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Review

Imaging from anatomic to molecular and atomic resolution scales: A review

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Imaging may be referred to as the 'eyes of science' as it provides scientists with highly informative multi-dimensional and multi-parameter data usually invisible to the naked eye. As instrumentation technologies and genetic engineering advances, it's possible in modern times to observe and image highly dynamic biochemistry processes. This paper reviews positron emission tomography (PET), magnetic resonance imaging (MRI), computed tomography (CT), ultrasound, visible light microscopy, bioluminescence (BLI) and fluorescence mediated tomography (FMT) imaging techniques, highlighting the principles behind the operation of each technique, their major strengths and drawbacks. With the enhancement of the existing techniques and evolution of new ones, the future possibility of refined view of systems invisible to naked human eye is promising. More so is when two or more techniques are combined in biological systems analysis.

Key words: Optical, imaging, microscopy, resolution, fluorescence.

INTRODUCTION

Imaging, whether macroscopic, microscopic or nanoscopic plays a unique role in sciences in that it aids to collect data that can be analyzed to provide useful insights during experimentation and postexperiment. In that respect, imaging may be referred to as the 'eyes of science'. Imaging provides scientists with highly informative multi-dimensional and multi-parameter data. Examples of physical parameters that could be imaged/measured are concentration e.g. of water (Pircher et al., 2003) and oxygen (Kurokawa et al., 2015; Papkovsky and Dmitriev, 2012a, b), tissue properties (Gao, 1996), surface area (Eils and Athale, 2003), molecular architecture (Fridman et al., 2012), protein binding dynamics (Marsh and Teichmann, 2015), protein diffusion rates (Day and Schaufele, 2008) and many others, all of which provide an insight on temporal biological functions. Traditionally imaging has been done using various techniques that for example freezes biological specimens during sample preparations, but with the recent advances in high-resolution microscopy and genetically engineered fluorescent probes that are fusible to cellular proteins (Day and Schaufele, 2008), live cell imaging is now possible, that is very useful to observe the highly dynamic cellular multi-parameters. In other words, events or parameters occurring on a large scale of time scales can be obtained in minimally invasive

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Table 1. Imaging from anatomical to atomic scales.

Imaging technique	Spatial resolution	Depth limit	Scan Time	Key use	References
PET/MicroPET/MicroSPECT	1-2 mm	No limit	Minutes	Metabolic imaging of molecules such as glucose, thymidine, imaging of probes such as antibodies, peptides etc.	Weissleder, 2002; Park et al., 2007; Koba et al., 2013; Moore et al., 2000; Jang, 2013
MRS	≈ 2 nm	-	Minutes-Hours	Detection of metabolites	Gabbay et al , 2007; Müller et al., 2014; Strobel et al., 2008
fMRI	≈ 1 nm	No limit	Seconds-Minutes	Functional imaging of brain activity	Kriegeskorte and Bandettini, 2007; Ciobanu and Pennington, 2004
MRI/MicroMRI	4-100 µm,	No limit	Minutes-Hours	Anatomical Imaging	Weissleder, 2002; Ciobanu and Pennington, 2004; Singh et al., 2002
CT/MicroCT	12-50 µm	No limit	Minutes	Lung and bone tumor imaging	Weissleder, 2002; Koba et al., 2013; Jang, 2013; Meng et al , 2006.
Ultrasound	≈ 50 µm	Millimeters	Seconds	Vascular and interventional imaging	Weissleder, 2002, Jang, 2013.
Visible Microscopy (various modern techniques are summarized in Table 2)					
Bioluminescence (BLI)	Several mm	cm	Minutes	Gene expression and cell tracking	Sadikot and Blackwell, 2005; Keyaerts et al., 2012)
Fluorescence mediated tomography (FMT)	1-2 mm	< 1 cm	Seconds-Minutes	Quantitative imaging of targeted fluorochromes in deep tumors	Ntziachristos et al., 2002; Ntziachristos and Weissleder, 2003
Atomic force microscopy	10-20 nm	-	Minutes	Mapping cell surface	Leonenko et al., 2007; Binnig et al , 1986.
Electron microscopy	≈ 5 nm	-	Seconds	Discerning protein structure	Murphy and Jensen, 2007

optical based techniques. Obtained parameters in biological systems are useful and applied to allow both therapeutic and diagnostic applications (Alivisatos et al., 2005; Michalet et al., 2005; Loo et al., 2004). In addition data acquired from imaging procedures forms the foundation for mathematical modeling e.g. of protein kinetics and biochemical signaling networks (Chen and Murphy, 2004). Imaging data can also be utilised to test computational models developed in computational biology (Kherlopian et al., 2008).

