidic traps were integrated with the electrode-based volume sensor, enabling the monitoring of real-time volume changes of single cells for several minutes [213]. In another embodiment, *Riordon et al.* integrated the electrodes with a pneumatic valve in order to engineer the sensitivity of the volume sensor; by pressurizing the valve, the buffer volume in the vicinity of the electrodes is modified, in turn modulating accordingly the sensor dynamic range and sensitivity [214].

Mass: In one of the earliest attempts for accurate mass determination of a single – hydrated – cell, the *Manalis* group developed the ‘*suspended micro-channel resonator*’. In this approach, a microfluidic channel is engraved in a vacuum suspended microcantilever; as a cell flows through the microchannel, the mass of the microcantilever and its resonant frequency are modified [215]. Thus, by detecting changes in the microcantilever’s resonance frequency, the ‘buoyant’ mass of a single cell can be accurately determined with the extraordinary sensitivity of 20-30 fg [216]. Recently, the same group integrated a single cell trap ([section 2.2.4](#)) for long-term

immobilization and measurements of the single cell mass variation under chemometric conditions [217]. More recently, MEMS resonant sensors have been developed, on top of which adherent cells are deposited for continuous mass monitoring [218]. In this embodiment, due to the resonator being in a hydrated state, the reported mass resolution is lower, on the order of 1 pg [219].

Interferometric optical techniques are also possible to infer mass information at the single cell level [220]. The operation principle is based on the optical phase delay experienced by light transmitted through a cell, which is proportional to the mass density of the cell. While initial attempts focused on the sensing of the refractive index of single cells [221], more recently, direct imaging techniques, such as the ‘*spatial light interference microscopy (SLIM)*’, have emerged [222]. These are label-free and non-invasive and, contrary to the aforementioned mechanical methods, they can perform rapid and sensitive mass measurements of individual cells even within populations [223].

3.1.3 Microspectroscopic Chemical Analysis

Microspectroscopy is a type of chemical analysis, realized by exciting the cell with a specific form of radiation, and analyzing the spectral response. Various such methods exist, categorized by the type of employed radiation, while imaging can also be achieved by spectral analysis at different (sub-cellular) locations. Common microspectroscopy methods for cell-physiology studies, such as Raman, infra-red and microbeam spectroscopy, will be reviewed separately in the following paragraphs.

Raman: In Raman microspectroscopy, laser light illuminates and scatters from a single cell at a different wavelength. This spectral shift depends on the vibrational frequencies of the intracellular molecules, which are distinct for specific chemical bonds. In this way, the

intracellular content, such as proteins, fatty acids, and nucleic acids, can be analyzed (**Fig. 8b**) [6]. Raman techniques emerged more recently in cell biology [224]. By addressing related technical challenges [225], Raman analysis became possible not only on dehydrated [226], but also on hydrated [227] cells (**Fig. 8c**).

One of the unique characteristics of Raman microanalysis is its compatibility with optical tweezers (**section 2.3.2**). This is frequently referred to as Laser Tweezers Raman Spectroscopy (LTRS) and is based on the use of a single laser beam that both traps and excites the target cell [228]. This has enabled unique bioprocessing investigations, such as ethanol and lipid biosynthesis [229, 230]. However, care needs to be taken to avoid cell photodamage, especially for prolonged single cell studies; the use of a near-IR excitation laser has been shown to only partially address this [231]. Metabolic analysis at the single cell level can also be performed by isotopic labeling, whereby incorporation of heavy isotopes shifts the Raman bands of the molecular bonds involved in the label incorporation [232].

Resonant Raman: In this microspectroscopy embodiment, the laser excitation wavelength is chosen to overlap with the absorption bands of fluorescent molecules inside the sample. In this way, the ‘resonance’ Raman effect is achieved, which greatly enhances the signal levels of the target by at least $10^3 - 10^4$ times. Typical examples involve the use of a UV excitation laser to detect the resonant Raman spectra of amino or nucleic acids [233, 234]. Due to the presence of autofluorescent compounds/pigments, resonance Raman has been particularly useful in the biochemical and metabolic studies of photosynthetic microorganisms [235]. Recently, the dynamics of *cytochrome c* under physiological and stress conditions were studied by resonance Raman, revealing its role in cell apoptosis [236]. Due to its enhanced signal levels, resonance Raman greatly enhances the measurement’s temporal resolution and minimizes photodamage;

however, photobleaching of the involved fluorescent molecules can occur, eventually leading to signal degradation [174].

