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Single Cell Spectroscopy: Noninvasive Measures of Small-Scale Structure and Function

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Abstract

The advancement of spectroscopy methods attained through increases in sensitivity, and often with the coupling of complementary techniques, has enabled real-time structure and function measurements of single cells. The purpose of this review is to illustrate, in light of advances, the strengths and the weaknesses of these methods. Included also is an assessment of the impact of the experimental setup and conditions of each method on cellular function and integrity. A particular emphasis is placed on noninvasive and nondestructive techniques for achieving single cell detection, including nuclear magnetic resonance, in addition to physical, optical, and vibrational methods.

Keywords

Spectroscopy; Single Cells; Nuclear Magnetic Resonance; Fluorescence Microscopy; Atomic Force Microscopy

INTRODUCTION

The study of the interaction of radiated energy (e.g. light, sound) with matter defines the broad field of spectroscopy. The nature of the interaction, including emission and scattering, often reveals spectra, or a distribution of energy emitted by a radiant source, and indicates physical properties of the matter studied. Spectroscopy has a deep history dating back at least several hundred years to experiments that revealed the distribution of color following the separation of light through a prism [1]. An example of an early spectroscopy instrument is the spectrograph that used components including a diffraction grating to separate visible light by wavelength or frequency. Modern spectroscopy can be separated into an extensive array of subfields including electron spin resonance, mass spectrometry, hyperspectral imaging, nuclear magnetic resonance spectroscopy, and photoacoustic spectroscopy.

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In biology, the study of single cells is important for investigating processes that can be confounded by heterogeneities often seen in large populations of cells within tissues of the body (e.g. [2–4]). Isolation and identification of single cells often requires defined protocols and unique techniques [5]. It is common to observe multiple cell types or cellular subpopulations that give rise to spatially complex tissues with multifunctionality. Moreover, cells are dynamic, sometimes changing (e.g. differentiating) in time depending on local microenvironmental cues [6]. For example, signaling gone awry may promote the onset of disease [7], which may be best targeted and treated if detected in an early initiation stage before extensive proliferation occurs.

The study of single cells, especially using modern methods of spectroscopy, is challenging in many applications due to inherent spatiotemporal resolution limitations coupled to the need to maintain cellular viability in a physiologically-relevant environment. Mammalian cells are typically on the order of 10 microns in diameter [8], and their morphology has been easily described in the living state by visible light microscopy for several hundred years [9, 10]. But interestingly, when cells are subjected to ultraviolet light, i.e. a slight shift in the electromagnetic spectrum, the energy exposure can result in altered chemical bonds in molecules leading to outcomes such as apoptosis [11, 12], collagen crosslinking [13], and cancer [14, 15]. Therefore, not all spectroscopy tools may be appropriate for the study of properties at the cellular scale, and a careful consideration of benefits and tradeoffs is required.

The purpose of this review is to outline the advantages and challenges of spectroscopy techniques for use in the study of single cells. Here, we define strengths and weaknesses of recent advances in spectroscopy measurements at the cellular scale, with a particular emphasis on modern methods including nuclear magnetic resonance (NMR), atomic force microscopy (AFM), fluorescence microscopy, and Raman spectroscopy. We note trends in technology that are leading to an increasing array of tools for single cell studies. Moreover, we describe combinations of spectroscopic modalities that provide information not attained by any single technique alone making available novel and detailed information from the basic unit of life.

METHODS: IDEAL CONDITIONS AND PRACTICAL CONSIDERATIONS

Each modern spectroscopic technique offers unique and specific information about the properties of the matter studied. But what constitutes an ideal spectroscopic method for measurements of single cells? Distinct spectral signals are needed to observe desired cellular components or dynamic processes, with minimal biological noise or dissipation due to the experimental setup, e.g., the cell carrier medium. In this context, the signal may represent a voltage, current or other message, with a variation that corresponds to information about the underlying properties (e.g. biomolecular concentration, self-diffusion, stiffness, etc). Well resolved spectral signals with high signal-to-noise ratio can assist in eliminating the burden of computationally-intensive post-processing and thus can result in more robust identification of underlying biomolecules and properties (e.g. [16–20]).

An ideal spectroscopic technique would provide biological information about the structure (e.g. location) and function (e.g. conformation or activity) of specific cell constituents, including RNAs and proteins. Specific intracellular activity, including the cascade of signal transduction pathway events [21], would ideally be identified and tracked in real time, with label-free methods (e.g., [22]) that do not disturb or disrupt the observed biology. One primary challenge is the ability to detect individual constituents, and many conventional spectroscopic techniques, e.g. NMR, currently lack the specificity required. Another challenge is the ability to detect small and low-abundance constituents, which may be

present at nano- or even pico-molar concentrations [23], without the aide of techniques such as microscopy-based antibody fluorescence labeling [24]. With increased demands on specificity, sensitivity, and real time detection, measurement throughput naturally diminishes and can impose practical limits on the ability to measure large populations of cells and responses.

Nevertheless, there are numerous counteracting factors that impose limitations on the capabilities of each spectroscopic technique. For single cell studies, maximizing spatiotemporal resolutions is typically a premium (Figure 1), illustrated by recent achievements in super-resolution microscopy (with spatial resolutions on the order of 10s of nanometers); however, this often comes with the tradeoff of increased time required for signal acquisition and image formation [25]. Furthermore, specificity is required to study actions of individual molecules within cells, often imposing challenges related to signal strength and labeling [26]. Signal specificity can be achieved by introducing impurities in the cell environment, such as fluorescent labels or metallic nanoparticles [27], that bind (or are already bound) to particular target molecules. These impurities, used as a means of labeling molecules for spectroscopic observation, can disrupt cell function resulting in misleading observations [28]. Processes that require the use of such impurities may be considered as invasive. On the other hand, noninvasive techniques, such as Raman spectroscopy, study the sample in a more natural environment (i.e., in solution), but the acquired spectral intensities are typically weak with overlapping spectral lines [17, 29]. Moreover, significant radiation that is often used to improve spatiotemporal resolution, in both invasive and noninvasive techniques, can damage the cell constituents and alter physiological functions [30]. Functional anomalies might also occur due to methods that require the observation of the cell to take place in artificial conditions, not emulating the natural environment, as in infrared spectroscopy where water can cause strong interference (in the mid-infrared spectral region) and thus the sample must be prepared in a way to reduce or eliminate this interference [29, 31]. The cellular microenvironment is an added complexity, noting that some cell populations cultured in two-dimensional monolayers express an altered phenotype compared to those maintained in a more physiologically-relevant three-dimensional state [32]. Additionally, cells are sensitive to the stiffness of the local environment, influencing distinct phenotypes depending on the surroundings and experimentally-defined test environment [6].

The acquired signal from spectroscopy methods depends on numerous parameters. Within a given technique, the location, intensity, and width of acquisition peaks can vary, since they depend on a variety of dynamic parameters including the testing medium, the cell growth and age, ambient temperature, and concentration of the observed molecules. As a result, quantitative analysis through the correlation of the spectral signatures to a standard reference library (e.g., [20, 33]) is not always a trivial process, and statistical numerical methods are often employed in order to attain an automated, unsupervised identification of the compounds (e.g., [34, 35]). In addition, spatial and temporal resolution enhancements often come at the expense of increased acquisition and post-processing time. Therefore, numerous factors need to be considered during the selection of the experimental method for single cell analysis.

METHODS: INDIVIDUAL AND COMPLEMENTARY BIOPHYSICAL MEASURES

Nuclear Magnetic Resonance (NMR)

Over the last few decades NMR has rapidly developed into a method well suited for probing biomolecules in living systems—applications range from characterization of

macromolecular structures at atomic resolution [36] to metabolic profiles in human cells, tissues and organs [37]. Observation of the NMR signal relies on nuclei with a nonzero spin quantum number (e.g. 1H, 13C, 15N, 31P); these nuclei are referred to as NMR-active because, in the presence of a magnetic field, an angular momentum of the nucleus is generated about the magnetic field resulting in a measurable net magnetic moment (Figure 2a).

