REVIEW

Imaging methods for elemental, chemical, molecular, and morphological analyses of single cells

Cyril Petibois

Abstract Combining elemental, chemical, molecular, and morphological imaging information from individual cells with a lateral resolution well below $1 \times 1 \ \mu m^2$ is the current technological challenge for investigating the smallest dimensions of living systems. In the race for such analytical performance, several techniques have been successfully developed; some use probes to determine given cellular contents whereas others use possible interactions between cellular matter with light or elements for characterization of contents. Morphological techniques providing information about cell dimensions have, when combined with other techniques, also opened the way to quantitative studies. New analytical opportunities are now being considered in cell biology, combining top-performance imaging techniques, applied to the same biosystem, with microscopy (nm–μm range) techniques providing elemental (micro-X-ray fluorescence, particle-induced X-ray emission, secondary-ion mass spectrometry), chemical (Raman, coherent anti-stokes Raman, Fourier-transform infrared, and near-field), molecular (UV–visible confocal and multiphoton), and morphological (AFM, ellipsometry, X-ray phase contrast, digital holography) information. Dedicated cell-culture methods have been proposed for multimodal imaging in vitro and/or ex vivo. This review shows that in addition to UV–fluorescent techniques, the imaging modalities able to provide interesting information about a cell, with high spatial and time resolution, have grown sufficiently to envisage quantitative analysis of chemical species inside subcellular compartments.

Abbreviations

AFM	Atomic-force microscopy
BAM	Brewster-angle microscopy
CARS	Coherent anti-stokes Raman scattering
CT	Computed tomography
DHM	Digital holography microscopy
ER	Endoplasmic reticulum
FOV	Field of view
FTIR	Fourier-transform infrared
GFP	Green fluorescent protein
MRI	Magnetic resonance imaging
OM	Optical microscopy
PET	Positron-emission microscopy
PIXE	Particle-induced X-ray emission
QDs	Quantum dots
SIMS	Secondary-ion mass spectroscopy
SNOM	Scanning near-field optical microscopy
SPECT	Single-photon emission computed tomography
STED	Stimulated emission depletion
UV-CF	Ultraviolet–visible confocal fluorescence
XR-PC	X-ray phase contrast
XRF	X-ray fluorescence

C. Petibois (✉)
Université Victor Segalen Bordeaux 2, CNRS UMR 5248, CBMN,
2 Rue Robert Escarpit,
33600 Pessac-Cedex, France
e-mail: c.petibois@cbmn.u-bordeaux.fr

Introduction

Cell biology is a fundamental field for investigating a pathological process, where any damage or behavioral change can be measured with accuracy, but also for determining the effectiveness of a return to the healthy condition. It is broadly assumed that most of the patholog-

ical processes start at the cell level, including carcinogenesis, viral infections, myopathies, leukemia, oxidative stress related diseases, … etc. Biochemical analyses at the cell scale are also mandatory for drugs approval, for determining toxicity of synthetic compounds, biocompatibility of surgical materials … etc. In this context, cell imaging may use a range of microscopy techniques that has been developed with the objective of investigating the basic and phenotypic functions of the smallest dimensions of complex living systems [1]. Considering all the imaging techniques now available, analytical applications on single cells may focus on any process determining its life, i.e., from initial growth to death, and any kind of information has become available, from trace elements to whole morphology. Cell–cell interactions may also be investigated by using imaging to monitor migration, fusion, tissue formation … etc. Some advanced techniques, for example UV–visible confocal fluorescence (UV-CF) microscopy, can even image contents of a single cell within a tissue, thus making sense of their behavior in their normal biological environment. However, the route is usually long from setting up an imaging technique to its routine use by non-specialists in instrumentation, i.e., by the end-users working in cell biology. The analytical techniques setup as microscopy/imaging tools able to reveal cell properties has been widely enlarged during the last two decades and combination of techniques is under way to furnish multimodal imaging systems. Every technique applied to cell imaging is defined by intrinsic limits, for example the lateral resolution achievable (sometimes diffraction-limited), sensitivity (source power), substrate compatibility (refraction index, transmissivity …) for cell cultures, sample presentation requirements … etc. Nevertheless, most of the available imaging techniques provide unique performances compared with the others, thus making their comparison useless out of the context of a biological issue, i.e., an analytical question about a given cell target.

Despite the enormous advances brought about by chemical, elemental, and scanning probe microscopy techniques, most microscopy investigations in cell biology are still carried out with conventional lenses and visible light. Taking advantage of the optical transparency of cells, light microscopy uniquely enables noninvasive imaging of the interior of cells in three dimensions (3D). Moreover, it enables detection of specific cellular constituents, for example proteins, nucleic acids, and lipids, by fluorescence labeling. Lens-based fluorescence microscopy would be almost ideal for investigating life at the subcellular level if it could discern details below 250 nm [2]. However, utilization of light in microscopy is limited by the Rayleigh criterion ($\lambda/2$ or diffraction limit). To overcome this optical limit of lens-based microscopes, technological development has focused on the study of the near field (NF), on collection of the evanescent wave that an illuminated sample produces at its surface. This has helped to bring the resolution well below the diffraction limit, to $\lambda/1000$ in some cases [3]. The consequence is that among the many techniques now available in microscopy, only some have reached the technological development needed to provide both lateral resolution and sensitivity sufficient for analysis of cellular content.

Optical microscopy gives basic 2D information about the morphology of the cell, but X-ray phase-contrast (XR-PC) microscopy may have 3D rendering, thus enhancing this information up to intracellular organization of the cell [4]. Elemental microscopy methods, such as those derived from the X-ray fluorescence (μXRF), particle-induced X-ray emission (μPIXE), and secondary-ion mass spectroscopy (μSIMS), enable mapping of metal ions and other inorganic contents at sub-micron resolution and at trace concentrations. Microscopy techniques based on vibrational spectroscopy, for example Fourier-transform infrared (FTIR), FT-Raman, coherent anti-stokes Raman scattering (CARS), and scanning near-field optical microscopy (SNOM), provide global chemical information about cell organic contents. Quantization of cell properties in respect of their morphology and dimensions is even envisaged at unprecedented lateral resolution (1×1 μm^2 or below) combining chemical and morphological imaging, thus opening the route to cytopathological examination at the same level of precision and accuracy as is currently performed routinely on tissues. Atomic-force microscopy (AFM) or ellipsometry imaging may delimit voxels in a cell volume with resolution in the nm range, thus well below the size of most intracellular organelles (Fig. 1). Furthermore, cell biology has recently started to turn its inherited practical habits to the development of new cell-culture methods dedicated to imaging analysis, thus accelerating the development and applicability of techniques. However, a continuous effort to enhance the performance of sources, detectors, optics, and image data management regularly redefines the limits of microscopy techniques and their analytical performance. The consequence is that the skills and experience required to use high-performance microscopy techniques and their related instrumentation evolve rapidly, inducing the setup of niche scientific communities. In this context, one major application of microscopy techniques, multimodality for increasing the amount of information obtained from a sample, is finally more difficult to organize and promote.