Surface Enhanced Raman (SERS): SERS is an alternative way to enhance the Raman efficiency and signal levels. In SERS, the cells are placed on either a rough, or a periodically structured metal surface [237]. The latter amplifies the local electromagnetic field; in turn, increasing signal sensitivity by a factor of up to 10^{15} [238]. However, SERS is strongly dependent on the distance of the metal surface to the target, with the enhancement levels decaying considerably even within 10 nm. This can be addressed by appropriate metal layer chemical functionalization ([section 2.2.4](#)), which enables the simultaneous cell capture and SERS analysis [239]. Metallic nanoparticles may also be used, which upon internalization enable intracellular SERS microspectroscopy [240, 241]. Tip-enhanced Raman spectroscopy is also based on the same principle, with the tip itself acting as the local optical amplifier, and overall enabling Raman images of very high spatial resolution (down to 10 nm) [242].

Coherent Raman: Coherent anti-Stokes Raman scattering (CARS) and Stimulated Raman Scattering (SRS) are two popular forms of coherent Raman microspectroscopy of single cells. In CARS, the sample is illuminated with two short-pulse (fs) laser beams instead of a single continuous wave like in spontaneous Raman. The frequency difference of the two excitation lasers needs to be equal to the vibrational frequency of the target bond, thus mapping specific chemical entities, such as lipids or proteins [243]. As a result, in comparison to spontaneous Raman, a significantly enhanced signal is obtained by more than 2 orders of magnitude [244]. CARS is a non-linear optical process (similar to two-photon absorption), hence exhibiting enhanced sectioning capabilities and making it ideal for 3D biological imaging. However, CARS

is mostly applicable for imaging abundant intracellular species, as its signal strength rolls off rather quickly at low target analyte concentrations.

In CARS imaging, the background noise contribution from non-resonant chemical species is usually high, resulting in reduced chemical contrast. This has been addressed by implementing phase sensitive detection in Stimulated Raman Scattering (SRS) [245]. As a result, multi-wavelength, rapid imaging of high resolution and specificity is possible, such as the biochemical and metabolic analysis of photosynthetic microorganisms (**Fig. 8d**) [246], and the internalization kinetics of small molecules [247].

Both CARS and SRS are highly promising in single cell chemical imaging due to their label-free nature, enhanced specificity, and sectioning ability. This is particularly pertinent in lipid imaging and spectroscopy at the single cell level. Such intracellular compartments are both abundant and exhibit a strong C-H vibration mode at 2850 cm^{-1} , making them readily detected with coherent Raman techniques with unsurpassed temporal resolution and chemical contrast. As a result, the areas of fundamental lipid biology and metabolism have experienced rapid growth due to the advent of such imaging platforms [248, 249]. Both CARS and SRS however, require complex optical set-ups, while the long term effects of the employed intense laser pulses on the cell physiology and integrity remain to be identified [250-252].

Infrared (IR) microspectroscopy: In this embodiment, the IR absorption spectrum of an individual cell is captured to directly identify the structure and function of intracellular macromolecules [253, 254]. The challenge in this strategy is primarily the signal saturation due to the strong IR absorption of water, which can be addressed by employing dehydrated samples [255]. Alternatively, ATR (attenuated-total-reflection) approaches can be employed to confine the excitation close to the substrate-medium interface, thus avoiding the water absorption, but

also likely exciting a partial volume of the cell [256]. A third successful approach to avoid water absorption is the use of tailored designed chambers that minimize the media thickness, such as low-thickness [257], or open access microfluidics [258].

Due to the employment of longer excitation wavelengths, IR microscopy exhibits a larger (diffraction limited) excitation area compared to Raman. Despite this, the IR absorption spectra of isolated cells can still be measured, as in the successful determination of metabolite (EtOH) formation dynamics [259] and DNA conformation [260] in isolated cells. The use of solid immersion optical elements can increase the resolution down to 2-3 μm [261]. True superresolution IR imaging can be achieved by coupling IR to an Atomic Force Microscope probe, a strategy that has been shown to successfully detect triglycerides in dried cells with a 100 nm spatial resolution [262].