NMR produces a spectrum of resonance lines over a range of frequencies (chemical shifts), the positions of which are dependent upon the local magnetic environment of the NMR-active nucleus. Such shifts in frequency are related to the chemical environment defined by the molecular structure. Thus, chemical groups (e.g. phosphates, phosphites, etc. in 31P NMR) produce identifiable patterns of resonance lines in the NMR spectrum and the chemical shift position of these patterns is influenced by the macromolecular structure of the group (Figure 3); this permits both detection of molecular structure and changes in conformation [38]. One of the most important attributes of NMR compared with other methods that provide atomic resolution structural information (e.g. X-ray crystallography and high-resolution electron microscopy) is that NMR can be performed *in vivo* and non-invasively, making it a very attractive modality to study cellular function.

Much of the early in-cell spectroscopy work focused on small molecules with high natural abundance such as metabolites. Smaller molecules tend to undergo more rapid molecular tumbling resulting in more well-resolved NMR spectral lines and thus increased sensitivity. Metabolites like glucose and high energy phosphates (ATP) can be readily studied using 1H and 31P NMR with simple one-dimensional acquisition methods [39, 40]. In addition to in-cell metabolite concentrations, isotope labeling of metabolic substrates (e.g. 13C labeled glucose) has been used to examine metabolic pathways [41].

While these early in-cell studies provide important insights into cell metabolism, they provide limited information on overall cell function. Parallel to in-cell NMR advances, observations of conformational changes of proteins in solution using NMR were being made based on the finding that 1H chemical shift differences are detectable between folded and unfolded proteins [38], and methods for *de novo* structural determination of proteins were ushered in through use of two-dimensional NMR techniques [36]. An additional major advance came with the production of recombinant proteins with stable isotopes (e.g. NMR-active 13C and 15N), paving the way for heteronuclear NMR studies and the ability to filter unwanted signals, an important step towards in-cell studies of macromolecules.

Studies of in-cell macromolecules were largely pioneered by Serber and Dotsch, who combined techniques of recombinant protein over-expression, isotope labeling of proteins and heteronuclear NMR experiments [42, 43]; each of these techniques were required for addressing specific challenges related to in-cell measurements. Above, we allude to the extensive amount and variety of molecules inside cells which give rise to NMR signals and thus of primary importance is the ability to discriminate between signals of interest and signals from the rest of the cell. Recombinant protein over-expression not only helps to increase the concentration of the protein of interest for improved detection, but it also helps to preferentially label the proteins of interest with NMR-active isotopes (e.g. through the use of labeled media) rather than molecules composing background structures [43]. Isotope labeling is critical in order to then filter proton signals from background proteins, nucleic acids, and intracellular structures by only probing those interacting with labeled nuclei through heteronuclear NMR methods. This is an effective approach due to the low natural abundance of NMR-active labeling isotopes like 13C and 15N (i.e. 1.1% and 0.2%, respectively). Protein over-expression, though effective in many applications (more for prokaryotic than eukaryotic applications) for addressing sensitivity issues, should be

carefully considered so as not to overcrowd the intracellular space as this would be expected to result in an unnatural environment and possibly influence protein structure and conformation compared with normal expression levels [44, 45].

NMR-based evaluation of cell function will likely rely to some extent upon a combination of *in vitro* and *in vivo* measurements in general. Much of the structural and functional NMR measurements of proteins prior to the in-cell work were made *in vitro* using highly purified proteins in solution—conditions that are ideal for maximum sensitivity. These *in vitro* experiments, though not identical to in-cell conditions, permit some control over environmental conditions to best match in-cell conditions, such as buffer content, pH, enzymes, and gas mixture, and serve as a first approximation of protein structure and function for detailed *de novo* characterization. Subsequent in-cell experiments can then explore the influence of protein interactions with other intracellular components. It is suggested that the complex environment inside cells including presence of organelles, sub-cellular compartmentation containing different physical and biological environments, and cellular differentiation results in heterogeneous and differential effects on protein structure [44]. Serber and Dotsch have demonstrated this through results showing more complicated in-cell spectra compared with *in vitro* spectra; this is attributed to possibly multiple conformational states *in vivo* [43].

As was mentioned above, molecular tumbling and line width are related in NMR measurements, which is both advantageous and problematic for in-cell measurements. The advantage is that changes in line-width can be used to indirectly detect dynamic intracellular processes modulated by cell signaling. For instance, binding events which modulate molecular mobility have been shown to result in such modulations [44]. The disadvantage is that the natural intracellular environment tends to be more viscous than that of the *in vitro* environment resulting in reduced sensitivity of NMR measurements which often limits the size of the molecule that can be reliably resolved with NMR.

In addition to the investigation of protein-mediated processes occurring within the cell, in-cell NMR has also demonstrated the ability to examine membrane transport of small molecules [46]. Since NMR can, in principle, detect any molecule containing 1H, it permits investigation of most organic small molecules eliminating difficulties related to common laboratory techniques such as use of radiolabeled substrates or transport currents. Such utility has been demonstrated for detecting transport of intracellular substrates in Xenopus oocytes [46].

To maximize sensitivity for given hardware limitations (e.g. probe size), in-cell NMR has primarily been applied to slurries of multiple cells by increasing the concentration of the molecule of interest and the filling factor of the probe. NMR measurements in individual eukaryotic cells have been primarily limited to larger cells (e.g. aforementioned Xenopus oocytes) with diameters of 1–2 mm [46]. For future extension of these methods to smaller mammalian cell types, it stands to reason that smaller diameter coils will be required as cell volume and thus molecular concentration decrease relative to the coil volume. Coils with diameters on the sub-millimeter scale are readily attainable through nanofabrication techniques and have been demonstrated in our lab [47] potentially permitting the application of these techniques to smaller (e.g. 20 μm diameter) human cells. Other improvements in sensitivity have been achieved using cryogenically cooled RF probes which have demonstrated increases in sensitivity up to a factor of 3 [48], which may be further considered for single cell studies. Moving forward, the ability to examine single cells by NMR will be important for improving our sensitivity to structural and functional characteristics that are likely to be heterogeneous between cells, such as in senescent or diseased cells.

Although this review is primarily focused on spectroscopic approaches it is worth brief mention of additional spatially resolved biophysical information that can be obtained through NMR imaging (MRI). The possibilities include intracellular structural information [49], maps of water content and mobility [50], water diffusion properties [51], membrane permeability [52], and lipid-specific spin-density maps [51]. Again, due to past and current hardware limitations, single-cell studies have been performed only with the Xenopus oocytes, due to their relatively large diameter. Future combination of both spectroscopic and imaging modalities in single cells has the potential for better understanding the relationship between intracellular environmental factors and cellular function.

Atomic Force Microscopy (AFM)

Atomic force microscopy (AFM) was invented by Binnig *et al*. [53] in 1986, and has since become a powerful tool for nanoscale imaging and force spectroscopy in numerous applications (Figure 2b). The first generation of AFM operated in the *contact mode*, wherein the tip of the AFM probe physically contacted the sample. In this mode, high scanning speeds can be achieved with a nearly constant cantilever deflection maintained by an electronic feedback loop as the AFM scans across the sample surface. Although time-efficient, this mode is not amenable to the study of many fragile (e.g. low stiffness) samples due to significant shear forces created by the AFM tip that can lead to irreversible damage of the sample. The *dynamic mode* AFM was a next generation and transformative technique [54, 55], wherein the AFM cantilever is vibrated by an external source so the AFM tip interacts intermittently with the sample. The amplitude, phase, or frequency shifts of tip oscillation may be used as a basis to extract information about surface properties or topography [56]. Since lateral forces are typically negligible when applied to the sample in dynamic AFM, the tip is less likely to detach samples that are weakly bonded to the substrate surface, which makes it especially useful for studying biological samples in liquids. Liquid dynamic AFM opens the door of studying biological samples *in situ*, including, for example, a topography map of a live neuronal growth cone from Aplysia imaged in L15 cell culture medium (Invitrogen/GIBCO, Carlsbad, CA) (Figure 4a).