In this review, every major technique is first considered for its analytical performance and the unique information provided for cell biology investigations (Table 1). The basis of future multimodal methods for enhancing our understanding of cell processes using high-performance analytical techniques is then discussed.
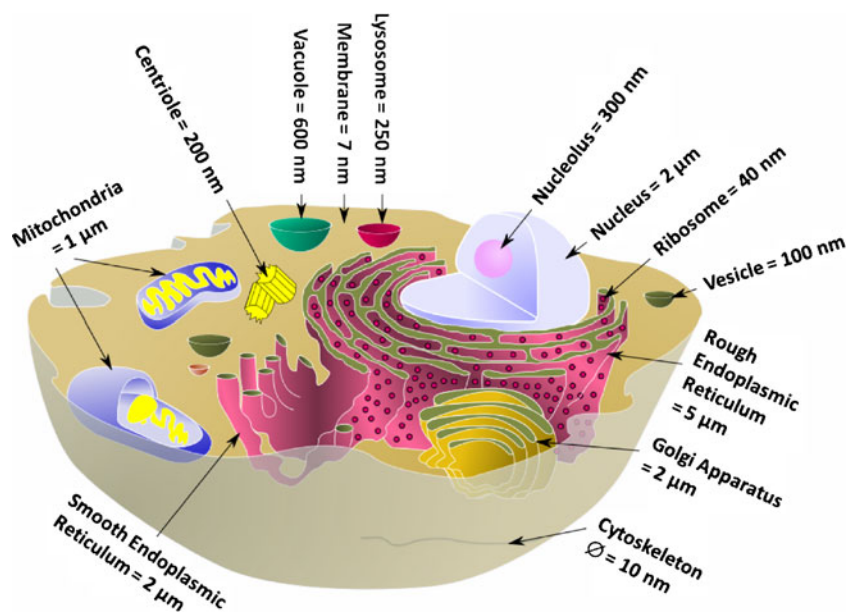
Fig. 1 Diagram of the components of a eukaryote cell with dimensions. The interior of a cell is a gel-like environment with filamentous cytoskeleton maintaining sub-cellular organelles and compartments in a morphological coherence. The cytosol is composed mainly of water and soluble molecules and inorganic elements. The main organelles have dimensions (d) of hundreds of nanometers up to a few microns, and their mobility inside the cell is limited in the short term. Spatial and temporal resolution for imaging a cell organelle requires focusing on it at $d/10$ for acquisition performed in 2D or 3D in less than 20 min in stable cell culture conditions (temp.=28–37 °C, normoxia, glucose=5.5 mmol L^{-1}, pH 7.7)

Expected features of cell-imaging techniques

Imaging techniques are considered for cell investigations once they fulfill some criteria of cell biology, i.e. lateral resolution better than 1×1 μm^2 for a field of view (FOV) $>50 \times 50$ μm^2, and sensitivity to chemical contents on the nmol to mmol scale. With such a lateral resolution, sub-cellular compartments and organelles may be discriminated, thus giving access to intracellular functions [5] (Fig. 1). Over these basic features, utilization of imaging methods for research or routine applications will be considered with regard to difficulty in the sample handling, image acquisition, interpretability (including data treatments), and reproducibility. As an example, electron microscopy-based methods are widely used to obtain images with nanometer-level resolution, but the relatively thick living cell cannot be observed because the sample must be chemically fixed, dehydrated, embedded in resin, then sliced ultra thin. Therefore, such a technique is not suitable for multimodal imaging investigations. For routine use, a technique must also provide high-quality images within a time scale of a few minutes, but also highlight automatically the expected information for immediate interpretation. Automation of post-processing tasks after image acquisition is undoubtedly the major factor limiting routine use of most modern imaging techniques. As an example, where UV-CF imaging is able to highlight the distribution of a given molecule inside a cell, because of the specific labeling of the molecule, spectroscopic techniques usually provide more global information about cell contents that must be treated, sometimes with methods requiring high skills in chemometrics [6] and with potential concerns about interpretability of the extracted results. Successful routine applications of cell-imaging techniques based on UV-CF means using fluorescent probes to target given molecules or green fluorescent protein (GFP). A widely diverse set of fluorescent probes has been developed with photophysical characteristics that are compatible with imaging biological systems. An important addition to this library of fluorescent probes has been the genetically encodable fluorescent proteins. Both fluorescent probes and fluorescent proteins have the enormous advantage of making UV-CF analyses perfectly specific for the targeted molecules [7]. In contrast, one may consider that routine use of such an imaging technique is intrinsically limited to the fixed conditions and thus to a defined understanding of cell properties. As a consequence, these routine techniques are blind to all other cell properties, which may have as much or even more importance to understanding cell behavior. This is possibly one of the major differences between routine and research techniques for cell imaging. Research instrumentation enables widening of the spectrum of cell properties that can be investigated but also requires more skills in different scientific fields. Most modern imaging techniques applied to cells (for

Table 1 Main analytical performances of imaging techniques applied to single cells. All performances have been tested on individual cells (U87 GM grown on different substrates for compatibility with the different techniques). Ex vivo condition are understood here as fixed cells (cryofixed, paraformaldehyde, paraffin …)

Technique	2D/3D	Source	Detection	Sensitivity/precision	Lateral resolution z-axis precision	Acquisition duration	Information
OM	2D	Light	Absorption	NA	500×500 nm NA	ms	In vitro/ex vivo Morphology
AFM	3D	Laser	Forces	1 nm (½Q)	250×250 nm 1 nm	20 min	In vitro/ex vivo Thickness
Ellipsometry	3D	Laser	Ellipsometry	0.2 nm	1×1 μm 0.2 nm	30 s	In vitro/ex vivo Thickness
XR-PC	3D confocal	X-rays SR	Water–carbon contrast	1%	15×15 nm 15 μm	SR=15 min CS=1 h	In vitro/ex vivo Morphology
μXRF	2D	X-rays SR	X-ray fluorescence	0.1 ppm	100×100 nm NA	SR=2 h	In vitro/ex vivo K/L Elements
μPIXE	2D	Electrons	X-ray fluorescence	1 ppm	1×1 μm NA	5 h	In vitro/ex vivo 10–30 K elements
μSIMS[a]	3D confocal	Ions	Secondary ions	nmol L^{-1}–μmol L^{-1}	50×50 nm 500 nm	10 min (2D) 2 h (3D)	In vitro/ex vivo Light elements
FTIR	2D	Globar SR	Absorption	μmol L^{-1}	2.5–20 μm NA	CS=30 min SR=2 min	ex vitro ν/δ Covalent bonds
Raman	3D confocal	Laser	Absorption	mmol L^{-1} (½Q)	250×250 nm 1 μm	20 min (2D) 1 h (3D)	In vitro/ex vivo
CARS	3D confocal	Laser C.Source	Absorption	μmol L^{-1}	100×100 nm 250 nm	1 min (2D) 20 min (3D)	In vitro/ex vivo
SNOM	2D	Laser SR	Scattered light	mmol L^{-1} (½Q)	50×50 nm NA	min–hours	In vitro/ex vivo
UV-CF	3D confocal	Laser	Fluorescence	nmol L^{-1}	250×250 nm 500 nm	s	In vitro/ex vivo Labeled molecules
STED	3D confocal	Laser	Fluorescence	nmol L^{-1}	15×15 nm 50 nm	s	In vitro/ex vivo Labeled molecules
Multiphoton	3D confocal	Laser	Fluorescence	nmol L^{-1}–μmol L^{-1}	250×250 nm $- 1 \times 1$ μm 500 nm $- 1$ μm	s	In vitro/ex vivo Labeled molecules

[a] 3D-μSIMS is regarded as confocal-like while eroding the sample surface at given lateral resolution

NA, not applicable; CS, conventional source; C.Source, coherent source; SR, synchrotron radiation

example UV-CF) use probes to determine given cellular contents, others use possible interactions between cellular matter and light waves (for example vibrational spectroscopy techniques and μXRF) or elements (for example PIXE) for characterization of cell contents. Development of these techniques to obtain high-quality images of cells and their conversion into functional images (revealing the distribution of given contents) may require the sharing of skills in physics (optics, sources, detectors, instrument setup …), mathematics (chemometrics, image processing …), chemistry (analytical, molecular probes …), and biology. On this basis, one may describe the transition between routine and research imaging techniques as follows: "A routine technique for cell imaging is a research technique which succeeded in integrating all skills into a press-button machine". At this level of instrumentation

development, biologists and clinicians can use the proposed technique for diagnostic purposes.

Methods for molecular imaging of cells

Ultraviolet-visible confocal fluorescence imaging

From the perspective of biology, fluorescence may be the most powerful contrast mechanism, because a wide variety of site-specific fluorescent probes have been developed, including some with emission sensitive to the local environment (for example pH or Na^+ concentration) [8]. Fluorescence microscopy, undoubtedly the most frequently used microscopy technique in modern biological research,

requires labeling of the molecules of particular interest with fluorophores. These chemically fixed labels have the potential to affect the native behavior of the labeled molecule. Chemical reactions limit the lifetime of the fluorophore and thereby the visualization process. Most importantly, however, molecules other than the labeled ones will not be visualized by optical methods. To overcome this limitation multicolor imaging by use of a variety of fluorophores enables simultaneous imaging of different biological structures. However, the number of colors that are simultaneously detectable is presently limited to a small number and the structures that may be observed must be selected carefully. Despite their substantial advantages in live cell imaging, organic fluorophores are subject to other limitations:

1. autofluorescence of sample contents (collagens … etc),
2. photobleaching, and
3. fluorophores efficiency and compatibility.