X-rays: Many types of X-ray microspectroscopy techniques for single cell elemental turn-over analysis exist, either in fluorescence or transmission mode [263]. The X-ray fluorescence microprobe is one of the first approaches in biology, well suited for the measurement of the concentration, location and turn-over rates of specific inorganic elements (**Fig. 8e**) [264]. A major application of this approach in the context of metabolism is the measurement of trace metal cycling at the single cell level for environmental applications [265, 266]. Soft X-ray microspectroscopy is a more recent technique, based on high-brightness synchrotron light sources and commonly operating in transmission mode (STXM) [267, 268]. 3D tomographic imaging is also possible in STXM by simply rotating the sample at discrete angles [269] (**Fig. 8f**). Overall, X-ray microspectroscopic imaging is ideal for structural imaging due to its 3D and nm resolution; long-term dynamic imaging is more challenging however because radiation dose can be damaging [270].

3.1.4 Electrochemical Analysis

Electrochemical methods are ideally suited for the analysis of the extracellular environment and the influence of cell secretion and cell-to-cell communication. To a similar note, microelectrophoretic approaches can also be applied to single cell analysis, as recently reviewed elsewhere [201]. Electrochemical methods are very attractive due to their low-capital investment requirements and microfluidic compatibility [203, 271-273]. Herein, our focus will be on single cell respiration, since oxygen – a common electron acceptor in metabolism – is rather electroactive and thus readily detectable. In the early attempts, scanning electrochemical microscopy (SECM) was employed, where a tip scanned over the cells and measured current modulations due to localized oxygen concentration variations [274]. While SECM has enabled very important findings, it was recently argued that the readings' accuracy is limited due to the cell's topography and associated challenges in maintaining the exact same scanning path during long-term measurements [275]. This challenge can be conveniently addressed by integrating the sensing electrodes with microfluidic control of the cell position, an approach that has recently enabled the precise quantification of respiration kinetics in single photosynthetic bacteria [276, 277].

3.2 Fluorescence Mediated Microanalysis

In this section, we will review fluorescent methods for registering metabolic activity and bioenergetics of single cells. This has been an established field for a many decades now, and numerous strategies and toolkits exist—a significant number of which are commercially available. Many excellent resources exist on this topic, such as the J. R. Lakowicz textbook for a complete background on fluorescence [278], as well as a few recent reviews on single cell

fluorescent imaging and spectroscopy [279-281]. Detailing all available methods is beyond the scope of this review; our aim instead is to highlight key available *methods* ([section 3.2.1](#)) and related *contrast agents* ([section 3.2.2](#)) of practical use in investigating energy flow and metabolism at the single cell level.

3.2.1 Methods

Fluorescent single cell methods are generally divided into cytometry, microscopy, and their modifications, such as Fluorescence Correlated Spectroscopy (FCS) and Fluorescent Recovery After Photobleaching (FRAP). These are briefly discussed below.

Cytometry: In cytometry – also described earlier in the context of single cell manipulation ([section 2.1.1](#)) – the cells flow in front of one or more laser beams and their scattering or fluorescent properties are detected by opposing detectors. Alternative embodiments include the *Laser Scanning* (LSC) and *Imaging cytometry*, both of which are also commercially available. In *LSC*, the laser excitation is scanned over cells deposited on slides or membranes, and thus is not limited to studies involving only cells in solution as in conventional flow-cytometry [282, 283]. *Imaging Cytometry* refers to a wide range of techniques of high-throughput cell microscopy [284]. In the most common embodiment, cells are deposited on a slide and imaged at the single cell level using basic or advanced microscopy modalities [285]. More recently, microfluidic image cytometry (MIC) was reported, where cells are contained in discrete microfluidic chambers and undergo growth and long-term imaging cytometry for high-content screening [286, 287].

Microscopy: The most common modality of fluorescent microscopy is the *epi-fluorescent* microscope using a laser (coherent) or a lamp (incoherent) excitation source [288]. To enhance the imaging contrast and enable 3D optical sectioning, techniques such as the *confocal* (laser scanning [289] or spin-disk [290]), and *multi-photon* [291] imaging, as well as *light-sheet* microscopy [292, 293] are currently available. Substantial accomplishments in *superresolution microscopy* have been recently reported, with even 10 nm resolution being possible [294]. The most common and commercially available strategies involve the *Structured Illumination Microscopy* (SIM), with a reported resolution of about 100-150 nm [295], and *Stimulated Emission Depletion Microscopy* (STED) with a 30 nm resolution limit [296]. 30-50 nm optical resolution can also be achieved by modulating the emission of single molecules, either stochastically, such as *Stochastic Optical Reconstruction Microscopy* (STORM) [297], and *Photoactivated Localization Microscopy* (PALM) [298], or, as more recently, deterministically using *polarization modulation* techniques [299].