IMAGING ON MULTIPLE SCALES

In contemporary times, using several techniques such as positron-emission tomography, magnetic resonance imaging, and optical coherence tomography, imaging is possible in a wide large of spatial resolution, ranging between 1 mm and 10 µm. Even much higher spatial resolutions at the molecular or at atomic levels (< 10 nm) are now possible with electron microscopy and scanning probe techniques, as summarized in Table 1.

Between these two resolutions extremes lies the resolving power of optical microscopy (that is, 10 nm to 1 mm) that utilizes visible light (≈ 400 to 700 nm). A summary of various modern visible range imaging techniques are summarized in Table 2. Imaging within this electromagnetic spectra region is beneficial since the radiation energy strikes a balance between being energetic enough to view fluorescent probes and fair enough to be minimally invasive, thus enabling live cell imaging.

However, a major drawback arises from the limits to spatial resolution that is famously represented by the Abbes equation (Abbe, 1873):

$$d = \frac{\lambda}{2\, n \sin\, \theta} \equiv \frac{\lambda}{2NA} \quad (1)$$

where λ is the radiation wavelength, n is the refractive index of the medium, θ is the half angle over which the objective can gather light radiation, NA is the numerical aperture of the objective. A direct implication of Equation 1, with $NA \approx 1.4 - 1.6$ possible with modern optics is that, the spatial resolution $d \approx \frac{1}{2}\lambda$, meaning that for green light of around 500 nm $d = \lambda/2 = 250$ nm (0.25 µm). In

Table 2. Modern innovative techniques that overcomes the Abbes resolution limit.

Visible microscopic technique	Principle	Resolution	Scan time	References
Near-Field (NSOM) -Scanning near-field optical microscopy -Wide-field near-field optical microscopy	Done by placing the detector very close to the specimen surface	20 nm (Lateral), 2–5 nm (axial)		Dürig et al., 1986; Oshikane et al., 2007: Novotny et al., 1995
-Photo-activated light microscopy (PALM) -Fluorescence PALM (FPALM) -Stochastic optical reconstruction (STORM)	Photoactivation/photo switching and localization of single fluorescent molecules	10-40 nm (Lateral) ~ 10-50 nm (Axial)	Seconds	Hess et al., 2006; Betzig et al 2006; Rust et al., 2006; Heilemann et al., 2008; Huang et al., 2008; Shtengel et al., 2009
Stimulated emission depletion (STED)	PSF shrinking by stimulated emission depletion	30-50 nm (Lateral) 30-600 nm (Axial)		Betzig et al., 2006; Rust et al., 2006; Donnert et al., 2006; Hell and Wichmann, 1994
Saturated structured illumination (SSIM)	Moiré pattern by spatially structured illumination	~ 100 nm (Lateral) < 300 nm (Axial)	Seconds	Gustafsson, 2000; Burnette et al., 2011; Andersson, 2008;
Ground state depletion (GSD)	Depletion of ground-state energy of out-of-focus molecules	~ 15 nm (Lateral)	Minutes	Hell and Kroug, 1995
4Pi and I^{5}M	Coherent addition of spherical wavefronts of two opposing high aperture angle lenses	~ 100 nm (Axial)		Bewersdorf et al., 2006; Egner et al., 2002; Egner et al., 2004; Egner and Hell, 2005; Geisler, 2009
Confocal microscopy	actively suppressing any signal coming from out-of-focus planes e.g. by use of a pinhole in front of the detector			Robert et al., 1996
Widefield and TIRF (total internal reflection fluorescence) Microscopy	Formation of evanescence excitation field as light is total internally reflected at an interface between a high- and a low-index medium	~ 100 nm		Chung et al., 2006
Optical coherence tomography	Use of low-coherence interferometry to produce a 2D image of optical scattering from internal tissue microstructures	Few µm	Minutes	Huang et al., 1991

addition, the limit implies that radiation of much lower wavelengths e.g. x-rays, can achieve higher resolutions, but this leads to the loss of the aforementioned advantage of visible light, that is, minimal invasiveness. As a consequence of tireless research, several techniques to innovatively overcome the Abbes resolution limit has been developed, as summarized in Table 2. Principles underlying the working of these techniques laid out in Table 2, have been presented in different articles and reviews (Weisenburger and Sandoghdar, 2015; Won, 2009; Hell et al., 2009).

IMAGING TECHNIQUES

It is projected that in the coming years, improvement in both spatial and lateral resolution combined with time-based sampling will be on the increase. In addition, more techniques will improve the ability to image highly dynamic molecular and cellular substructures. These innovative techniques will most probably lie on improvement of the existing microscopy methods, ultrasound, computed tomography (CT), magnetic resonance I maging (MRI), positron emission

Table 3. Comparison of micro-, mini- and clinical-scale CT.