To overcome these limitations, the use of fluorescent quantum dots (QDs) has been proposed. QDs are inorganic fluorescent nanocrystals that provide a useful alternative for studies that require long-term and multicolor imaging of cellular and molecular interactions. For labeling specific cellular proteins, QDs must be conjugated to biomolecules that provide binding specificity. Bioconjugation approaches vary with the surface properties of the hydrophilic QD used [9]. Despite several advantages of QDs, for example their enhanced brightness and resistance to metabolic degradation and photodamage, there are a few impediments to their successful use. Two of these are the tendency of QDs to aggregate in the cytosol and the tendency of single QDs to bind multiple molecules. An alternative is also the use of the green fluorescent protein (GFP), a protein composed of 238 amino acids which emits green fluorescence when exposed to blue light. The intense fluorescence of GFP is because of the nature of a chromophore composed of modified amino acids within the polypeptide. Hence, because the gene product is easily detectable because of its intense fluorescence, the GFP cDNA has become a unique reporter system [10]. Thus, the GFP gene can be introduced into organisms and maintained in their genome by breeding, injection with a viral vector, or cell transformation [11]. This avoids labeling molecular targets after cell culture, for example by use of organic fluorophores or QDs. There is thus no chemical change applied to cells before UV-CF imaging. In this way, even if UV-CF imaging provides information about only the labeled targets, the sample remains available for analysis by other imaging methods. Although UV-CF is diffraction-limited, for the best possible 3D rendering of 250 nm in the x, y, and z dimensions, new developments of laser-based microscopy systems now push this type of imaging towards nanoscopy, i.e. down to 15-nm

spatial resolution for stimulated emission depletion (STED) and other optical microscopy-derived systems [12]. STED has been proved to enable non-invasive in vitro real-time imaging of labeled molecules in cell sub-compartments, thus defining the future standards of analytical performances expected for cell biology investigations. Although such instrumentation is not yet widely available for routine measurements, cell biologists will rapidly shift to such high-resolution technology. However, one must remember that all UV–visible confocal methods remain limited by the number of cell properties they can reveal at the same time.

Multiphoton fluorescence imaging

In the most commonly used case of two-photon excitation, a fluorophore molecule is excited by the near simultaneous absorption of two photons of infrared laser light, each approximately twice the wavelength (half the energy) required for more conventional single-photon excitation used in confocal or wide-field fluorescent microscopy [13]. As fluorescence emission is a function of the square of the laser excitation intensity, this non-linearity provides an inherent optical sectioning capability that eliminates the generation of out-of-focus light. The high photon density necessary for multi-photon excitation occurs only at the focal point, and as photons disperse from this point, fluorescence excitation rapidly falls off also. In addition, the long infrared wavelengths in multi-photon excitation (700–1000 nm) provide several advantages over the shorter, higher energy single-photon wavelengths (350–500 nm) used to excite the same fluorophore: they penetrate deeper into the scattering tissue (ca. 500 μm), enabling thicker intact tissue preparations and even whole animals to be imaged. In addition, the infrared excitation is inherently less phototoxic to cells and the surrounding tissue, and photobleaching effects are constrained to the focal point, rather than extending throughout the entire exposed sampling area [14]. Biological samples strongly scatter light, making high-resolution deep imaging impossible for traditional confocal-fluorescence microscopy systems. Two photon-excited fluorescence microscopy thus overcomes this limitation, providing large-depth penetration mainly because even multiply scattered signal photons can be assigned to their origin as the result of localized nonlinear signal generation. The main limitations of multi-photon excitation are:

1. Slightly lower resolution with a given fluorophore compared with confocal imaging. This loss in resolution can be eliminated by use of a confocal aperture at the expense of a loss in signal. Thus, depending on the cell property being imaged, a compromise will

turn toward higher sensitivity/lower resolution when molecular concentration is low or lower sensitivity/ higher resolution when molecular concentration is high.

2. Thermal damage can occur in a specimen if it contains chromophores that absorb the excitation wavelengths.

3. It only works with fluorescence imaging (the exception being a CARS setup).

Methods for morphological imaging of cells

Optical microscopy

OM is undoubtedly the basic and key technique for single-cell analysis. It uses visible light (bright field) and a system of lenses to magnify images of small samples, and possibly a CCD camera for digital storage of images. In cell biology, inverted microscopes are useful for observing living cells or organisms at the bottom of a large container (e.g. a culture flask or Petri dish) under more natural conditions than on a glass slide.

One major limitation of OM is that at very high magnification with transmitted light, point objects are seen as fuzzy discs surrounded by diffraction rings (Airy disks). The resolving power of a microscope is taken as the ability to distinguish between two closely spaced Airy disks. It is these effects of diffraction that limit the ability to resolve fine detail. The extent of and magnitude of the diffraction patterns are affected by the wavelength of light (λ), the refractive materials used to manufacture the objective lens, and the numerical aperture (NA) of the objective lens. There is thus a finite limit beyond which it is impossible to resolve separate points in the objective field, known as the diffraction limit. Assuming that optical aberrations in the whole optical set-up are negligible, the resolution d, is given by: $d = \lambda/2NA$. Usually, a λ of 550 nm is assumed, corresponding to green light. With air as medium, the highest practical NA is 0.95, and with oil, up to 1.5. In practice, the lowest value of d obtainable is approximately 0.2 μm or 200 nm. This lateral resolution is sufficient for single-cell studies. However, one major disadvantage of optical microscopy is the lack of image contrast usually found on transparent systems, for example cells, and contrast is ultimately achieved at poorer lateral resolution. There are thus major limitations for discriminating sub-cellular components. Alternatively, the use of electrons and X-rays instead of light enables much higher resolution; the wavelength of the radiation is shorter, so the diffraction limit is lower. Another possibility is to use phase-contrast microscopy, an optical microscopy illumination technique in which small phase shifts in the light passing through a transparent specimen are converted into amplitude or contrast changes in the image. This is different from light microscopy, because the human eye measures only the energy of light arriving on the retina, so changes in phase are not easily observed. In fact, optical microscopy is used routinely only to verify the quality of cell cultures (cell shape, viability, growth …).

X-ray phase-contrast

Phase contrast has become a widely used technique that shows differences in refractive index converted into a difference in contrast. However, it fails to provide true 3D rendering because contrast amplitude is not directly correlated with distances between objects. This drawback may be overcome by XR-PC, where an X-ray microscope uses electromagnetic radiation in the soft to hard X-ray band to produce images of very small objects. Here, the contrast is given by the different absorption of soft X-rays in the water window region (wavelength region: 2.3–4.4 nm, photon energy region: 0.28–0.53 keV) by the carbon atom (main element composing the living cell) and the oxygen atom (main element in water). The contrast achieved on biosamples of small dimensions, for example cells, is very high because the two major chemicals present are water and organic molecules. The resolution of X-ray microscopy lies between that of the optical microscope and the electron microscope. It has a clear advantage over conventional electron microscopy because it can view biological samples in their natural state, i.e., in water of the culture medium for cells. Until now, resolution of 30 nm or better has been possible by using the Fresnel zone plate lens which forms the image using the soft X-rays emitted from a synchrotron. Recently, it has also been proposed to use soft X-rays emitted from laser-produced plasma rather than synchrotron radiation.

Application of XR-PC to cell imaging has been shown to provide true 3D rendering with all details of subcellular compartments [15]. In fact, any compartment being spatially well defined inside the cell, i.e., separating inner and outer spaces by a membrane, shows sufficient carbon–water contrast (>1%) to be delimited in 3D. The resulting image reveals darker objects with higher carbon density, thus identifying most of the cell organelles, for example vacuoles, vesicles, mitochondria, and the nucleolus [16]. Over the last two decades, continuous efforts to enhance nanofabricated X-ray optics and detectors has enabled the launch of the very first XR-PC laboratory instruments with nanoscale capability (e.g. www.xradia.com), thus enabling more routine utilization than with systems found in synchrotron radiation facilities. This availability should popularize access to XR-PC in cell biology.