Fluorescence Lifetime Imaging (FLIM): In FLIM, the image contrast is derived by the fluorophore excited-state lifetime at each pixel, rather than its intensity. Generally, there are two FLIM types, one operating in the frequency domain (frequency modulated illumination and detection) [300] and one that operates in the time domain by counting photons using fast lasers and detectors [301]. FLIM is a unique imaging modality, because it enables fluorophore discrimination based on their conformation and their local chemical microenvironment, both of which directly affect their excited state lifetime [302]. A common challenge in FLIM is the low photon budget at the pixel of each image, which however has been recently overcome by processing the image using the *phasor analysis* method [303]. As a result, FLIM has found many applications in metabolic imaging, especially by discriminating free and protein bound NADH

(short and long lifetime respectively). This has in essence revolutionized *in-vivo* metabolic imaging applications, such as the discrimination of proliferative stem cell in living tissue [304].

Fluorescence Correlated Spectroscopy (FCS): In FCS, a laser beam illuminates part of the cell and the intracellular fluorescence is continuously monitored. The latter exhibits fluctuations which decipher intracellular molecular dynamics, such as the concentration and diffusion constant of fluorescent proteins [305]. *Fluorescent Recovery After Photobleaching (FRAP)* is a similar technique, where a location of the cell is continuously illuminated until bleached; subsequently, the dynamics of fluorescence recovery is monitored; namely, the diffusion in the observation volume of fresh, fluorescent molecules [306]. Similar to FCS, FRAP provides information about the diffusion and binding kinetics of fluorescent proteins.

3.2.2 Contrast Agents

Contrary to the aforementioned label-free techniques, in fluorescence, chromophores need to be implemented that absorb light at a specific wavelength and remit at a different one. Such molecules or probes that are crucial in exploring the physiology or metabolic state of single cells will be discussed in this section, including fluorescent proteins, enzymes and biosensors.

Fluorescent Proteins (FP): Since the advent of the green FP (GFP) cloned from the jellyfish *Aequorea victoria* [307], FPs have arguably become the workhorse contrast agent in modern bioimaging applications [308]. These genetically encoded chromophores exhibit now a wide variety of emission wavelengths (**Fig. 9a**), as well as enhanced photostability and brightness [309]. FPs are commonly used as reporters of other non-fluorescent proteins. This is achieved by fusing them to the protein of interest through a short peptide linker, which in turn is realized by cloning the FP sequence in a continuous open reading frame of the gene of interest [310]. FPs

have found numerous single cell applications. One example involves the long-term single cell imaging of expression of genes linked to metabolic regulation. These investigations have been pioneering in elucidating metabolic gene regulation under perturbations [83], as well as the nature of stochastic transitions in metabolic networks, such as the ‘all or non’ behavior reported for the lactose [311], or galactose utilization [312]. FP fusions can also be used to tag specific intracellular organelles [313]; a few recent examples of this type specific to metabolism involve the high-throughput imaging of mitochondrion network size scaling during proliferation, [314] and the role of triacylglycerol enzymes in lipid droplet biosynthesis [315].

Physiological Fluorophores: Fluorescent (non-genetically encoded) probes capable of accumulating in specific intracellular organelles belong to this category [279]. There is a wide variety of such probes, each targeting a specific intracellular moiety. One of the earliest examples of this class is the stain of nucleic acids, such as the Hoechst or propidium iodide dyes [24, 316], with the latter being selectively internalized upon apoptosis, thus also functioning as a fitness indicator. Specific organelles may also be targeted, such the mitochondrion, [317] or the cell membrane [318], the latter also functioning as a vesicle-mediated internalization pathway indicator. Another important class of physiological fluorophores in bioprocessing are lipophilic dyes that selectively partition within lipid droplets [319], enabling thus the direct visualization of lipid droplet metabolism and strain selection [320].

Biosensors: These are a special class of probes that their optical properties are altered depending on variations of the local microenvironment [321]. Calcium sensors [322] belong to this category, as well as membrane potential [323] and hydrogen ion sensors [324]. The latter are ideal as metabolic activity indicators, operating on the simple principle that pH homeostasis cannot be maintained at low ATP production [325]. Such pH sensors were very recently

employed to reveal subpopulations of distinct metabolic profile in isogenic cultures [21]. Other types of biosensors also exist, such as membrane potential and FRET ones; the interested reader is re-directed to recent reviews on the topic [29, 323].