Parameter	Suitable for	Resolution μm)	Transaxial scan field-of-view	Time of scan	References
CT	Large animals up to humans	> 450	> 20 cm	Few seconds	Weissleder, 2002; Meng et al., 2006; Thomasson et al., 2004.
Mini-CT	Mice, primates, rats , rabbits	100-450	5-20 cm	Sub seconds to few seconds	Meng et al., 2006; Ritman, 2011.
Micro-CT	Tissue samples , insects, mice and rats	5-100	1-5 cm	Seconds to hours	Meng et al., 2006; Ritman, 2011.

tomography (PET), microCT, microMRI, fMRI, MicroPET among others. This review paper briefly discusses in this section the imaging techniques across a multiple resolution scale, giving a summary of each technique's underlying principles and discussion of their advantages and limitations.

PET/MICROPET/MICROSPECT

Positron emission tomography (PET) is an imaging technique that enables performance of *in vivo* measurements of the anatomical distribution and rates of specific biochemical reactions (Phelps and Mazziotta, 1985). In this technique, radioactive decaying nuclides such as ^{15}O, ^{13}N, ^{11}C, ^{18}F, ^{124}I and ^{94m}Tc are incorporated into metabolically active molecules and then intravenously injected into the animal (Massoud and Gambhir, 2003; Kherlopian et al., 2008; Weissleder, 2002). These metabolically active molecules diffuse in the target tissue, after a brief window period and the nuclides begin to decay, emitting positrons that collide with free electrons. This interaction of positrons and free electrons results to the conversion of matter into two 511 keV γ-rays emerging in opposite directions (Nelson et al., 2002; Phelps et al., 2002) which are detected and observed by detector rings. By using radioactive tracers, reconstruction of 3-D images to show the concentration and locations of metabolic molecules of interest is made possible (Gambhir, 2002). For example, molecular events in the course of Cancer development, during therapy or recurrence can be monitored (Phelps et al., 2002; Gambhir, 2002) by PET. Applied that way, PET is a highly sensitive and minimally-invasive technology. An additional strength of PET is that quantitative kinetic data of highly dynamic biochemistry can be acquired repetitively. However, due to the same decay type of the different radioactive tracers, it is only possible to trace one molecular species in a given imaging experiment or clinical scan. Single photon emission tomography (SPECT) also involves the detection and quantification of gamma-emitting radionuclides. This technique is commonly used in experimental oncology to track individual molecules or cells. The molecule or cell of interest is labelled with a gamma-ray-emitting nuclide such as, ^{99m}Tc, ^{111}In, ^{123}I or ^{125}I, injected into an animal and followed using sodium iodide gamma cameras or solid-state cadmium-zinc telluride (CZT) detectors (Groch, 2001). Gamma emitting nuclides are cheap and differ in their decay time, energy and mode of attachment and hence different nuclides are used for different applications. By placing Lead collimators between the animal and detectors, spatial information is obtained, which when obtained in various projections are finally tomographicaly reconstructed.

MRI/MICROMRI AND FMRI

When imaging an object by Magnetic Resonance Imaging (MRI), the object is placed in a strong magnetic field. This magnetic field aligns the spins of the hydrogen nuclei, predominantly in water and fat, in a direction parallel to the field. Then using a radio frequency pulse, the sample's protons spins can be made to tilt and precess (Nazarian et al., 2013). A radio frequency receiver records the resulting signal, which is can readily be constructed to produce detailed pictures of organs, soft tissues, bone and virtually all other internal body structures (Curie et al., 2013). At much higher magnetic field strengths, which improves resolution, the method is referred to as MicroMRI. Functional magnetic resonance imaging or functional MRI (fMRI) is a functional neuroimaging procedure based on MRI, and used to image brain activity in response to specified stimuli. When a stimuli requires a response in a particular area of the brain, metabolism and hence demand for blood flow and more oxygenated hemoglobin increases in that area. The ratio of oxygenated and deoxygenated brain is altered in

that region and hence an image taken will have a different contrast, especially when compared with the baseline measurements (Parrish et al., 2000).