Atomic force microscopy

AFM is a very high-resolution type of scanning probe microscopy, with demonstrated resolution below a nanometer, more than 1000 times better than the optical diffraction limit. The precursor to AFM, scanning tunneling microscopy, was developed in the early 1980s. The AFM is one of the foremost tools for imaging, measuring, and manipulating matter at the nanoscale. The information is gathered by "feeling" the surface with a mechanical probe, a cantilever with a sharp tip at its end that is used to scan the specimen surface. When the tip is brought into the proximity of the sample surface, forces between the tip and the sample lead to a deflection of the cantilever according to Hooke's law. Depending on the instrument setup, forces that are measured in AFM on biological material include mechanical contact force, van der Waals forces, chemical bonding, and electrostatic forces. As well as force, additional quantities may simultaneously be measured, for example the thickness of a biosample relative to its support.

For imaging, AFM may be used in static or dynamic modes. In the static mode (named the "contact mode"), the tip deflection is used as a feedback signal to collect information from the sample. However, the attractive forces close to the surface of the sample can be strong, causing the tip to touch its surface. The consequence for biosystems, which are mainly constituted by water and very soft organic compounds, is that sample can be damaged or destroyed by tip contacts. In the dynamic mode (also referred to as both "tapping mode" and "non-contact mode"), the cantilever is externally oscillated at or close to its fundamental resonance frequency. The oscillation amplitude, phase, and resonance frequency are modified by interaction forces between the tip and sample. These changes in oscillation relative to the external reference oscillation provide information about the sample's characteristics. In tapping mode, the amplitude of cantilever oscillation is greater than 10 nm, typically 100 to 200 nm. Because of the interaction of forces acting on the cantilever when the tip comes close to the surface, Van der Waals force or dipole–dipole interaction, electrostatic forces, … etc. cause the amplitude of this oscillation to decrease as the tip gets closer to the sample. An electronic servo in the Z axis uses the piezoelectric actuator to control the height of the cantilever above the sample. A tapping AFM image is therefore produced by imaging the force of the oscillating contacts of the tip with the sample surface. Tapping AFM has been shown to preserve lipid bilayer morphology, thus ensuring biosystems surfaces can be scanned in vivo without damage. The non-contact mode is even better for very fragile biosystems, such as cells. Here, the cantilever is oscillating at a frequency slightly above its resonance frequency, the amplitude of oscillation being typically a few nanometers (<10 nm). The van der Waals forces, which are strongest from 1 nm to 10 nm above the surface, act to reduce the resonance frequency of the cantilever, which, combined with the feedback loop system, maintains a constant oscillation amplitude or frequency by adjusting the average tip-to-sample distance. Measuring the tip-to-sample distance at each (x, y) data point enables construction of a topographic image of the sample surface (Fig. 2) [17].

In contrast with OM, the exceptionally high signal-to-noise ratio of the AFM enables the observation of single unlabeled macromolecules in a heterogeneous environment. AFM imaging of biological samples may be performed in buffer solution and at ambient temperatures, which enables the imaging of living cells and single proteins at work. Such experiments can be performed over a time range of several hours without destruction of individual proteins or disturbing their inherent assembly. In such a case, the AFM must be performed in tapping mode (with applied force <100 pN) or, better, in non-contact mode, thus ensuring that sample is not touched by the tip of the cantilever [18]. Furthermore, the AFM provides a true three-dimensional surface profile and samples do not require any special treatment (for example embedding, fixation …) that would
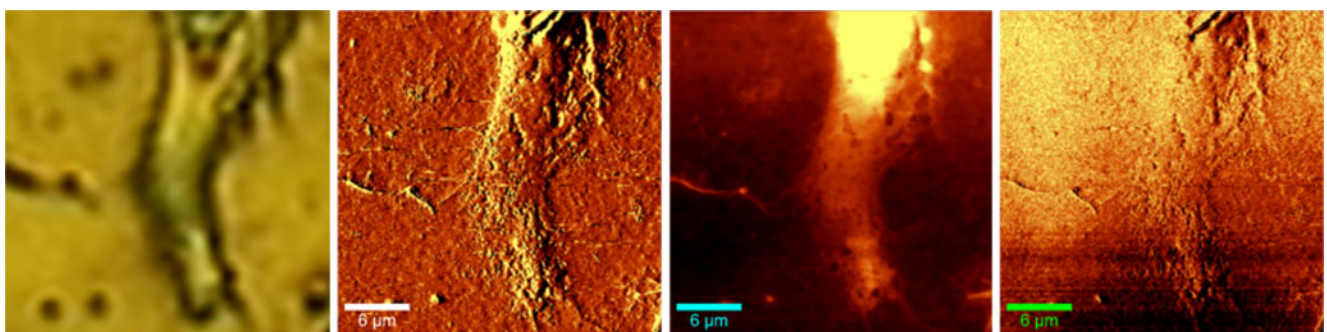

Fig. 2 Ex vitro AFM images of an individual cell in non-contact mode. Total dimension of the frame is 25×25 μm². AFM images obtained at 250×250 nm² lateral resolution with 1-nm z-axis precision for a 22 min acquisition. *From left to right*: optical image of a cell cytosolic extension, corresponding force max (*white bar*), stiffness (*green bar*), and topography (*blue bar*) AFM images

irreversibly change or damage the sample. Thus AFM imaging may be regarded as suitable for multimodal imaging of cells. On the other hand, the AFM can only image a maximum height of a few μm for a maximum FOV of 150×150 μm^2 [19]. There is also a limitation in AFM imaging applications for some in vivo processes because of the relatively slow scanning for large areas (in minutes).

Ellipsometry imaging

Ellipsometry measures the change of polarization upon reflection or transmission. Typically, ellipsometry is done only in the reflection setup. Electromagnetic radiation is emitted by a light source and linearly polarized by a polarizer. The exact nature of the polarization change is determined by sample properties (thickness, complex refractive index, or dielectric function tensor). Although optical techniques are inherently diffraction-limited, ellipsometry exploits phase information and the polarization state of light, and can achieve angstrom resolution. In its simplest form, the technique is applicable to thin films with thickness less than a nanometer to several micrometers. The sample must be composed of a small number of discrete, well-defined layers that are optically homogeneous and isotropic.

Single-wavelength ellipsometry employs a monochromatic light source. This is usually a laser in the visible spectral region, for instance, an HeNe laser with a wavelength of 632.8 nm. The advantage of laser ellipsometry is that laser beams can be focused on a small spot size. Furthermore, lasers have a higher power than broad-band light sources. Thus, ellipsometry can also be set up as imaging ellipsometry by using a CCD camera as a detector. Advanced imaging ellipsometer technology operates on the principle of classical null ellipsometry and real-time ellipsometric contrast imaging. This provides a real-time contrast image of the sample, which provides information about film thickness and refractive index [20]. Despite evident advantages over many other imaging techniques, ellipsometry imaging has received little attention from cell biologists. The lateral resolution of ellipsometry imaging is not better than 1×1 μm^2, but its precision in the z-axis may reach 0.1 nm (or 1 Å). As an example, such z-axis resolution enables analysis of ECM components and its interface with the cell membrane. With the ability to scan a 300×300 μm^2 FOV in a few seconds, this technique gives access to cell morphology changes (dynamics) in real time. Furthermore, this optical microscopy technique does not perturb the sample and, as such, can be considered for multimodal imaging, notably using UV-fluorescence means (epifluorescence of confocal microscopy) at the same time for real-time multimodal measurements.