Respiration activity is another important metabolic indicator, which can be optically detected, in addition to the previously discussed electrochemical methods ([section 3.1.4](#)). For the optical readout of respiration, specific phosphorescent sensors need to be employed. These, upon collision, transfer non-radiatively their energy to molecular oxygen and their intensity decreases [278]. Optical oxygen sensing has been applied both to microfluidic cultures [326], but also at the single cell level trapped in microwells [327, 328] ([Fig. 9b](#)). Indirect respiration detection is also possible, involving sensors such as the Redox Sensor Green dye [329]. Their operation is based on the active electron transport system during respiration, which reduces the sensing moiety and modifies its emission spectrum [330]. There are also many types of *genetically encoded biosensors* capable of detecting a variety of intracellular entities such as hydrogen peroxide [331] and calcium [332]. These do not need to undergo cell internalization in order to operate, thus substantially simplifying their use. Such sensors have recently found many applications in detecting intracellular concentrations of specific metabolites. In these, the metabolites react with transcriptional activators, initiating the expression of fluorescent proteins. Such recent biosensor examples involve the dynamic monitoring of amino acid production in the industrially relevant strain *C. glutamicum* ([Fig. 9c](#)) [333, 334].

Enzyme activity: Biosensors also exist for assaying enzyme activity [335]. Specific to single cell analysis, non-fluorescent and cell-permeable substrates can be employed, which are then enzymatically converted into a fluorescent one. Two very basic examples are Calcein-AM [336] and Alamar Blue (resazurin) [337] that become fluorescent or lose their emission respectively

upon enzymatic reaction. Fluorescent substrates adhered on the cell membranes have also been reported; these have been useful in selecting protease variants by FACS: desired variants cleaved the fluorescent unit, thus exhibiting no fluorescence and enabling FACS sorting [338].

With the advent of microfluidics, it has been recently possible to assay secreted metabolites in intact cells, as well as lyse cells and quantify their intracellular metabolite content. Regarding metabolite secretion, single enzyme studies in pneumatically isolated microfluidic chambers, [152] or population screening in microfluidic droplets have been reported [20]. In the latter report, the use of microfluidic droplets enabled the concentration of the secreted metabolite, with the impressive ability to identify 1 desired variant in a population of 10^4 cells (**Fig. 9d**) [20]. A *coupled enzymatic assay* was employed based on the indicator Amplex UltraRed, which converts into a fluorescent compound in the presence of horseradish peroxidase and hydrogen peroxide (H_2O_2), the latter being the product of oxidase mediated metabolite oxidation [339]. In regards to assaying lysed cells, single cells were trapped in a microfluidic chamber, and underwent several washing steps and eventually lysis. Lysate targets were then surface immobilized and quantified by a sandwich ELISA assay [340].

3.3 Mass Spectrometry Methods (MS)

Specific to metabolomics, much recent technological advancement has enabled operation at the single cell level. These technologies have been reviewed recently in many excellent reports [341-343], so we will only summarize key recent findings and remaining challenges. In regards to challenges, one is the tremendous variability of metabolites, as well as their rapid turnover rates--even at the single cell level [10]. As recently reviewed by *Heinemann et al.*, several strategies have been developed to address this, including chemical separations prior to detection,

microfluidic cell handling and nanophotonic signal enhancement [343, 344]. *The use of complementary detection methods in addition to mass-spectrometry has also gathered considerable excitement. One such example is the selective analysis of either the cytoplasm or of the intracellular compartments by combining nano-electrospray ionization (ESI-MS) with video microscopy [345] as illustrated in Fig. 11a-b.*

Achieving high-throughput and statistically significant single cell data is another challenge in metabolomics. To address this, one approach developed by the *Zenobi* group is microarrays for mass spectrometry (MAMS). Contrary to microwell approaches ([section 2.2.1](#)), MAMS contain an array of hydrophilic spots on a hydrophobic surface, thereby isolating one or a few cells in a spatially organized matrix [346]. This approach enabled the group to characterize metabolic population variability in yeast using MALDI MS [347]. In an alternative approach, to achieve ultra-high throughput, the *Nolan* and *Tarren* groups developed the mass cytometer, whereby single cells are stained with isotopically labelled antibodies and subsequently analyzed in an ICP-MS [39]. The technique – termed *CyTOF* – enabled the simultaneous detection of 34 different parameters. CyTOF was primarily used for immunophenotyping of single cells. Given appropriate antibodies, the technique is also ideal for high-throughput single cell metabolomics. Single cell MS imaging is also possible, by analyzing specific locations of the cell sequentially. The most traditional approach has been the MALDI-MS, which exhibits a spatial resolution of approximately 1 μm [348]. By using an ion instead of a laser beam, the more recent ‘secondary ion mass spectrometric’ imaging (SIMS) exceeds the spatial resolution limit of MALDI-MS by more than one order of magnitude [232, 349] ([Fig. 10c](#)). However, compared to MALDI, SIMS exhibits lower chemical specificity, which can be addressed by supplying the targets with

isotopically labelled nutrients. Sampling at different times also enables the turn-over determination of specific macromolecules [350, 351] (**Fig. 10d-f**).