Besides an imaging technique, AFM has also been used as a tool for force spectroscopy to measure the interactions between the tip and sample with piconewton resolution. In a quasi-static force spectroscopy measurement, the AFM cantilever is brought vertically down to the sample surface and then in the opposite direction; a plot of cantilever deflection as a function of the vertical displacement of the piezoscanner (towards or away from the sample surface) is then recorded (as shown in Figure 4b). This is called a force-displacement curve or F–Z curve. Applications of F–Z curve include nano-indentation, adhesion, molecular interactions and lithography. The basic principles of this methodology with relevant examples are well described in [57]. If an array of F–Z curves is collected over the entire scan area, a force volume mode image is generated; force volume imaging provides a map of the sample's material properties simultaneously with the topography map. For an oscillating cantilever, the tip-sample force can affect the amplitude, phase, or frequency of the cantilever oscillation; dependence of these characteristics upon tip-sample distance can provide useful information about the local material properties or specific molecular interactions, which are also regarded as spectroscopic data.

AFM has been widely used in cell imaging since it can work in cell culture medium without the need of cell fixing or staining, and it provides higher spatial resolution than optical microscopy. For example, AFM was used to visualize the movement of stress fibers within live cells in a time-lapse series [58]. *In vitro* high-resolution AFM imaging revealed the germination-induced changes in spore coat topography and structure of Bacillus atrophaeus spores [59]. A recent study used a high-speed AFM to image the molecular dynamics of live magnetotactic bacterial cell surfaces at sub-molecular resolution [60]. Although high

resolution and high speed have been achieved in imaging relatively stiff bacterial cells, imaging live mammalian cells is still a challenge because they are much larger (resulting in low spatial resolution for a given acquisition matrix) and softer (resulting in a slow change in force-gradient responses, feedback control and scan speed).

A significant advantage of AFM over the optical microscope is that AFM provides information on the nano-scale mechanical and chemical properties of sample surface [61, 62]. Those properties play important roles in cellular process such as morphogenesis [63], metastasis [64], focal adhesion [65], mechano-transduction [66] and drug delivery [67].

The AFM cantilever can be used as an indenter to probe surface elasticity. By fitting the force-indentation curves to a contact mechanics model such as Hertz [68], Johnson-Kendall-Roberts (JKR) [69], or Derjaguin-Müller-Toporov (DMT) [70–72], the elastic modulus can be extracted. Techniques based on the quasi-static force spectroscopy (F-Z curve) have been used to map the elastic properties of the cell surface [73–76], however they lack the spatial and temporal resolution for capturing the dynamic processes such as diffusion processes or active transport with relevant details. A recent technique was described based on dynamic force spectroscopy to map quantitatively the stiffness and viscoelastic dissipation of live cells, which is ~10–1,000 times faster than the quasi-static AFM techniques [77].

Chemical force microscopy (CFM) is an imaging technique that uses a functionalized tip to probe chemical groups and their interactions at nanoscale resolution [78]. For example, CFM was used to map the hydrophobicity of Aspergillus fumigatus with nanoscale resolution [79]; a follow-up study shows surface hydrophobicity was lower after germination [80]. CFM is based on quasi-static force spectroscopy so it has relatively low scan rate.

A quantitative knowledge of cell adhesion is important to analyze processes in biology including development [81], cellular signaling and wound healing [82], metastasis [83], and infection [84]. AFM-based single cell force spectroscopy (SCFM) has been developed to quantify adhesion of cells to each other, wherein a cell is attached to an AFM cantilever and then positioned to interact with another cell on the substrate at a given location with help of optical microscopy. The interaction forces can be measured by the cantilever deflection. Many receptor-ligand interactions have been studied by SCFM and the results are summarized in [85]. Limitations of SCFM include long acquisition time, thermal drift, high cost associated and difficulties in data interpretation.

Although powerful and versatile, there are still several technical challenges in AFM imaging and force spectroscopy of single cells. One important concern is fast scan speed. On one hand, live cells are often characterized by transient dynamic processes; on the other hand, the response of live cells, once out of an incubator or tissue, varies with a limited lifetime. Currently, AFM imaging of soft mammalian cell usually takes more than 10 minutes, which is insufficient to capture many transient dynamic processes, and which also limits the number of images acquired per sample. Another challenge is the load force control. Live cells are extremely soft with relatively low Young’s modulus values (i.e. on the order of tens of kPa [86]), and even force applications in the sub-nanonewton will cause significant deformation or act as a mechanical stimulus for biological activity in the cell. Ideally, a non-contact scan is desired to minimize loading of live cells. Moreover, quantitative material property measurements are limited by both the experimental techniques (like the cantilever stiffness and tip radius calibration) and theoretical modeling (for example, the commonly used Hertz contact model fails when the tip starts to ‘feel’ the hard substrate).

Finally there is rising interest to combine AFM with other techniques to obtain complementary information on structure and composition of materials and molecules. The

main development is to integrate optical microscopes to AFM [87]; for example, combined AFM and epifluorescence microscopy was used to correlate cellular stiffness with membrane potentials [88]. AFM and Raman spectroscopy is another combined technique; the interface of an AFM with a Raman spectrometer has been demonstrated to have improved sensitivity, selectivity, and spatial resolution over a conventional Raman microscope [89].

Fluorescence Microscopy and Spectroscopy

Fluorescence microscopy is a well-established method in life sciences used for the study of molecules or proteins that emit photons of specific energy (fluorophores) as a response to the excitation of a particular wavelength (Figure 2c). Fluorophores can already reside (or enter by diffusion) in the region of interest (exhibiting autofluorescence) or, most commonly, are manually inserted (example shown in Figure 5). Fluorescence microscopy is a diverse field, encompassing many techniques that provide unique biological and functional information of the cells of interest. Imaging modalities include conventional fluorescence microscopy and emerging super resolution techniques, while spectroscopy methods include fluorescence correlation and hyperspectral methods.

Recent advancements, notably the reduction of background noise and the improvement of imaging resolution, have enabled the detection and observation of single molecules in individual cells. The optical diffraction limits of conventional microscopy have been exceeded by super-resolution techniques that allow imaging of features smaller than 200 nm. Unfortunately, these techniques currently rely on costly imaging hardware and are often impeded by the temporal lag of storing and processing large data, with the exception of some recent "smart" optimization methods (e.g., [90]).

The development, however, of new techniques has not lost its momentum over the last years, with the advances of semiconductor technologies acting as a catalyst with faster and more sensitive cameras, solid state drives for storage, and stronger, power-efficient illumination sources. Furthermore, a strong foundation has been set with a number of commercially available tools, including structured illumination spectroscopy (SIM), stimulated emission depletion microscopy (STED), photoactivated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM), and total internal reflection microscopy (TIRFM), among others. Additional information on super-microscopy methods is provided in a comprehensive review by Toomre and Bewersdorf [91].

In structured illumination microscopy (SIM) a stripe pattern between the objective and the sample is employed in order to differentiate between the fluorophores. The stripe pattern shifts and rotates while frames are acquired, so that signal levels become more distinguishable in the unmasked parts of the image. The resulting resolution is at ~115 nm [92], while, a variation of this technique, the saturated structured-illumination microscopy (SSIM) has exhibited resolution of < 50 nm on a bead sample [93]. Increase of resolution due to saturation in SSIM bears, however, the danger of photodamage.

In stimulated emission depletion microscopy (STED) [94] the active area of fluorescence emission in a particular spectrum (e.g., 650–730 nm) is confined by a doughnut-shaped laser beam that follows the excitation beam and forces emission of higher wavelength photons from the surrounding fluorophores. A point-spread function of 5.8 nm [95] has been achieved, but with a large required laser power of 8.6 GW cm^{-2}.