Digital holography microscopy

Digital holography microscopy (DHM) is a newly developed imaging technique suitable for cell analyses which generates in real time a high-resolution 3D image of a sample in transmission or in reflection [21]. DHM provides precise and strictly non-invasive morphology measurements of microsamples without the addition of any contrast agent and with very low illumination power, thus avoiding perturbation or modification of the specimen [22]. It is characterized by the absence of a scanning mechanism, enabling real time measurements of moving samples. It provides nanometer scale vertical precision for x,y resolution of 290×290 nm^2 (diffraction limited). DHM is thus very sensitive to small shape modifications or intra-cellular composition changes associated with drugs, or mechanical or electrical stimulation. They are ideal instruments for high-throughput screening, time-lapse measurements, and diagnostics. DHM may be also completed with a fluorescence module enabling simultaneous DHM–epifluorescence measurements. The imaging system of DHM makes use of a video (CCD) camera to record a hologram produced by the interference between a reference wave and a wave emanating from the surface of the specimen. The captured image is transmitted to a computer where numerical procedures are used to reconstruct a 3D image of the specimen. Application to life sciences has shown that DHM is well suited to non-invasive and real-time in vitro measurements, thus offering access to cell morphology dynamics [23].

Methods for elemental imaging of cells

X-ray fluorescence microscopy

X-ray fluorescence microscopy (μXRF) is the emission of characteristic "secondary" (or fluorescence) X-rays from a material that has been excited by bombardment with high-energy X-rays or gamma rays. Besides X-rays and gamma rays, protons are also used to produce the characteristic X-rays (see PIXE section). The phenomenon is widely used for elemental and chemical analyses. To excite the atoms, a source of radiation is required, with sufficient energy to expel tightly held inner electrons. Conventional X-ray generators are most commonly used, because their output can readily be "tuned" for the application, and because, relative to other techniques, higher power can be deployed. When the energy source is a synchrotron, or the X-rays are focused by optics such as a polycapillary, the X-ray beam can be very small and very intense, and atomic information on the sub-micrometer scale can be obtained. X-ray generators in the range 20–60 kV in order to use the K

line, which enables excitation of a broad range of atoms. When X-rays are absorbed, an electron bound in the absorbing atom is ejected in what is known as the photoelectric effect. The hole this electron leaves behind is filled by a less strongly bound electron, and the surplus energy is commonly released by the emission of another X-ray. This phenomenon results in X-rays with energy that is characteristic of the element that emitted it. By detecting and analyzing the energy of these X-rays, the elemental make-up of the irradiated sample can be determined with high sensitivity. Because specimens prepared for X-ray fluorescence do not require sectioning, they can be investigated close to their natural, hydrated state with cryogenic approaches [24]. Combined with the ability to provide information about the oxidation state and coordination environment of metal cations, μXRF is ideally suited to study of the intracellular distribution and speciation of trace elements, toxic heavy metals, and molecule–metal complexes [25]. These characterize the coupling of metal species with biomolecules by covalent bonding, for example for metalloproteins. Trace elements have an important function in biological systems and the variations in their concentration play an important role in the development of diseases. Many pathological disorders arise as a consequence of trace element deficiencies or excesses. Combined with morphological techniques, for example XR-PC, μXRF is able to determine metal species distribution within cells, thus giving information about metal-driven processes and structural abnormalities in metallo-molecules (notably proteins having two or more different metal species) … etc. Synchrotron radiation X-ray sources enable the analysis of metal species at 0.1 ppm (a few thousand atoms) sensitivity with 100 nm lateral resolution [26] for the K emission lines of Si, P, S, Cl, Ar, K, Ca, Ti, Cr, Mn, Fe, Co, Ni, Cu, Zn, Se, Br, Kr, and Sr, and the L emission lines of Cd and Pb [27]. μXRF is thus the only technique available for quantitative elemental imaging of whole cells.

The main limit of this technique is the current availability of μXRF systems, which are located in synchrotron radiation facilities having hard X-ray beamlines, and no laboratory instrumentation is currently available for single-cell analyses at spatial resolution below 1×1 μm^2. This unavailability is mainly because of the lack of conventional X-ray sources sufficiently powerful to achieve the required sensitivity (0.1 to 1 ppm for most of metal species) at the expected lateral resolution for cell analyses. There is thus a strong demand from cell biologists for a μXRF laboratory system [28], which would be unique for determining inorganic elements at trace concentration. New generation of X-ray sources and nanofabricated optics should even open the route to the first industrial initiatives for laboratory systems combining XR-PC and μXRF into a single instrument, thus providing, for the first time, 3D morphological imaging correlated with elemental mapping of cells.

PIXE microscopy

An alternative to μXRF, particle-induced X-ray emission (PIXE) is another technique used for determining the elemental make-up of a sample. When exposed to an ion beam, atomic interactions occur that give off electromagnetic radiation at wavelengths in the X-ray part of the electromagnetic spectrum specific to an element. PIXE is a powerful but destructive elemental analysis technique for investigating biosystems. Only elements heavier than fluorine can be detected. Whole-cell and tissue analysis is possible using a microscopic PIXE beam, this method is also referred to as nuclear microscopy because it uses a device called a microprobe. A microprobe is a device that uses electromagnetic or electrostatic lenses to focus an ion beam, down to a few hundred nanometers at the best lateral resolution. In this way a microprobe is very similar to a scanning electron microscope. Some differences are that the nuclear microprobe beam is composed of, usually, but not exclusively, protons and alpha (He ions) particles [29]. Some of the most advanced nuclear microprobes have beam energies of 2 MeV and up. This gives the device very high sensitivity to minute concentrations of elements (approx. 1 ppm). However, cells naturally contain trace concentrations of metals, thus PIXE microscopy cannot provide high-sensitivity mapping at lateral resolution better than 1×1 μm^2 for a 5 to 10 h acquisition time. Therefore, as for μXRF, PIXE cannot be regarded as a routine technique for elemental mapping of cells, but rather as a research tool for fundamental studies in cell biology [30].

SIMS microscopy

Secondary ion mass spectrometry microscopy (μSIMS) is an alternative technique to XRF and PIXE for element-specific and molecule-specific imaging experiments [31]. μSIMS uses a focused energetic ion beam on the nanometer scale for bombarding a solid sample, combining this with a time-of-flight mass spectrometer for simultaneous detection of the sputtered particles. The feasibility of μSIMS studies on cells depends on special preparation techniques, which must be adapted to the needs of any particular project. In each case, the preparation technique must preserve the chemical and structural integrity of the living cell. Thus, cryopreparation is a necessary step for the analysis of substances which are potentially diffusible [32]. Moreover, instrumental setup for SIMS requires that cells must withstand the ultra-high vacuum in the analysis chamber and ion beam bombardment. There is thus evidence that μSIMS cannot be regarded as a routine technique for cell

biology but rather as a powerful elemental imaging method for research purposes.

The 3D chemical imaging capability of μSIMS is an advantage over XRF and PIXE. 3D μSIMS provides a potentially powerful approach for biological analysis, because it can resolve the chemical composition of a sample in the x,y,z dimensions by gradually eroding the cell surface. However, the utility of 3D analysis with SIMS is sample type-dependent. For example, the nucleus and cytoplasmic compartments of interphase cells in cell cultures and tissue sections can be reasonably studied in SIMS images without sequential recording of multiple images of the same mass. The spatial resolution achievable with 3D-μSIMS is about 500 nm, thus competing well with μXRF, but without the need for a synchrotron radiation source. Despite advantageous analytical performance over μXRF and PIXE, μSIMS applications are much more difficult to perform, notably because of to sample handling requirements and the critical aspects of analyses in a vacuum. Furthermore, the availability of μSIMS systems remains very limited and its utilization is truly restricted to physicists as pure specialists of mass spectrometry, thus far from cell biologists' environment.