Finally, it is worth noting that single-cell proteomics is also currently pursued with MS; however, little progress has been achieved to this end, primarily due to the small number of proteins in individual cells, and the inability to amplify such analytes as commonly done in gene studies and sequencing. LTQ-Orbitrap MS is the most promising strategy to address this; however, extremely efficient sample handling needs to be perfected towards this task [341].

3.4 Nuclear Magnetic Resonance Methods (NMR)

Single cell metabolomics with NMR is still a field at its infancy, exhibiting still insufficient detection limits. However, both the information content that can be extracted with NMR and the fact that it is a non-invasive technique are unique and very attractive features [352]. NMR of very large cells such as oocytes [353] and neurons [354] has been possible, albeit at an incomplete metabolome coverage. Recent progress however in designing novel microcoil probes, [355] and the integration of NMR with microfluidics. Both are very promising approaches that have so far enabled further decrease of the observation volume and enhanced the sensitivity down to the *nmol* regime [356].

4. Conclusions

Single cell analyses, such as imaging and sequencing, have been of crucial importance in modern life sciences and particular systems biology. More recently, such analyses were applied to metabolic investigations and engineering, enabling unique observations, such as the discovery of subpopulations with distinct metabolic profile [20, 21, 357], and the quantification of metabolic

variability in populations [347]. The importance of such findings spans not only systems biology, environmental microbiology and the biomedical field, but also industrial applications of synthetic biology, where such phenotypic variations are known to negatively impact productivity [330, 358]. Further insight in this domain will clearly lead to better understanding of environmental challenges, as well as identifying novel therapeutic and metabolic engineering approaches.

With this review, our aim is to outline presently available techniques for isolating and probing the bioenergetics and metabolic potential of individual cells. While each individual category (manipulation and probing) are promising by themselves, their co-integration is likely to enable a deeper insight in to the origins and effects of cellular metabolic heterogeneity. This is evident by the recent discoveries enabled by the fusion of droplet microfluidics with advanced fluorescence techniques [20], and that of single cell surface patterning with mass spectrometry [347]. Emerging computational and microanalytical techniques will also play a critical role; the latter involving ‘omics’ analysis of microbial cells with enhanced coverage and ‘correlative microscopy’ [359] by fusing MS with superresolution microscopy [360] for both analyzing metabolic network function and structure.

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6. Figure Captions

Figure 1: *From many down to the single cell;* (a) An industrial scale fermenter with an approximate height of a few meters (credit U.S. Department of Energy). (b) A bioreactor growing algae; the vertical dimension of the instrument is a few cm containing approximately 10^{12} cells (credit U.S. Department of Energy). (c) A budding *Yarrowia Lipolytica* yeast, with the daughter cell exhibiting an approximate 2 μm diameter. (c) Schematic representations of the 2D, 1D and 0D confinement types discussed in this review.

Figure 2: *Flow-through single cell analysis;* In (a), the schematic of a conventional single cell flow cytometer is illustrated (Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology [361], Copyright (2008). (b) Loading of single cells in droplets at a microfluidic T-junction; the black arrows point to the individual cell in the droplet (reproduced from [362] with permission from The Royal Society of Chemistry). (c) Single hybridoma cell encapsulation in arrested droplets (reprinted by permission from Macmillan Publishers Ltd: Nature Protocols [55], Copyright (2013).

Figure 3: *Confinement in 3D microcavities;* (a) A schematic of the fabrication and seeding of PDMS microwell arrays; the procedure involves pouring and curing the prepolymer on to a patterned master, and subsequently peeling off and placing the microwells in a Petri dish; finally, the cell suspension is introduced and allowed to sediment onto the microwells, and excess cells from the top surface are rinsed (reprinted from [66]; Copyright (2005) American Chemical Society). (b) An SEM image of single trapped Jurkat T cells in an array of cell retainers etched in SiO_2 ; scale bar is 20 μm (reproduced from [68] with permission from The Royal Society of Chemistry). (c) Mammalian cells trapped in microwell arrays at the bottom of a microfluidic

channel; the microwells are the circular structures and the microfluidic channel walls are denoted by the straight lines (reproduced from [78] with permission from The Royal Society of Chemistry).