ULTRASOUND

Detection of reflected sound waves (20-60 MHz; 2-10 MHz in humans) as echoes by probes forms the basis of ultrasound imaging. A handheld probe that sends pulses of sound into a patient's body is moved over the body using water-based gels. The gels are used to avoid intense sound reflections at the borderlines between ultrasonic probe and the patient's skin resulting from air pockets. The depth of the tissue reflecting the sound waves is inferred from the time it takes for the echo to travel back to the probe. Computer Algorithms are then used to interpret these echo waveforms to construct an image (Jensen, 2007). Ultrasound major strengths are that it is less expensive compared to other techniques, it is more patient friendly e.g. there is no claustrophobia, and it is dynamic making real time observation possible which makes artifacts detection easier than in other techniques (Lento and Primack, 2008). A major drawback arises due to techniques' dependence on body's general constitution; resolution is compromised in obese and muscular patients for example (Lento and Primack, 2008). However, improvement of visualization and resolution of deeper structures is improving with recent refinement of tissue harmonics (Rosenthal et al., 2001).

CT/MICROCT AND MINI-CT

X-ray computed tomography (CT) measures the absorption of X-rays as they pass through tissues. Intrinsic differences in absorption between bone, fat, air and water result in high-contrast images of anatomical structures in CT. Due to the CT relatively poor soft-tissue contrast and inability to differentiate between tumors and surrounding tissue, iodinated contrast agents, which perfuse different tissue types at different rates, are commonly used to delineate organs and tumors. Practically, a low X-ray source and a detector rotate around the subject, acquiring volumetric data.

The detectors are typically charged coupled devices (CCD) and act to photo transduce incoming X-rays (Kalender, 2006). Micro- and mini-CT are scaled down CT-imaging modalities for small animals, which in principle provide the same information about morphology and disease status or disease progression for animals as clinical-scale CT does for humans. However, several major differences compared to clinical CT scanning exist as reviewed by Bartling et al. (2007). For animal studies, microCT machines can be used which typically operate with higher energy X-rays when compared to human scanners. The increase in energy improves resolution, but exposes the specimen to more ionizing radiation which has adverse health effects. A comparison of the three is shown in the Table 3.

A key advantage of CT is its high spatial resolution, 12 to 50 µm (Weissleder, 2002; Meng, 2006) which is needed to visualize fine anatomical details. CT can also be combined with functional imaging technologies that provide dynamic and metabolic information. The radiation dose of CT, however, is not negligible and this limits repeated imaging in human studies due to health risks (Thomasson et al., 2004).

OPTICAL MICROSCOPY APPROACHING TO NANOSCOPY

Optical microscopy could be regarded as the most significant tool to visualize objects that are usually invisible to the naked eye. It's a technique with the capability to measure surface morphology and localize molecular and protein distributions *in vivo*. Through microscopy, our knowledge about the 'micro-world' has been greatly enhanced. Technological improvement, coupled with the advent of green fluorescent protein (GFP) leading to rise of fluorescence microscopy has been a major step forward in the study of living cells. Traditional fluorescence microscopy suffered from the need to use high energetic laser light, hence photobleaching the samples and detection of out of focus excitations, further impairing the resolution already limited by Abbes equation. Improvement of traditional fluorescence microscopy, has led to development of new microscopy techniques such as multi-photon and confocal and microscopy to deal with the two drawbacks of traditional microscopy respectively (Yuste, 2005). Following continuous and ongoing research, in effort to break the diffraction barrier, other techniques like stimulated emission depletion (STED) (Betzig et al., 2006), photo-activated localized microscopy (PALM) (Hess et al., 2006; Betzig et al., 2006) and stochastic optical reconstruction microscopy (STORM) (Rust et al., 2006) has been developed. These methods have improved resolution to, 30 to 50 nm for STED and 10 to 40 nm for PALM/STORM (Heilemann et al., 2008, Huang, 2008; Meyer et al., 2008). In STED microscopy two pulsed lasers are used, one laser pulse is chosen to have the wavelength that excites fluorophores, and a second donut shaped laser pulse that depletes fluorescence follows instantaneously. The wavelength of the second laser is tuned to be longer than the fluorescence emission. Consequently, photons from the second laser induce electrons to drop to a lower energy level (stimulated emission) averting typical fluorescence, and hence achieving emission depletion. The final result would be a very small area (center of the donut shaped depleting beam) from where the fluorescence is detected. This area is smaller than a diffraction-limited spot. The

principle behind STORM and PLAM is the ability to stochastically switch on and off (Huang et al*.,* 2009; Huang et al*.,* 2010) a small subset of fluorescent molecules, the only difference is that PALM uses photo-activatable fluorescent proteins and STORM uses photo-switching of pairs of cyanine dyes (Bates et al., 2007; Rust et al., 2006; Heilemann et al., 2008). If the number of activated fluorophores is kept low enough so that the distance between the molecules is larger than the resolution limit, each fluorophore molecule can be localized. A super-resolved image is then obtained by the superposition of many wide-field images containing information on the location of different single molecules (Hess et al*.,* 2006; Betzig et al*.,* 2006; Rust et al*.,* 2006).