Methods for chemical imaging of cells

FTIR imaging

Fourier-transform infrared (FTIR) microscopy and imaging techniques achieve contrast via the intra-molecular vibrational modes, similarly to well recognized X-ray microscopy methods in which contrast is achieved by recording spectra before and after the absorption edges of an element contained in the specimen [33]. In biology, IR spectroscopy has important advantages compared with other techniques: global organic information is obtained [6], high contrast without staining, the use of non-ionizing radiation, and no damage to samples. Whatever the source used, a Globar or synchrotron radiation, IR radiation is focused through, or reflected from, a tiny spot on the sample and then analyzed with a spectrometer. FTIR imaging of individual cells remains very limited because of t the lack of sensitivity of imaging systems close to diffraction limit [34]. However, the use of synchrotron radiation has been demonstrated to be a benefit for cell imaging, notably using a focal plane array (FPA) detector [33], which enables the acquisition of images within minutes. Another major limit in the utilization of FTIR imaging for analyzing cells is the poor lateral resolution achieved, 2.5 to 25 μm in the mid-infrared spectral range (4000–500 cm^{-1}) with respect to the diffraction limits, or, more precisely, 3 μm for compounds made of fatty acyl chains (cell lipids), 6 μm for amides and

amines (cell proteins), and 10 μm for osidic residues (cell sugars). However, modern IR optics and FPA detectors enable 1×1 μm^2 resolution ($36 \times$ magnification of 40×40 μm^2 individual detectors in FPA) or even better ($74 \times$ magnification level at 540×540 nm^2) to be achieved. Because such lateral resolution is well below the diffraction limit, interpretation of IR spectra extracted from cell FTIR images does not correlate with the dimensions of a pixel, i.e., the spectrum contains sample information at the dimensions of the diffraction limit whereas the pixel from the optical image is smaller (e.g. 6×6 μm^2 spectral information from the FTIR spectrum compared with 1×1 μm^2 from the optical image pixel). However, an effort is under way in different laboratories to manage this limit, notably by modifying interferograms to correspond to the dimensions of the final image pixel. The possibility of using immerged optics is also envisaged for live cell imaging at the surface of an ATR crystal coupled to the FPA detector, thus promising to open new opportunities for FTIR applications [35]. Another expected enhancement driving FTIR toward inclusion among the tools used for routine imaging of biology inside the cell is an increase of signal-to-noise ratio (SNR) of one order of magnitude to obtain high-quality spectra from cell FTIR images for extracting quantitative chemical information [5, 33]. This analytical performance enhancement is likely to come from detectors rather than sources. Synchrotron radiation IR sources are 2–3 orders of magnitude brighter than the Globar, but the increase in the photon flux at the sample location when using high lateral resolution (e.g. 1×1 μm^2) is only a few percent superior [34]. Thus, the gain in SNR will primarily benefit from higher sensitivity of IR detectors for small differences in photon flux (typically 1–5% between background and sample spectra).

Raman microscopy

The Raman shift chemical imaging spectral range spans from approximately 50 to 4000 cm^{-1}; the actual spectral range over which a particular Raman measurement is made is a function of the laser excitation frequency. The basic principle behind Raman spectroscopy differs from the mid-infrared in that the x-axis of the Raman spectrum is measured as a function of energy shift (in cm^{-1}) relative to the frequency of the laser used as the source of radiation. Briefly, the Raman spectrum arises from inelastic scattering of incident photons, which requires a change in polarizability with vibration, as opposed to infrared absorption, which requires a change in dipole moment with vibration. The end result is spectral information that is similar and in many cases complementary to the MIR. The Raman effect is weak, because only about one of 10^7 photons incident on the sample undergoes Raman scattering. However, Raman

spectroscopy has a clear advantage over FTIR in that both organic and inorganic materials have a Raman spectrum and they generally produce sharp bands that are chemically specific. Therefore, despite the higher sensitivity of FTIR, Raman microscopy is a powerful method for elucidation of chemical species and their dynamics in biological sample, for example living cells (see example in Fig. 3), because of its noninvasive and nondestructive capability. High-sensitivity stimulated Raman scattering microscopy is even setup as a three-dimensional multiphoton vibrational imaging technique offering background-free and readily interpretable chemical contrast [36]. This technical evolution of Raman confocal microscopy enables mapping molecular species in 3D with the ability to follow their dynamics in living cells and organisms, on the basis of the wealth of Raman spectroscopy. More generally, Raman has significant advantages over other techniques because it is a scattering technique, thus specimens do not need to be fixed or sectioned and, thus, in vitro studies are possible for living cell analyses. Raman spectra can be collected from a very small volume (<1 μm in diameter); these spectra enable identification of species present in that volume. Furthermore, water does not interfere with Raman spectral analysis. Raman imaging has been proved to be powerful in the chemical analysis of cells, with hyperspectral images showing the distribution of cholesterol, proteins, nucleic acids, and fatty acids. Using a confocal setup, Raman imaging reaches lateral and depth resolution of about 250 nm and 1 μm, respectively, and 50×50 μm^2 FOV

may be mapped within 10 to 30 min. Because the objective lenses of microscopes focus the laser beam to several micrometers in diameter, the resulting photon flux is much higher than achieved in conventional Raman setups. However, the high photon flux can also cause sample degradation, and for this reason some setups require a thermally conducting substrate (which acts as a heat sink) in order to mitigate this process. By using Raman imaging, in vitro time and space-resolved Raman spectra of microscopic regions of cells can be measured. Actually, Raman imaging is regarded as the best vibrational spectroscopy-based technique for imaging lipids in biosystems, because the sensitivity to C–H groups is very high and specific (considering C = C–H, CH$_2$ and CH$_3$ groups). With scanning durations of several tenths of a minute, Raman imaging is not yet ready for measurements of whole cell dynamics, but as for FTIR imaging, the next generation of detectors should speed up the process.

Coherent anti-stokes Raman scattering microscopy

As mentioned previously, spontaneous Raman microspectroscopy requires a long exposure time because of the small cross section of the Raman scattering. As an alternative approach, coherent Raman imaging methods have been developed. CARS may be described as a four-wave mixing process, i.e. three pulses interact with a sample creating molecular coherence, the decay of which is recorded as the directionally irradiated CARS signal. It thus uses multiple
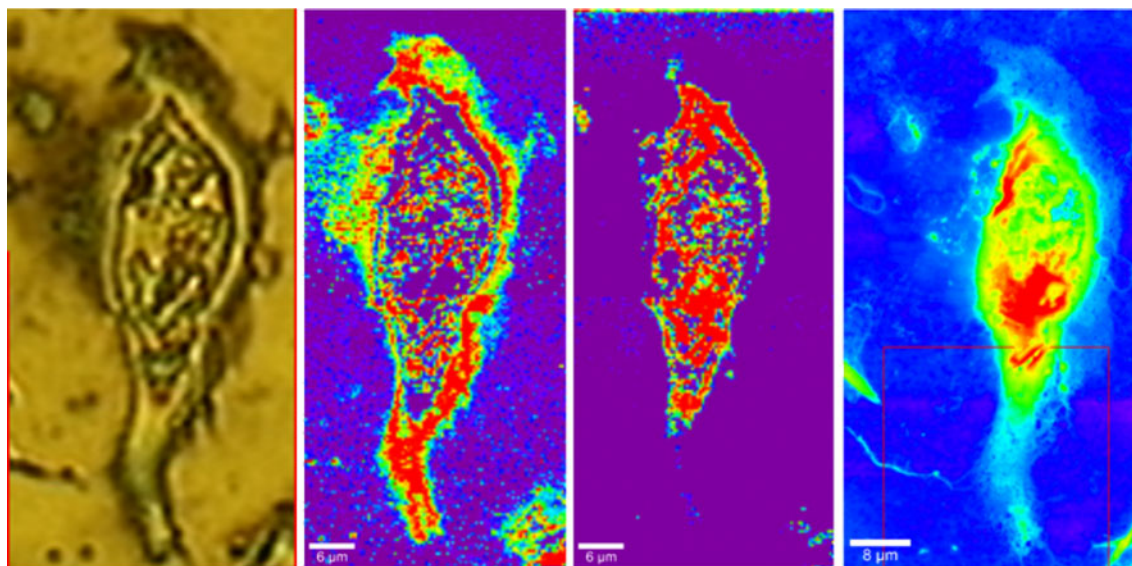