Figure 4: 2D and 1D Confinement; A zoomed-in view of the Tesla microchemostat (a), illustrating the ‘diode loop’ with the trapping (grey) and loading (black) regions; in (b) the shallow trapping region of Tesla microchemostat is shown, where cells are confined in 2D; the scale bar is 20 μm [82] (Copyright © 2005 EMBO and Nature Publishing Group). (c) A bioreactor with a shallow circular 2D growth area placed inside a deeper supply channel; note the radially arranged channels that enable nutrient supply and waste removal (reproduced from [89] with permission from The Royal Society of Chemistry). (d) 1D bacterial growth in 1D patterned agarose (lower) in contrast to agarose pads where bacterial crowding takes place (upper); reproduced from [97] with permission from The Royal Society of Chemistry.

Figure 5: 0D Confinement; (a) Microfluidic single cell trapping (*left*), where cells are trapped at the stagnation point and kept there immobilized by flow driven hydrostatic pressure (denoted by white arrows). A bright field image of an individual trapped Jurkat T-cell (*right*); adapted from [103]; Copyright (2003) American Chemical Society. In (b), a single cell trapping array is illustrated (*left*, scale bar is 500 μm); a higher resolution image of the trapping array (*middle*), along with an individual trapped cell (*right*); reproduced from [104] with permission from The Royal Society of Chemistry. (c) Monolithic PDMS pads for trapping yeast cells, while releasing the daughter cells under the continuous flow of media (adapted from [131]). (d) A schematic diagram of a microfluidic ‘lateral percolation’ trap; the operation principle is based on the geometric relationship between paths 1 and 2. When the trap is empty, the resistance of path 1 is

lower until a cell occupies, after which subsequent cells will follow the bypass loop (adapted from [110]; Copyright (2007) National Academy of Sciences, U.S.A.).

Figure 6: *Dynamic Confinement 1*; (a) A schematic representation of a pneumatically isolated microcavity; cell trapping occurs by compressing the flow channel by the two control channels (reprinted by permission from Macmillan Publishers Ltd: Nature [152], Copyright (2006). (b) A fluorescently labelled *E. coli* trapped inside a submicrofluidic indentation; release takes place by increasing the flow rate (reproduced from [106] with permission from The Royal Society of Chemistry). (c) A single cell hydrodynamic trap by generating microeddies around a solid cylinder at low frequency oscillations of flow (reproduced from [158]; Copyright (2006) American Chemical Society). (d) A stagnation point generated at the junction of two perpendicular microchannels; active feedback flow control ensures the stabilization of the stagnation point and the immobilization of a single cell therein (reprinted with permission from [160]; Copyright (2010), AIP Publishing LLC).

Figure 7: *Dynamic Confinement 2*; Schematic representation of single cell trapping by OT (a) and a ‘dual beam trap’ (b). In (c), radiation pressure forces enable selective release of microwell loaded cells (reproduced from [178]; Copyright (2007) American Chemical Society). (d) DEP trapping of a Pollen grain in an eight electrode electric field cage (reprinted from [189] Copyright (1993), with permission from Elsevier). In (e), the cell loading and retrieval is illustrated for the magnetic microrafes (reprinted with permission from [198]; Copyright (2011), AIP Publishing LLC).

Figure 8: *Label-free microanalysis*; (a) Single cell *proliferation* in a microchemostat (reprinted from [206]). In (b), the *Raman spectrum* of a bacterium is plotted, including the major bands

attributions (reprinted from [226]; Copyright (2000) American Chemical Society). (c) *Raman images* of yeast at different wavenumber regions, marking specific intracellular molecular fractions (adapted from [363]; Copyright © 2005 John Wiley & Sons, Ltd). In (d) a multiplex *SRS image* of single algal cells, depicting photosynthetic pigments (red), lipids (green) and protein (blue); (adapted from [246]; Copyright (2000) American Chemical Society). (e) Elemental maps from *X-ray emission* of the marine species *D. norvegica* using a nuclear microprobe at a 5 μm spatial resolution (adapted from [264]). (f) A budding yeast cell imaged by soft X-ray tomography, where different colors represent different organelles (reproduced with permission from [269]).