Two techniques that use switching but in a random manner until deactivation due to bleaching takes place are the stochastic optical reconstruction microscopy (STORM) [96] and the photoactivated localization microscopy (PALM) [25]. Applied in three-dimensions,

sub-20-nm spatial resolution was achieved in the reconstruction of protein organization through interferometric PALM (iPALM) [97], and mitochondrial morphologies as well as mitochondria-microtubule contacts were resolved with 20–30 nm lateral and 60–70 nm axial resolution through whole-cell 3D STORM [98].

A near-field microscopy technique that is not limited by the diffraction laws is the total internal reflection microscopy (TIRFM). This technique exploits the evanescent field that is produced at an interface of two media with different refractive indexes. It can achieve resolution of 30–100 nm [99]. An inherit issue of this technique, is that it can only study emission at (or close to) the interface [100], therefore it cannot be used for imaging inside the cell.

Two associated techniques for the analysis of intercellular interactions are the Förster (or fluorescence) resonance energy transfer (FRET) and the fluorescence lifetime imaging microscopy (FLIM). In FRET, the transition of an externally excited fluorophore (donor) provides the energy to an acceptor fluorophore whose emission decay is collected. In order for FRET to take place, the emission spectrum of the donor should be larger than the absorption spectrum of the acceptor, their separation should be less than 10 nm, and the orientation of the relative transition moments should be appropriate [101]. A method of estimating FRET is through the observation of the fluorescence lifetime of the donor. FLIM can be employed to provide the difference of the donor lifetime in the presence of an acceptor and therefore estimate the FRET efficiency. In live cell environment, FRET biosensors can be employed for the study of mechanotransduction through a pair of fluorophores that are connected either to the same domain or to separate domains that may interact with each other. An in-depth review of these methods and their application in live cell imaging through fluorescence proteins is given by Wang, Shyy, and Chien in [102]. The disadvantage of using FRET/FLIM is the requirement for careful experimental set-up, since factors such as donor-acceptor orientation and stoichiometry reflect on the parameters that define the energy transfer and therefore demand complex mathematical modeling for the measurement interpretation.

Even though the aforementioned techniques provide the means for the observation and understanding of biological events, a more direct, quantitative measurement of the interactions within live cells is required. A trivial way of calculating the number of fluorescent probes and their diffusion time, is provided by the fluorescence correlation spectroscopy (FCS; [103]). This method measures the fluorescence intensity as a function of time in a confocal volume. The autocorrelation function is then applied to the spectrum in order to extract diffusion time, number and concentration of the molecules in the particular volume. Binding relationships between two or more fluorophores in the confocal volume can also be extracted through the correlation of their spectra (fluorescence cross-correlation spectroscopy). A limiting factor on the resolution of these methods is the volume size of the confocal element. It is however apparent, that they can be combined with some of the super-resolution microscopy techniques, in order to miniaturize the observed voxel.

An additional enhancement to fluorescence microscopy is achieved through the use of hyperspectral imaging (HSI) [104, 105]. The addition of a dispersing element enables the collection of full spectral information for each scanned pixel as opposed to the typical bandpass filtering which allows only a narrow, fluorochrome-specific band of frequencies to be collected. Precise mapping and distinction of fluorochromes is therefore possible, allowing for the analysis of areas where significant overlapping is present. Hyperspectral imaging has aided in distinguishing normal, precancerous, and cancerous cells [106], determining the cell cycle status of live cells *in vitro* [107], and also in molecular profiling of individual tumor cells [108]. A large number of fluorophores can be identified; e.g., in the

latter study, 10 different tumor marker-specific antibodies were labeled to provide a distinctive cellular profile. Apart from the need of a high-resolution CCD (charge-coupled device) camera, the motorized stage and the imaging spectrograph, an additional important requirement of HSI is the large computational power and storage space.

A technique that uses fluorescence, scattered light, as well as spectroscopy in order to differentiate and perform analysis of micron-level sized particles is flow cytometry (FC). A typical FC system is comprised by a microfluidic network that ensures single cell (or particle) pass through a chamber exposed to one or more laser diodes and a number of optical paths to collect the fluorescence emissions, the spectra, and the scattered light. FC can only analyze suspended cells, which is its main disadvantage. However, it can be coupled with a variety of fluorescence and spectroscopy imaging techniques (e.g., FRET [109] and hyperspectral imaging [110]) providing a powerful tool, especially with the potential for high-throughput analysis [111]. For more information on the important field of flow cytometry, which is beyond the scope of the present review, the reader is referred to the reviews by Robinson [111, 112].

Research on the development of image processing techniques for commercial fluorescence microscopes has enabled 3D image reconstruction and opened the way for high-content analysis of single cells. Initially, using widefield microscopy, distinction based on phenotype became possible [113]. Later on, the employment of confocal microscopy techniques enabled high-resolution 3D reconstruction of subcellular structures and allowed the study of the subcellular reorganization of the nucleus, the Golgi apparatus, and centrioles [114]. The image processing framework is laid out comprehensively in [114] and the main issues and practical solutions for successful high-content cell screening are discussed.

While the advancements in fluorescence microscopy and spectroscopy have been very promising, there are concerns when these methods are used towards single cell analysis. First, it needs to be ensured that the experimental methods will not cause a disruption in the cellular physiology (e.g. through overexpression), and that the formed protein fusion vector will perform the expected cellular function. In addition, the increase of light intensities for saturation (e.g. SSIM) or photobleaching (e.g. in STED) bears the danger of phototoxicity that can damage the subcellular components. Since this can be caused from the emission of fluorophore molecules as well, they need to be chosen such that they produce long wavelength excitations.

Raman Spectroscopy

Raman spectroscopy is capable of acquiring spectroscopic signatures of cell constituents through Raman scattering effects that occur when a photon (typically from a laser source) promotes a molecule from a low vibrational state, i.e. the ground state, to a higher, virtual state (Figure 2d). Since a shift in electronic states does not take place, the system cannot reside at the virtual state and therefore returns to one of the low vibrational states. The energy difference between the initial and the final vibrational state is expressed through the wavelength of the emitted Raman photon, mainly due to the Stokes scattering radiation.

Raman spectroscopy can be applied to aqueous solutions since water does not cause interferences, in contrast to infrared spectroscopy. Moreover, labeling of the molecules is not required as is the case in fluorescence microscopy, with the emitted spectra relying exclusively on the biochemical composition of the observed voxel. This also underlines an important advantage, which is the ability of *in vivo* studies of single cells without the need for introduction of dyes. Furthermore, the volumetric resolution is similar to fluorescent microscopy, since the excitation and acquisition of the scattered light occurs in similar fashion. Interestingly though, fluorescence expression can also be recorded in Raman

spectrometers enabling studies like the fluorescent *in situ* hybridization while acquiring Raman spectra (Raman-FISH), which ca*n* improve the understanding of subcellular processes and functions by providing both phenotypic (Raman) and phylogenetic (FISH) information [115, 116].

Two important impediments hamper the direct application of Raman spectroscopy for the study of single cells. First, since Raman scattering is a weak effect, a long acquisition time is required in order to achieve a high signal-to-noise ratio. Secondly, the variance between the acquired spectra that typically contain a large number of bands requires statistical analyses and the comparison to a database of standard signatures of compounds, such as nucleic acids and proteins [20, 33]. Commonly used methods for the analysis of acquired spectra are the principal component [35], discriminant function [34], and hierarchical cluster [34] analyses.

An improvement of the power of emitted Raman signal is achieved through the excitation at frequencies close to the region of the electronic absorption bands of certain molecules. This technique is called resonance Raman spectroscopy [17] and can lead to six orders of magnitude increase in the signal-to-noise ratio. Selective spectrum enhancement can be provided by adjusting the excitation wavelength, which is the case in UV-resonance Raman spectroscopy (UVRR). The range of 200–260 nm of the UV excitation results in signals mainly from nucleic and aromatic amino acids. Another advantage of using this excitation spectrum is that the background signal due to the native cellular fluorescence is eliminated since it is instigated at higher excitation wavelengths [117]. The application of UVRR for single cell spectroscopy was first reported by Sureau et al. in 1990 [118].