Fig. 3 Raman confocal microscopy of an individual cell. Total dimension of the frame is 100×35 μm^2. Raman images obtained in confocal mode at a 250×250 nm^2 spatial resolution (first layer of the cell at support location) and within 20 min. *From left to right*: optical image of a cell, mapping of Raman shift for lipids (3000–2800 cm^{-1}), phosphates (1250–1100 cm^{-1}), and proteins (1700–1500 cm^{-1}) with ECM components. The *red square in the right image* was further analyzed by AFM (Fig. 2). Raman microscopy images obtained in confocal geometry on a WITec 300R system equipped with a 532-nm laser powered at 8 mW at the focal point to avoid sample heating

photons to address the molecular vibrations, and produces a signal in which the emitted waves are coherent with one another. As a result, CARS is several orders of magnitude stronger than spontaneous Raman emission. The Raman signal is detected on the red side of the incoming radiation where it might have to compete with other fluorescence processes. The CARS signal is detected on the blue side, which is free from fluorescence, but it comes with a non-resonant contribution. The differences between the signals from Raman and CARS arise from the fact that Raman relies on a spontaneous transition whereas CARS relies on a coherently driven transition. This nonlinear variant of Raman spectroscopy combines signal enhancement by more than four orders of magnitude with further advantages such as directional emission and narrow spectral band-width. Another advantage of CARS microscopy is its inherent three-dimensional imaging capability without the need to insert a confocal pinhole into the detection path. This is because of its ability to use high numerical aperture and very tight focusing, thus providing lateral and axial resolution with precision of a few tenths of a nm only. Compared with two-photon excited fluorescence imaging with lipophilic dye labeling, CARS microscopy provides sharper contrast and avoids photobleaching [37]. Although CARS setups are just becoming available and cell imaging applications started a few years ago, there is no definitive overview of the analytical performance achievable at present. Nevertheless, one may consider that CARS imaging has significant advantages over FTIR and Raman techniques, notably in terms of the sensitivity and spatial resolution achieved, but will remain limited to specific applications, because global analysis of samples is not yet possible on large spectral intervals (tenths of cm^{-1} for CARS vs. thousands of cm^{-1} for FTIR and Raman).

Scanning near-field optical microscopy

SNOM has versatile applications according to its illumination field, providing topographic and/or chemical information about the sample. A THz time-domain spectroscopy enables exploration of the rich spectroscopic information on molecular vibrations, rotations, and other low-energy transitions in biological and organic compounds, and semiconductor structures. Spatial resolution of THz imaging is limited by the long wavelength used (0.3 mm at 1 THz). Several approaches have been proposed for breaking the diffraction barrier ($\sim\lambda/2$). When a near-field technology is applied to THz imaging, the interaction between the THz wave and sample surfaces is limited effectively to a small area, making it possible to achieve microscopic imaging with sub-wavelength resolution. There have been several approaches to sub-wavelength resolution for example the sub-wavelength aperture, the dynamic aperture, and, re-

cently, apertureless THz near-field microscopes. The first near-field image was obtained using a metal cone. The end of this metal cone was polished in order to produce a very small aperture at its extremity. This small aperture, in close proximity to the sample surface, acts like a very small optical hole through which the THz propagates and illuminates the sample surface. The size of the hole gives the resolution of the near-field images obtained, typically smaller than $\lambda/4$. The second approach for near-field imaging in the THz domain uses a sub-wavelength object instead of a hole. When illuminated, the small object acts like a tiny antenna that scatters light in all directions. The end of a metal tip is used as a small scattering object. The metal tip end being smaller than few nanometers, the tip-sample approach may be very well controlled down to the nanometer scale. With a metal tip, resolution down to few hundred nanometers in the THz ($\lambda/1000$) has been demonstrated [38].

As the name implies, information is collected by spectroscopic means instead of imaging in the near-field region. By use of near field spectroscopy (NFS) one can probe spectroscopically with subwavelength resolution. Raman SNOM and fluorescence SNOM are two of the most popular NFS techniques because they enable identification of nanosized features with chemical contrast. Some of the common near-field spectroscopic techniques are:

1. Direct local Raman SNOM: Aperture Raman SNOM is limited by very hot and blunt tips, and by long collection times. However, apertureless SNOM can be used to achieve high Raman scattering efficiency factors (around 40). Topological artifacts make it hard to implement this technique for rough surfaces.
2. Surface enhanced Raman spectroscopy (SERS) SNOM: This technique can be used in an apertureless shear-force SNOM setup, or by using an AFM tip coated with gold. The Raman signal is found to be significantly enhanced under the AFM tip. This technique has been used to give local variations in the Raman spectra under a single-walled nanotube. A highly sensitive optoa-coustic spectrometer must be used for detection of the Raman signal.
3. Fluorescence SNOM: This highly popular and sensitive technique makes use of the fluorescence for near field imaging, and is especially suited to biological applications. The technique of choice here is the apertureless back to the fiber emission in constant shear force mode. This technique uses merocyanine-based dyes embedded in an appropriate resin. Edge filters are used for removal of all primary laser light. Resolution as low as 10 nm can be achieved using this technique.
4. Near field infrared spectrometry for implementing IR-SNOM is also under development with the use of

powerful IR sources (such as synchrotron radiation or FEL) to enable collection of chemical information from the sample surface [39].

Limitations of SNOM primarily include:

1. very low working distance and extremely shallow depth of field;
2. limited to study of surfaces;
3. not conducive to study of soft materials, especially under shear force mode; and
4. long scan times for large sample areas or high-resolution imaging.

Multimodality in cell imaging

Multimodal imaging may refer to two different imaging approaches:

1. the use of hybrid imaging systems enabling the combining of at least two modalities on the same sample possibly, simultaneously; and
2. the use of several techniques applicable in sequence on the same sample.

The setup of hybrid instrumentation is limited by the physics of imaging, i.e., by sources, optics, and detectors. There are thus very few hybrid systems available for simultaneous imaging of a sample. In biology, single-photon emission computed tomography (STECT) imaging is the most popular hybrid imaging, enabling the acquisition of three-dimensional images of metabolic functions offered by the SPECT with the structural images provided by CT [40]. PET may also be combined to MRI for functional imaging, but with limitations inherent to each modality, notably in terms of sensitivity and spatial resolution [41]. Another example of hybrid imaging includes an X-ray and MRI system in which the X-ray tube and detector lie within the MR scanner. Hybrid imaging is now regarded as the future of pathology diagnostics in clinics, notably for cancers, where morphological and chemical information must be combined for staging or gradation [5]. However, technological solutions for the simultaneous application to cells of several types of imaging remain rare, notably because of the lack of compatibility between optics. In cell biology, the most powerful systems combine interferential phase contrast with UV-CF microscopy, with time-resolved acquisitions for in vitro dynamics of cells. Setups using several modalities in the same instrumentation for sequential imaging of samples on the same sample stage have been proposed. These systems enable combination of AFM with Raman confocal microscopy and even UV-SNOM, or FTIR imaging with UV epifluorescence microscopy. These

setups now seem to be credible alternative to UV-CF imaging because of the global information (FTIR, Raman) they may provide.

Another advantage is that morphological information (e.g. AFM) may be coupled with chemical data, thus opening the way to quantitative imaging with high spatial resolution for defining functional voxels. This is a critical feature of future multimodal imaging systems developed for cell biology, with three basic issues:

1. Analyzing chemical species inside a voxel means determining a cellular volume (false 3D imaging) or being able to focus on a subcellular compartment (true 3D imaging). Actually, only XR-PC is able to image cells with true 3D rendering, easily defining cell organelles but only ex vivo. AFM, ellipsometry imaging, and DHM provide 3D rendering in vitro, but with respect to a surface; of these, two (ellipsometry imaging and DHM) have the advantage of time and z-axis resolution, AFM being more versatile in the cell data obtained. XR-PC may determine a voxel with 15-nm x,y,z dimensions, thus providing access to any subcellular organelle. Topographic information provided by AFM enables definition of a voxel from a surface with a lateral resolution of 5×5 nm^2 and with a z-axis precision of 1 nm, but time resolution is a few tenths of a minute for one cell. By contrast, DHM enables determination of a voxel with 290×290 nm^2 lateral resolution (1 nm precision in the z-axis), but in a few seconds only. Ellipsometry has equivalent time resolution with lower lateral resolution (1×1 μm^2), but with the highest z-axis precision (0.2 nm). Thus, depending on the cell property being determined, every technique may provide at least one significant advantage for quantitative analyses.
2. The sensitivity expected is the ability to detect tens to hundreds of species of a given chemical compound inside the voxel. Here, there is competition between spatial resolution and signal sensitivity achievable by the combined techniques, i.e., the better is the resolution, the lower is the sensitivity and vice versa. This is the most challenging objective of multimodal setups for cell imaging, because an individual cell cultured on a flat support is characterized by a $5–10 \times 10^3$ μm^3 volume for a 5–10 ng weight. Therefore, a 100-nm x, y,z voxel (the average dimension of cell organelles) will represent a 1×10^{-6} ng weight. Considering that cells are composed of 80% water, the total chemical matter will weight 0.2×10^{-6} ng, with 60% proteins, 20% lipids, 5% saccharides, 3% salts, 0.1% metal ions … etc.
3. A multimodal imaging system makes sense of available cell data when these can be superimposed on a final "multimodal" image. This means that data-treatment