Figure 9: *Fluorescence mediated microanalysis:* (a) Chromophore classes found in fluorescent proteins and their respective emission bands (reprinted by permission from Macmillan Publishers Ltd: Nature Chemical Biology [309], Copyright (2014). (b) A phosphorescent oxygen sensor at the bottom of a microwell plate containing two cells (reprinted from [328], Copyright (2009), with permission from Elsevier). (c) Population heterogeneity in the production of L-valine, revealed by an FP-fused genetically encoded biosensor (reproduced from [333]). (d) Microfluidic high-throughput screening platform of secreted metabolites in microfluidic droplets; reprinted by permission from Macmillan Publishers Ltd: Nature Biotechnology [20], Copyright (2014).

Figure 10: *Mass spectrometry:* Sequential clips illustrating the metabolic analysis of single cells using nano-electrospray ionization (ESI-MS); in this instance, video microscopy enables the selective analysis of the cytoplasm (a) or individual granules (b); reprinted from [345]; Copyright © 2008 John Wiley & Sons, Ltd. (c) Schematic illustrating the NanoSIMS imaging of a single cell: the secondary ion beam rasters over the sample removes material to be analyzed by MS. Quantitative raster NanoSIMS images of the *R. palustris* bacterium, illustrating the

distribution of $^{12}\text{C}^-$ (d) and $^{13}\text{C}^-$ (e); image (f) illustrates the $^{13}\text{C}^-$ enrichment at one pole of the cells (reproduced from [351]).

Table 1: *A table summarizing single cell manipulation strategies as discussed in section 2. The ‘method’ column describes the associated technique, including the characteristic ‘type’ (ie. flow through, dynamic, permanent), its ability to ‘recover’ cells, its ‘capacity’ (in number of cells), the ‘expertise’ requirements (+, -: low, high), its ability to control the chemical ‘microenvironment’, its ‘surface noise’ properties (arising from non-specific surface adhesion and leading to optical or chemical noise), and finally the related ‘section’ and ‘references’.*

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Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

Figure 10

method	type	recovery	capacity	expertise	micrenvironment	surface noise	section/references
flow-cytometry	flow-through	+	$10^4 - 10^5$ cells	+	not available	+	2.1.1 / [22 - 39]
microfluidic droplets	flow-through	+	$10^3 - 10^4$ cells	-	not available	+	2.1.2 / [40 - 63]
microwell	permanent	-	$10^2 - 10^3$ cells	+	-	-	2.2.1 / [64 - 75]
microchemostats	permanent	-	$10^2 - 10^3$ cells	-	microfluidics	-	2.1.2 / [76 - 81]
2D confinement	permanent	-	$10^2 - 10^3$ cells	-	microfluidics	-	2.2.2 / [82 - 92]
1D confinement	permanent	-	$10^2 - 10^3$ cells	-	microfluidics	-	2.2.3 / [93 - 100]
axial percolation	permanent	-	$10^1 - 10^3$ cells	-	microfluidics	-	2.2.4 / [103 - 109]
lateral percolation	permanent	-	$10^1 - 10^3$ cells	-	microfluidics	-	2.2.4 / [110 - 115]
hydrogels	permanent	-	$10^1 - 10^3$ cells	+	microfluidics	-	2.2.4 / [116- 125]
surface functionalization	permanent	-	$10^1 - 10^3$ cells	+	microfluidics	-	2.2.4 / [126 - 149]
pneumatic microcavities	dynamic	+	$10^0 - 10^2$ cells	-	microfluidics	-	2.3.1 / [150 - 155]
hydrodynamic	dynamic	+	$10^0 - 10^2$ cells	-	microfluidics	-	2.3.1 / [106, 156 - 161]
acoustic	dynamic	+	$10^0 - 10^2$ cells	-	microfluidics	-	2.3.1 / [162 - 166]
optical tweezers	dynamic	+	$10^0 - 10^2$ cells	+	microfluidics	+	2.3.2 / [167 - 174]
radiation pressure	dynamic	+	$10^0 - 10^2$ cells	-	microfluidics	+	2.3.2 / [175 - 180]
optoelectronic tweezers	dynamic	+	$10^0 - 10^4$ cells	+	microfluidics	-	2.3.2 / [181 - 184]
photothermal/catalytic	dynamic	+	serial	+	-	-	2.3.2 / [110]
dielectrophoresis	dynamic	+	$10^0 - 10^2$ cells	-	microfluidics	-	2.3.3 / [187 - 195]
magnetic tweezers	dynamic	+	$10^0 - 10^2$ cells	+	microfluidics	-	2.3.4 / [196 - 200]