An additional method that increases the sensitivity (up to 14 orders of magnitude) is the surface-enhanced Raman spectroscopy (SERS). This technique employs the plasmon resonance emitted from SERS-active substrates (e.g., AgNP-decorated silicon substrate; [119]) where the cells are cultured with metal nanoparticles, referred to as SERS tags (e.g. red-resonant gold nanorods; [27], and gold colloids; Figure 6), that are adsorbed to the Raman-active tag of interest. However, the preparation burden of the active substrate or the nanoparticles raises practical concerns especially since the effect of these on the normal biological function of the cells is not fully understood.

Among the numerous spin-off technologies based on the Raman effect is the coherent anti-Stokes Raman spectroscopy (CARS). With CARS, the tuned, mixed frequency of a laser pump and the Stokes scattering matches the Raman vibration frequency of the molecule, leading to an amplified anti-Stokes signal. This method has been exploited for the study of the transport and motility of lipid droplets in living cells [120], proving its value as a noninvasive tool for the study of dynamic processes in single cells. CARS also provides the capability of 3D imaging [121]. An excellent report on the method is given by Evans and Xie [121].

It is apparent that Raman spectroscopy, especially through its developments, should be among the favorable technologies for single cell imaging. The required peripheral tools and postprocessing for the amplification and assessment of the scattering signal introduce the limiting factors of cost and time, but nevertheless, the non-invasive observation and quantification capabilities render this method very useful for biochemical studies.

ON THE HORIZON

Major improvements in single cell analysis have been noted in techniques that rely on complex instrumentation and computationally-heavy post-processing, noting especially phenomenal solid-state and nanotechnology advancements during the last several years in optics-related tools (lasers, cameras, and detectors) and sensing and processing hardware

(spectrometers, processors, hard drives). Improvements of nanotechnology fabrication techniques have also led to more sensitive and precise instrumentation, for example, AFM cantilevers designed for high-speed dynamic mode imaging [122]. Another noticeable example is NMR, where nanofabrication techniques have produced probes that permit measurements on single cells rather than cell suspensions or slurries commonly used with more standard probes.

While methods of spectroscopy each provide unique information about the structure and function of single cells (as shown in Table 1), combined methods enable a new array of information that is not achievable from any individual method alone. For example, coupling of Raman spectroscopy and fluorescence microscopy has enabled Raman-FISH simultaneous studies [115]. More recently, we fabricated micro radiofrequency coils for NMR sensing on the tips of AFM cantilevers for the combined acquisition of biophysical and biochemical signals at the cellular scale [47]. Because the spectral data offered by different physical detection processes and acquisition modalities may often be complementary, the combination of these data are expected to yield new insight into the structure and function of cells that represent a variety of physiological states, from disease and damage to regeneration and healing.

CONCLUSION

Through this review, we presented several of the most prominent intercellular techniques used to unveil the internal mechanisms and their correlation to biophysical signatures with a focus on single-cell analysis. The discussion of recent achievements, in addition to the disclosure of specific limitations, provide not only a guide for the researcher to select the most appropriate tool but also help overcome challenges required for qualitative and quantitative measurements in single cells from heterogeneous populations. A natural direction is to combine the features of established technologies through novel hybrid tools that can overcome individual restrictions (e.g., through the abovementioned merging of NMR and AFM techniques) and provide more versatile and sensitive tools for examination of biological phenomena, therapeutic development, and rapid diagnoses.

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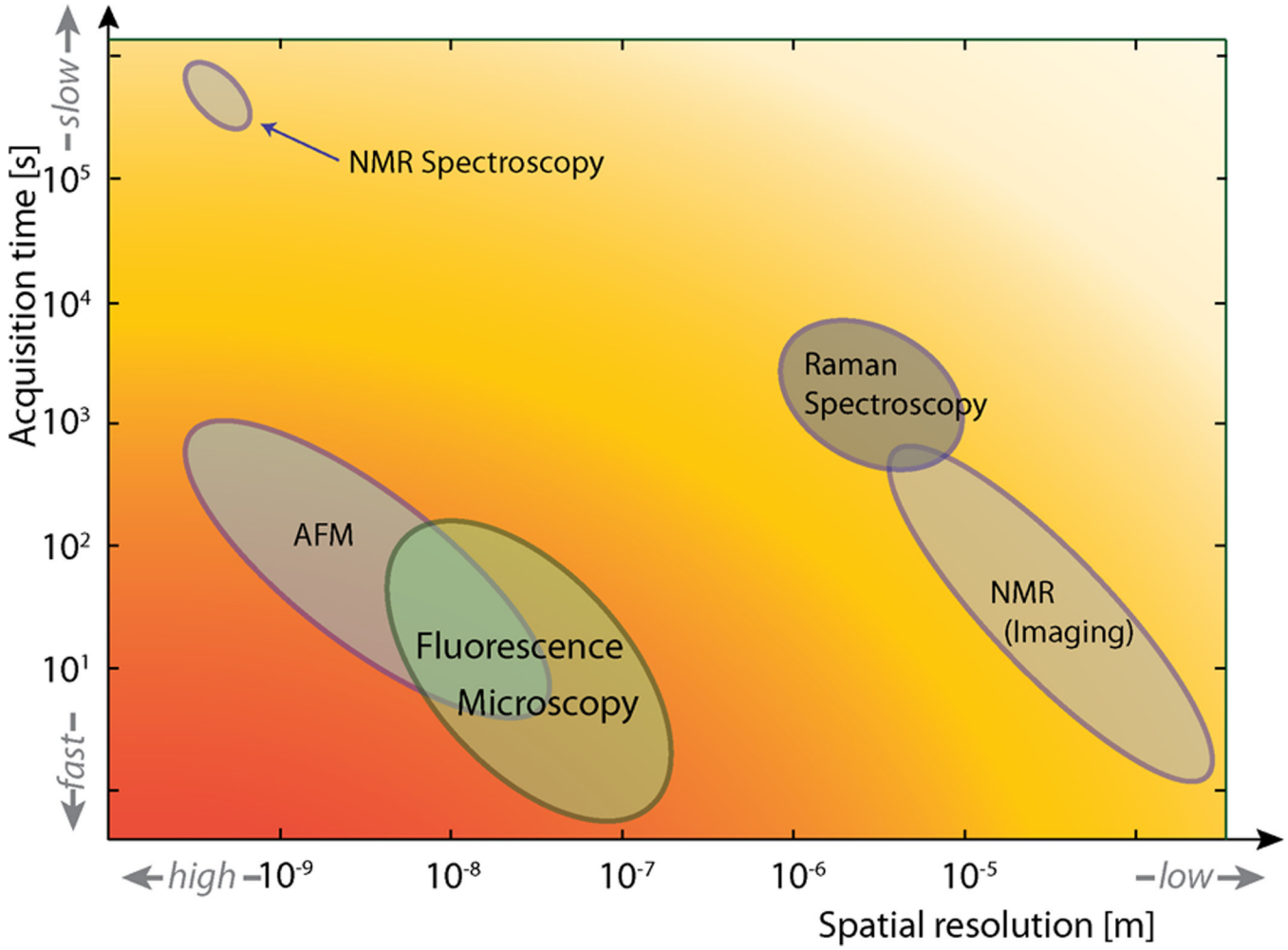

Figure 1. Spectroscopy and imaging methods span distinct orders of magnitude in spatial resolution and acquisition time

For nuclear magnetic resonance (NMR), atomic force microscopy (AFM), fluorescence microscopy, and Raman spectroscopy, Improvements in spatial resolution often come at the cost of longer data acquisition time. The gradient fades to a lighter color as spatial resolution decreases to levels that cannot resolve in-cell features and acquisition times that are too long to capture transient effects or rapid dynamic processes.