methods must be performed at the same lateral spatial resolution to correlate a morphological dimension with chemical information. For imaging modalities providing 2D maps, the process is simply interpolation of pixels to the largest dimension found in the techniques to be compared. For modalities providing 3D rendering, the process may be much more complicated, with voxels obtained using techniques in confocal or non confocal geometry. Then, interpretation of final images combining multimodal data from cells may suffer from large errors. Now that several techniques are able to image cells on the nanometric scale, and possibly with confocal geometry, there is a true opportunity to unravel subcellular dynamics with appropriate time resolution. Examples of future major cell biology applications include combination of XR-PC with XRF and UV-CF for investigating the role of metalloproteins (30% of total proteins) in neurodegenerative diseases [42], STED and DHM for synaptic trafficking related to major brain function development [43], AFM and CARS for cell cycle processes [37], ellipsometry imaging with ATR-FTIR imaging [44] of the ECM-membrane interface in tissue formation, … etc.

Conclusion

Cell imaging is dominated by UV-fluorescence techniques because of the very specific results they provide. Alternative techniques have emerged and give access to a broader range of cell properties, giving the illusion that everything may be analyzed in a cell—chemicals, molecules, elements, structures, and dimensions. However, because these techniques are characterized by intrinsic limits (sensitivity, resolution, and signal specificity) and accessibility, their utilization for imaging biosamples has often suffered from drawbacks that reduced their use to very restricted applications in cell biology. It is becoming evident that several techniques have been sufficiently developed and opened to more routine applications to envisage crossing or combining their results into a single view of a cell. The most challenging is to couple chemical and morphologic data, thus giving access to quantitative analysis of the cell, and even down to single subcellular compartment. Although, the coupling of high-performance analytical techniques for subcellular quantitative imaging is in its infancy, several major high-tech companies are now developing instrumentations combining morphological and chemical microscopies. Future microscopy systems will by sufficiently sensitive to determine cell compounds in small voxels and in real-time, thus offering unprecedented opportunities to investigations of cell biology.

References

1. Stephens DJ, Allan VJ (2003) Science 300:82–86
2. Hell SW (2007) Science 316:1153–1158
3. Betzig E, Trautman JK (1992) Science 257:189–195
4. Meuli R, Hwu Y, Je JH, Margaritondo G (2004) Eur Radiol 14:1550–1560
5. Petibois C, Cestelli Guidi M (2008) Anal Bioanal Chem 391:1599–1608
6. Petibois C, Déléris G (2006) Trends Biotechnol 24:455–462
7. Sample V, Newman RH, Zhang J (2009) Chem Soc Rev 38:2852–2864
8. White JG, Amos WB, Fordham M (1987) J Cell Biol 105:41–48
9. Jaiswal JK, Goldman ER, Mattoussi H, Simon SM (2004) Nat Methods 1:73–78
10. Prasher DC (1995) Trends Genet 11:320–323
11. Heim R, Cubitt AB, Tsien RY (1995) Nature 373:663–664
12. Willig KI, Kellner RR, Medda R, Hein B, Jakobs S, Hell SW (2006) Nat Methods 3:721–723
13. Helmchen F, Denk W (2005) Nat Methods 2:932–940
14. Flusberg BA, Cocker ED, Piyawattanametha W, Jung JC, Cheung EL, Schnitzer MJ (2005) Nat Methods 2:941–950
15. Weon BM, Je JH, Hwu Y, Margaritondo G (2006) Int J Nanotechnol 3:280–297
16. Son SW, Park SY, Park GM, Ha SH, Lee GW, Lee OS, Hwu Y, Kim AR, Je JH, Oh CH (2008) Skin Res Technol 14:13–17
17. Chaudhuri O, Parekh SH, Lam WA, Fletcher DA (2009) Nat Methods 6:383–387
18. Novak P, Li C, Shevchuk AI, Stepanyan R, Caldwell M, Hughes S, Smart TG, Gorelik J, Ostanin VP, Lab MJ, Moss GW, Frolenkov GI, Klenerman D, Korchev YE (2009) Nat Methods 6:279–281
19. Lapshin RV (2004) Nanotechnology 15:1135–1151
20. Malmsten M, Siegel G, Becker A (2001) J Colloid Interface Sci 240:372–374
21. Colomb T, Kuhn J, Charriere F, Depeursinge C, Marquet P, Aspert N (2006) Opt Express 14:4300–4306
22. Rappaz B, Marquet P, Cuche E, Emery Y, Depeursinge C, Magistretti P (2005) Opt Express 13:9361–9373
23. Emery Y, Cuche E, Colomb T, Depeursinge C, Rappaz B, Marquet P, Magistretti P (2007) J Phys Conf Ser 61:1317–1321
24. Paunesku T, Vogt S, Maser J, Lai B, Woloschak G (2006) J Cell Biochem 99:1489–1502
25. Finney L, Mandava S, Ursos L, Zhang W, Rodi D, Vogt S, Legnini D, Maser J, Ikpatt F, Olopade OI, Glesne D (2007) Proc Natl Acad Sci USA 104:2247–2252
26. Yang L, McRae R, Henary MM, Patel R, Lai B, Vogt S, Fahrni CJ (2005) Proc Natl Acad Sci USA 102:11179–11184
27. Corezzi S, Urbanelli L, Cloetens P, Emiliani C, Helfen L, Bohic S, Elisei F, Fioretto D (2009) Anal Biochem 388:33–39
28. Fahrni CJ (2007) Curr Opin Chem Biol 11:121–127
29. Rokita E, Mutsaers PHA, Quaedackers JA, Taton G, de Voigt MJA (1998) Nucl Instrum Methods Phys B 319:180–185
30. Kemner KM, Kelly SD, Lai B, Maser J, O'Loughlin EJ, Sholto-Douglas D, Cai Z, Schneegurt MA, Kulpa CF Jr, Nealson KH (2004) Science 306:686–687
31. Chandra S (2004) Appl Surf Sci 231–2:467–469
32. Arlinghaus HF, Kriegeskotte C, Fartmann M, Wittig A, Sauerwein W, Lipinsky D (2006) Appl Surf Sci 252:6941–6948

33. Petibois C, Piccinini M, Cestelli-Guidi M, Marcelli A (2010) J Synchrotron Radiat 17:1–11
34. Petibois C, Deleris G, Piccinini M, Cestelli Guidi M, Marcelli A (2009) Nat Photonics 3:179
35. Kuimova MK, Chan KL, Kazarian SG (2009) Appl Spectrosc 63:164–171
36. Freudiger CW, Min W, Saar BG, Lu S, Holtom GR, He C, Tsai JC, Kang JX, Xie XS (2008) Science 322:1857–1861
37. Fu Y, Huff TB, Wang HW, Wang H, Cheng JX (2008) Opt Express 16:19396–19409
38. Wang K, Mittleman DM (2004) Nature 432:376–379
39. Vobornik D, Margaritondo G, Sanghera JS, Thielen P, Aggarwal ID, Ivanovc B, Tolk NH, Mannid V, Grimaldi S, Lisi A, Rieti S, Piston DW, Generosi R, Luce M, Perfetti P, Cricenti A (2005) J Alloys Compounds 401:80–85
40. Phelps ME (2000) Proc Natl Acad Sci USA 97:9226–9233
41. Miller JC, Thrall JH (2004) J Am Coll Radiol 1:4–23
42. Higgins CM, Jung C, Xu Z (2003) BMC Neurosci 4:16
43. Lin HH, Lai JS, Chin AL, Chen YC, Chiang AS (2007) Cell 128:1205–1217
44. Kazarian SG, Chan KL (2006) Biochim Biophys Acta 1758:858–867