An additional innovative microscopic technique that achieves resolution at the nano-scale (< 10 nm) is the Förster Resonance Energy Transfer (FRET). The technique has been reviewed extensively by Sekar and Periasamy (2003), and Roy et al. (2008) among others. The technique is based on resonance energy transfer whereby, an excited donor fluorophore in close proximity (< 10 nm) to an acceptor fluorophore, whose absorption spectrum overlaps with the emission spectrum of the donor, non-radiatively transfers some of its excited state energy to the acceptor in a manner dependent on dipole-dipole resonance coupling (Pietraszewska and Gadella, 2010). Hence a positive FRET occurrence estimates that the two fluorophores are within 10 nm (Berney and Danuser, 2003). With a confocal microscope, one way FRET is performed is by acceptor-bleaching method as described in earlier publications by former colleagues (Orthaus et al., 2008; Orthaus et al., 2009) in use to study the structure of the human Kinetochore. Recently, the technique has demonstrated, to be a valuable tool in our study the centromeric chromatin (Llères et al., 2009). This is the chromatin site where the kinetochore structure is anchored by the presence of CENP-A histone and longer links made possible by the CENP-C terminal tails as we have reported in Abendroth et al. (2015). A combination of the STORM and FRET in the study kinetochore structure and centromeric chromatin is expected to be a major breakthrough in understanding of these highly dynamic structures in the cell cycle.

BIOLUMINESCENCE IMAGING

Bioluminescence imaging (BLI) is a technique similar to Fluorescence Optical imaging, the difference is that here, the few photons being measured emanate from cells that have been genetically engineered to express luciferases (Sato et al*.,* 2004). Luciferases are enzymes that catalyze the oxidation of a substrate to release photons of light (Greer and Szalay, 2002). These enzymes are isolated from a variety of species for example, luciferase from the firefly catalyze the oxidation of luciferin, whereas luciferase from the sea pansy *Renilla reniformis* catalyze the oxidation of coelenterazine causing the release of a photon (Sadikot and Blackwell, 2005). Because mammalian tissues do not naturally emit bioluminescence, *in vivo* BLI has considerable appeal because images can be generated with very little back-ground signal. Credit to genetic engineering, a number of luciferases that results to a spectral shift of released photons have been created (Loening et al*.,* 2010). This, coupled with high sensitive optical filters, imaging of two or more cellular proteins, or their mutants can be tracked simultaneously. BLI is inexpensive, a good alternative of PET imaging, however, it is not likely to be used in a clinical setting, because it can only monitor transgenically modified tumour cells and because it is difficult to detect photons released from deep tissues (Weissleder, 2002).

FLUORESCENCE-MEDIATED TOMOGRAPHIC IMAGING

In this technique, fluorescently labeled probe of interest is exposed to light from different sources. The resultant fluorescence is detected by various detectors positioned in a spatially defined order in an imaging chamber. The obtained information is reconstructed using an algorithm, giving a 3-D reconstructed tomographic image that makes it useful for clinical measurements. The potential of FMT for laboratory use is great since it does not require radioactive labeling and it can make use of the increasing number of fluorescent tags (Ntziachristos and Weissleder, 2003). As a major strength of this technique, it has been demonstrated that a combination of measurements of fluorescent and of intrinsic contrast can provide self-calibrated tomographic data that could yield absolute fluorochromes concentration reconstructions (Ntziachristos et al., 2002). In addition the technique can do in vivo observations (Ntziachristos et al., 2002).

CONCLUSION

A review of the diverse imaging techniques used for diagnosis and research has been presented though the list is not exhaustive. It is very clear that no single technique can achieve everything that needs to be observed, each technique has its own strengths as well as limitations. These unique strengths and drawbacks dictate where and when to apply a technique e.g. ultrasound for its low associated risks is applied in viewing fetus, but it is of no use to image lungs and bones for example because of the very high reflections at air-tissue and bone-tissue interfaces. Another major factor that makes each technique unique is the resolution. This is a consequence of biological systems being complex, and so is the radiation interaction with these systems. Infact there is a resolution gap between techniques that are suitable for experimental research

and clinical imaging, that is, there is a gap between anatomic and microscopic scales. To address the resolution gaps, a combination of imaging techniques is recommended and indeed these combinations are being tried out. For example there have been successful attempts to bridging fluorescence microscopy and electron microscopy. Another example is the combination of PET and CT scanners which makes it possible to acquire metabolic information recorded with higher resolution anatomical CT images.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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