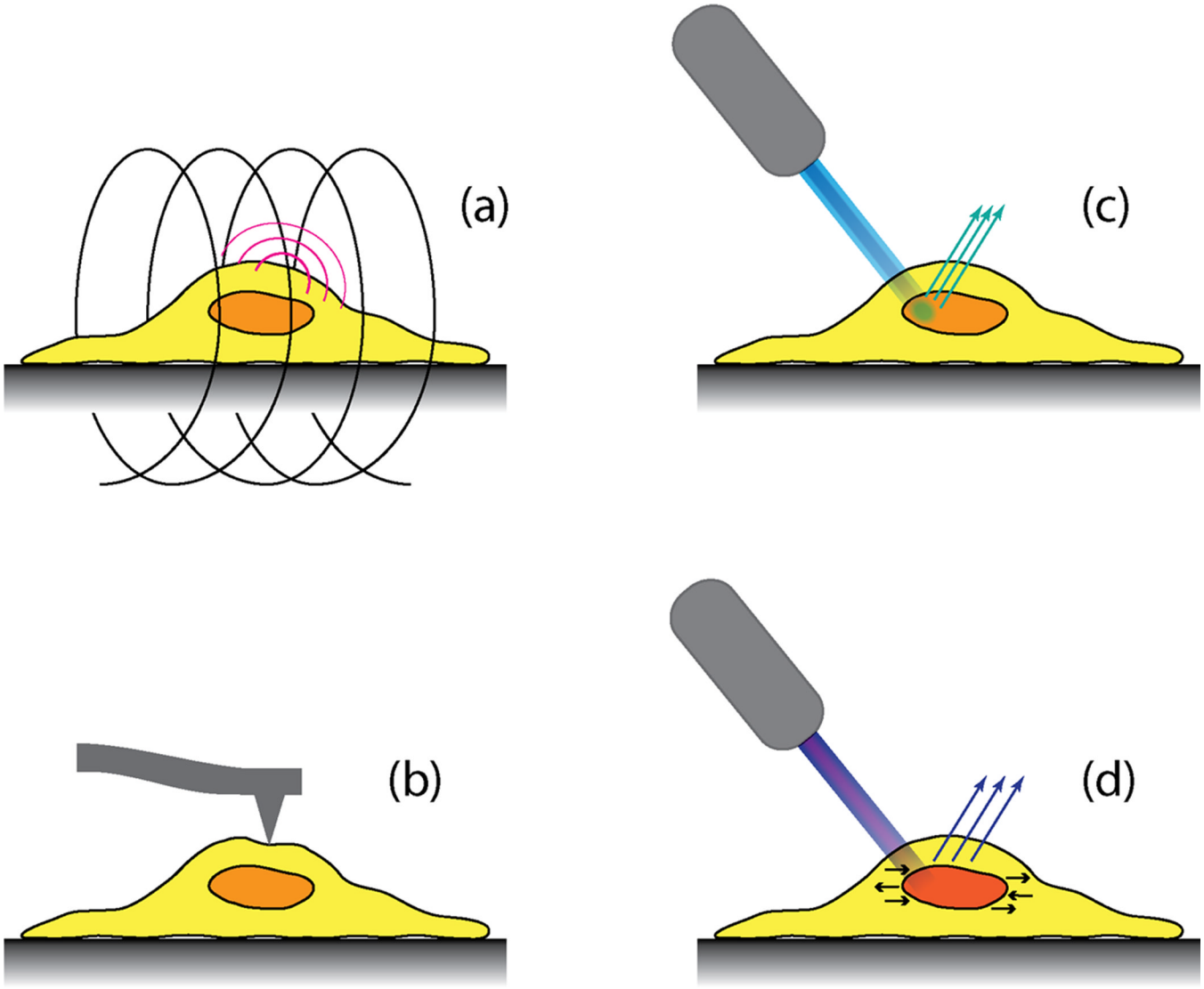

Figure 2. Spectroscopy and imaging methods characterize the structure and function of single cells

Quantitative measurements of cellular substructures and dynamic processes are attained by fundamentally different detection mechanisms. In particular, a) nuclear magnetic resonance which takes advantage of electromagnetic radiation absorption and reemission in the presence of a magnetic field, b) atomic force microscopy, where a cantilever deflection is used to study topography and biophysical properties, c) fluorescence microscopy and spectroscopy that exploits light-matter interactions, and d) Raman spectroscopy where information is extracted through the monitoring of vibrational energy transitions.

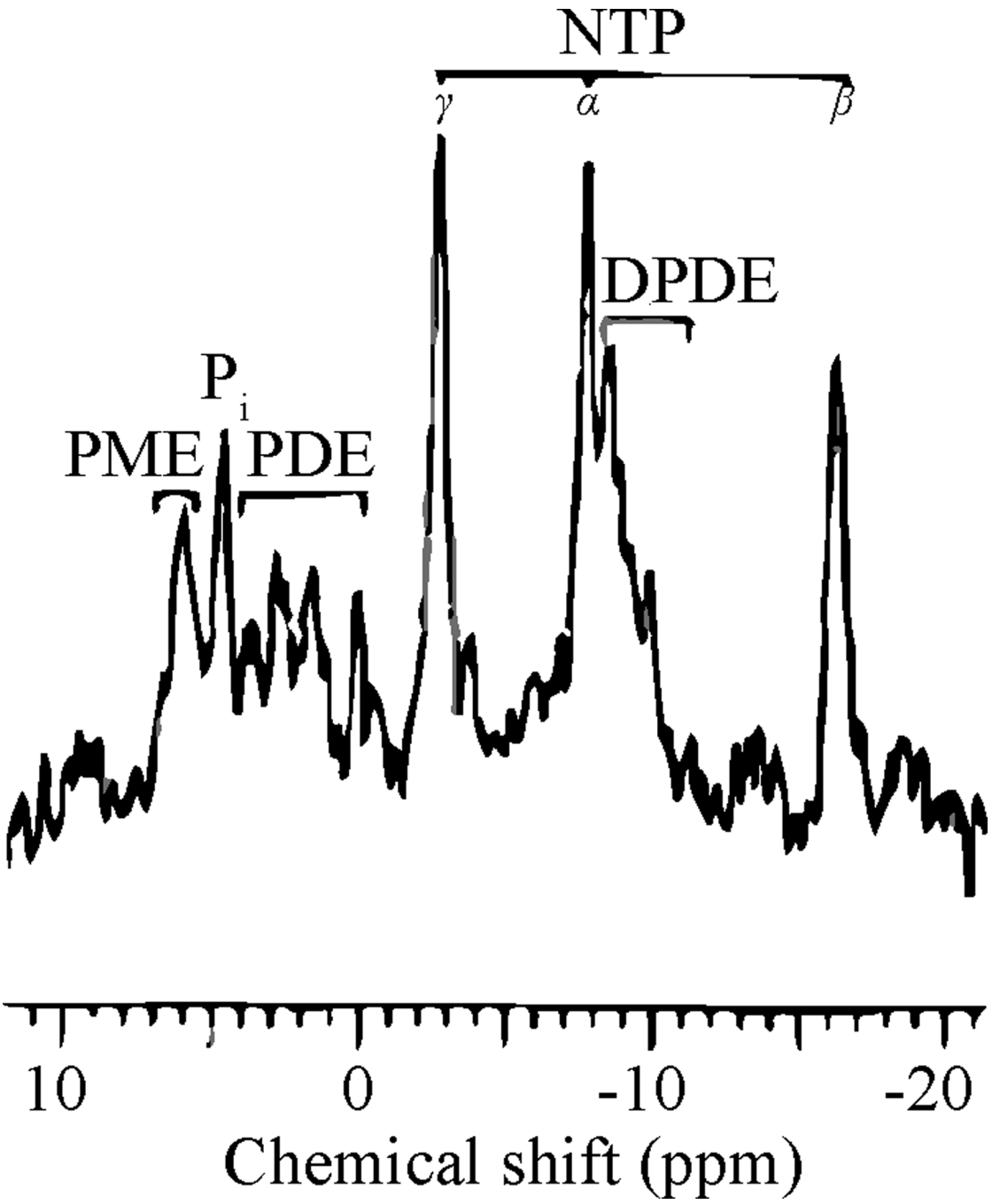

Figure 3. NMR spectroscopy produces a spectrum of resonances whose positions correspond to the local molecular environment

Fourier transformed 31P spectrum obtained from a superfused horse articular sample. Labeled peaks belong to: phosphomonoesters (PME), inorganic phosphate (P_i), phosphodiesters (PDE), nucleoside triphosphates (NTP), and diphosphodiesters (DPDE). Reprinted from [132], with permission from Elsevier.

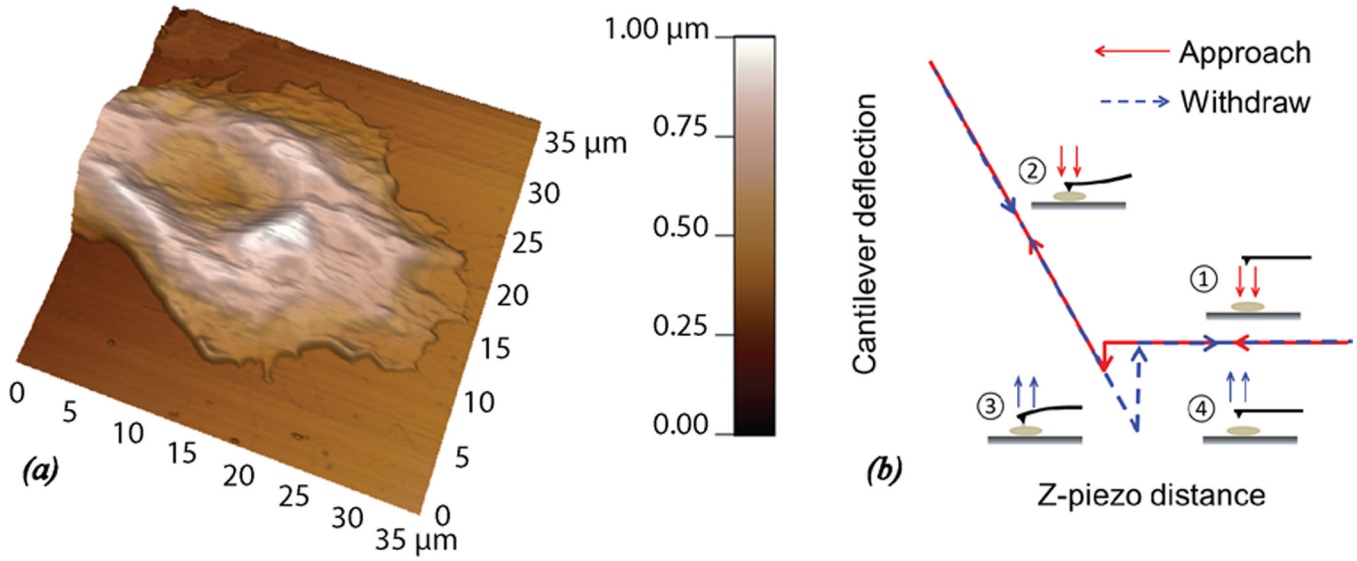

Figure 4. Atomic force microscopy maps topography and probes material properties of live cells

(a) Dynamic mode AFM topography image of a live neuronal growth cone from Aplysia Image field of view = 35 × 35 um, matrix size = 256 × 256 pixels. (b) Schematic diagram of a quasi-static force spectroscopy measurement (F-Z curves) on a cell.

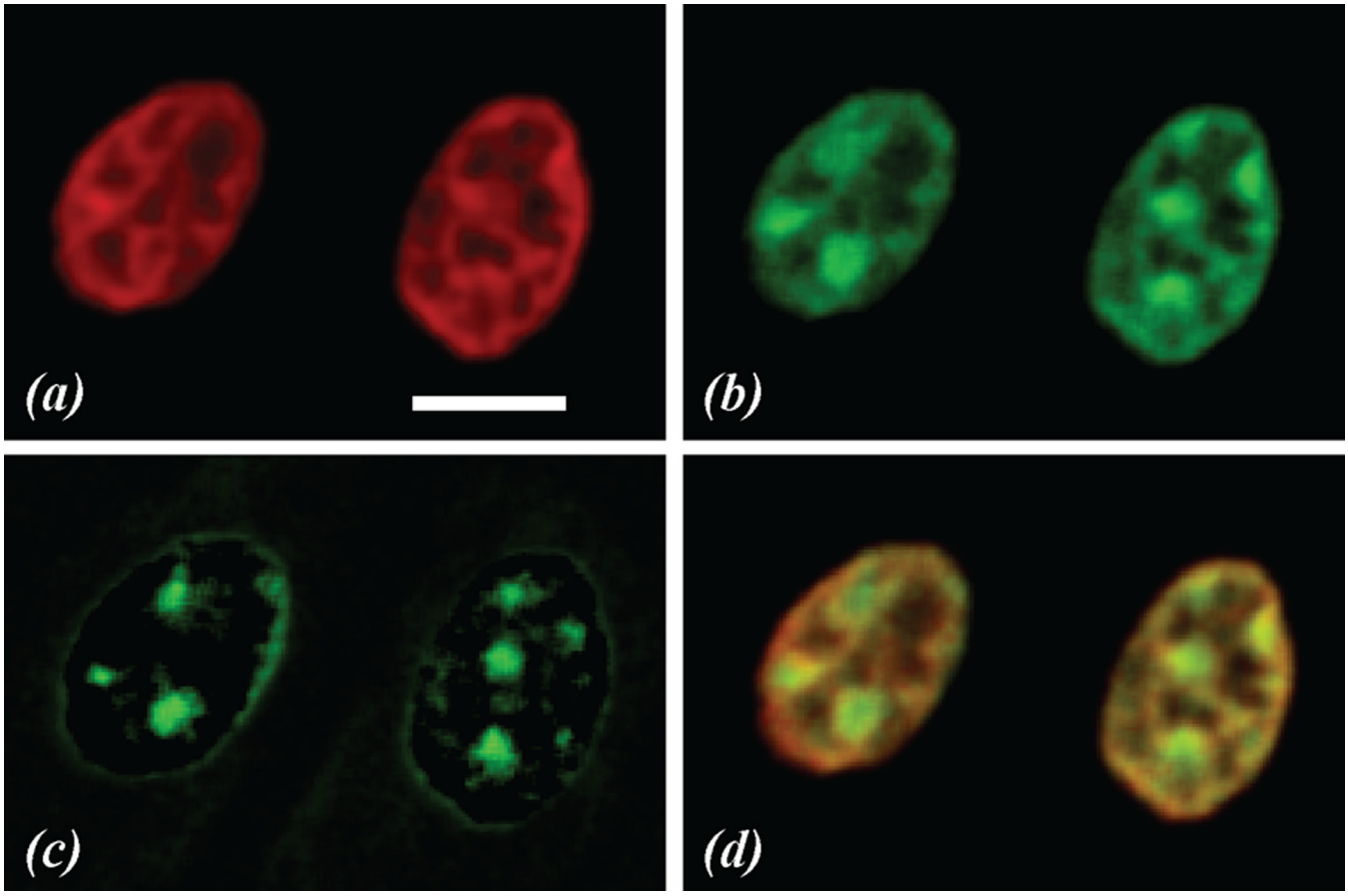

Figure 5. Fluorescence microscopy visualizes detailed and submicron cellular structures

Representative nuclei are shown in two adjacent cartilage cells (chondrocytes) maintained in their native three-dimensional extracellular matrix. Nuclei were visualized using multiphoton fluorescence microscopy (40× objective; numerical aperture = 0.95; spatial resolution = 0.12 μm/pixel) with a Mai Tai DeepSee tunable IR laser (700–1000 nm) for detection of fluorescent dies DAPI (*a*) and SYTO® RNASelect™ (*b*; Invitrogen). The nuclear components DNA (*a*; red) and RNA (*b*; green) are shown in false colors to aide in contrast, noting that difference (*c*) and merged *(d)* images reveal regions of colocalization. Scale bar = 5 μm. While submicron structures can be identified in single living cells, as depicted in the figure, the most recent advances include descriptions of protein organization and mitochondrial cross-sections [97, 98].

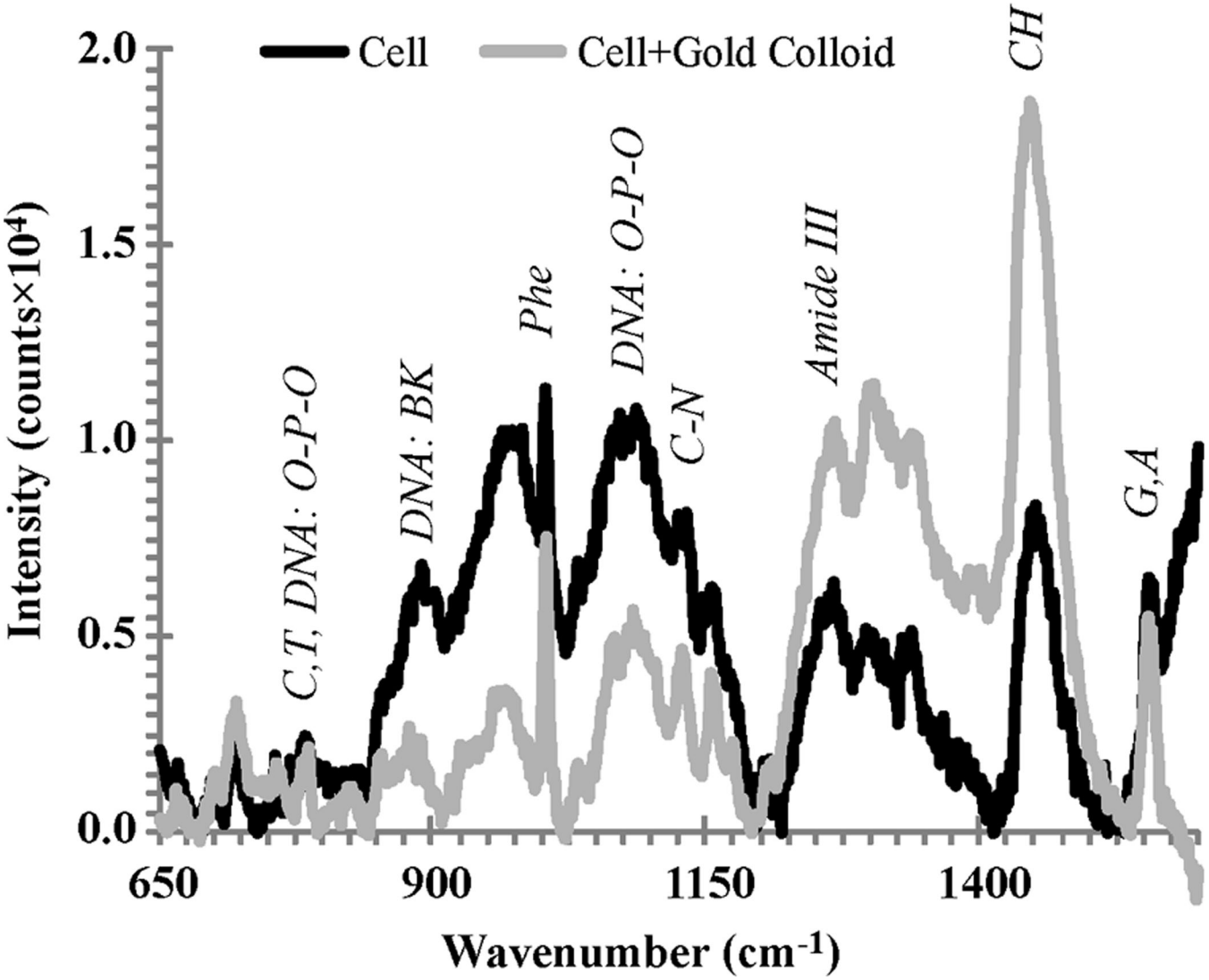

Figure 6. Raman spectra reveal characteristic peaks of molecular constituents and structures in single living cells

Spectral information includes contributions from various cell compartments, including cytoplasm and nucleus, in addition to DNA [133], with signal enhancements aided through the addition of gold colloid (10 nm diameter; Sigma; 20% final concentration). In this representative study, a 660 nm laser was focused on a single cell (tenocyte) from a micromass culture (density = 80,000 cells / 10 μL) plated on the surface of a glass slide. The spectrum was collected with a 50× objective, 600 g/mm grating, 10 minute acquisition, and wave number range from 650 cm^{-1} to 1600 cm^{-1}While constituents and structures can be identified in single living cells, as shown, recent advances include studies of lipid metabolism and tissue structure [120, 121].

Table 1

Spectroscopy methods provide unique and complimentary measures of single cell structure and function

Characteristic requirements and applications are provided for specific techniques, in addition to their latest resolution limits.

Modality		Detection	Technique	Resolution	Special requirements	Suggested for; application example(s)
NMR	Spectroscopy	net nuclear magnetic moment	HSQC/HMQC	50 μM intracellular concentration/0.5±0.007 nm	labeling (15N, 13C), heteronuclear RF probe	live cell suspensions; protein structure [123], modification (e.g. phosphorylation [45], protein-protein interaction [124], and acetylation [125]) and solute transport [46])
	Imaging	net nuclear magnetic moment	e.g. spin echo and gradient echo	3–4 μm^3	magnetic field gradients	live cells; mapping of biophysical properties (e.g. water relaxation [50] and diffusion [51]), lipid mapping [51])
AFM	Spectroscopy	viscoelasticity, adhesion	F-Z curve, force volume	sub-10nm	long acquisition time	live cells; human platelets [73], lactococcus lactis [126]
	Microscopy	friction	contact	sub-10nm	loading force control	fixed cell; cartilage [127]
		viscoelasticity	dynamic	sub-10nm	complex data interpretation	live cells; E. coli, fibroblasts, red blood cells [77]
Fluorescence	Spectroscopy	intensity colleration in confocal voxel	FCS	limited by confocal microscope optics	complex instrumentation	live cell imaging; protein-DNA interactions [103, 128]
	Microscopy	fluorophore (FP) molecules	SSIM 3D	100 nm (lateral) < 300 nm (axial)	diffraction grating	slow acquisition time; synaptonemal complex [129]
		nitrogen-vacancy defects	STED	≈6 nm	high illumination intensity	solid-state fluorophores; type IIa diamonds [95]
		continuous FP activation and bleaching	iPALM STORM	2 – 25 nm	long acquisition time	sparse FPs in active region; gag protein in HIV-I [25], mitochondria and mitochondrial-microtubule contacts [98]
		evanescent field detection	TIRFM	100 nm	refraction interface	membrane-bound molecules;plasma membrane of mouse myoblastic cell [100]
Raman	Spectroscopy	vibrational resonance	UVRR	5 μm diameter observation area	strong dependence on excitation frequency	nuclear DNA; nucleus of T47D living cell [118]
		plasmon resonance	SERS	10^{14} SNR improvement over stimulated RS	fabrication complexity	multiplexed arrays, cell sorting; cell apoptosis [119], cytometry [27]
		anti-Stokes Raman emission	CARS	diffraction limited	background noise	lipids; lipid biology [130], single lipid vesicles [131], LD trafficking in cells [120]