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Optical imaging probes for biomolecules: An introductory perspective.

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5 An overview of optical biomolecular imaging is provided. Following a brief history of the development of probes and technologies in this area, general approaches used image biomolecules in current commercial systems are discussed. A brief summary of research challenges in this area – in terms of both the chemistry and technique development - is introduced. Finally, areas rich for possible future development are suggested.

10 Introduction

Attributed to the Apostle St Thomas, the familiar phrase "*Seeing is believing*", is the philosophical basis of all sensor and image probe technologies. Although, the design of synthetic, molecular-based, targeted sensors only really emerged as a clear research 15 discipline in the last few decades, as is often the case, Nature got there first.

Research into the molecular basis of the five senses has revealed the exquisite sensitivity of these biological systems: a rod cell within the eye can detect a single photon, olefactory 20 receptors (which, interestingly, can only detect molecules with molecular weights below 300 Da^1) can immediately detect the presence of specific chemical functional groups, and the mechanoreceptors of the human cochlea can detect sound driven vibrations of only 0.3 nm and differentiate 1 Hz differences in 25 tones pitched at around 1000 $Hz.^2$ In fact, cellular functions throughout the cell cycle are predicated on a complex network of signalling systems triggered by the detection of specific molecular substrates that consequently up- or down-regulate biological pathways and events.

30 In all these natural biological sensor systems the same protocol is used: Perception, Signal Transduction, and Response – a sequence that has become familiar in the design of synthetic systems. While the generation of intentionally designed small ion and molecule sensors following these principles has greatly relied 35 on developments of specific macrocycles, optical imaging probes for cellular components and biomolecules have a much longer history.

Optical microscopy stains – a brief history

Since most cells are transparent and largely colourless, detailed 40 cellular studies could only truly begin after the development of differential cell stains; this process began in earnest in 1858 when Joseph von Gerlach outlined how dilute solutions of carmine could be preferentially taken up by, and stain, the nuclei of brain tissue cells. [3] In the following decades, a number of significant 45 breakthroughs in this area were made. Despite the fact that most stains were generally non-specific, and had affinities only for certain broad categories of molecules - such as proteins, nucleic acids, or lipids - several imaging protocols developed in this period are still important today.

50 Notably, Hans Christian Gram delineated the staining methodology that bears his name. Gram staining - which is still used as one of the first steps in classifying bacteria - relies on the fact that, whilst the purple stain crystal violet is retained in peptidoglycan-rich cell walls of Gram-positive bacteria, a 55 counterstain is required to image Gram-negative strains. [4]

Perhaps more remarkably, one of the cell staining systems developed during this early period of discovery, over a century ago, [5] is still the most commonly used imaging agent for medical diagnostics.
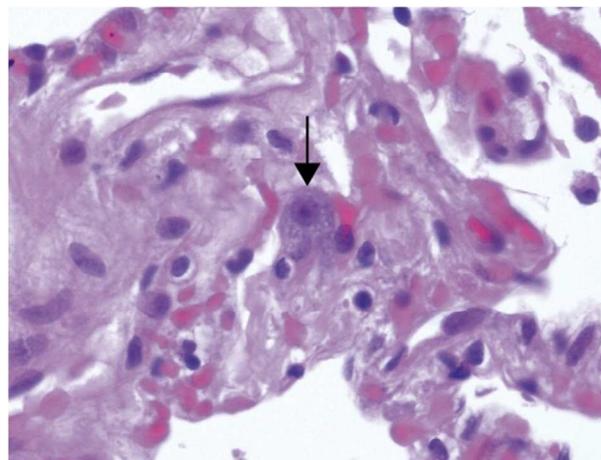
60 **Fig. 1** H&E staining used in a lung biopsy of a SARS patient - arrow marks an enlarged pneumocyte. Image from Nichols *et al*, [6] with permission from Elsevier.

As its name implies, the haematoxylin and eosin, H&E, stain is 65 a combination of two stains. Haematoxylin, which is a derivative of the Central American logwood tree, is the only natural product stain still commonly in use. When haematoxylin is dissolved in water and oxidised, addition of Al^{3+} ions produces haemalum, a dye that stains nucleic acids blue. The second component of

H&E, eosin, is a general counterstain that produces red and pink colours when it non-specifically binds to proteins.

Although the use of H&E was first described almost 140 years ago, it is still considered to be "*the standard morphological* ⁵ *staining method for just about every histological laboratory in the world*" [7] and is used millions of times a day in general histology and cancer diagnosis - see Fig 1.

Apart from the fact that classical stains like those used in the Gram and H&E protocols are generalized stains with low ¹⁰ selectivities against specific biomolecules, they image cellular structures through absorbing transmitted light and thus the contrast they provide is finite. In contrast, luminescent dyes can theoretically provide infinite contrast, thus potentially providing an advantage of higher sensitivity and image resolution. ¹⁵ Consequently, following the introduction of fluorescence-based microscopes in the early twentieth century and the identification of many luminescent dyes for specific biomolecules and cellular structures, this became the preferential optically-based microscopy technique. [8]

²⁰ In the twenty-first century, research in cytology, molecular biology, and medicine and diagnostics has become more and more reliant on optical microscopy and as new techniques are developed, this trend can only continue. Furthermore, the desire to move from non- or low specificity *stains* of tissues and cells to ²⁵ optical *probes* designed to bind to intracellular targets with high specificity has motivated much research in this area.

Taken together the reviews in this special issue present detailed snap-shots of the present state of play in many aspects of research in this rapidly expanding area. This article provides a ³⁰ brief curtain-raising introduction into some general aspects of this area.

Biomolecular probes – general design principles

Broadly speaking, two main approaches have been employed in the construction of these systems. In one approach luminescent ³⁵ small molecules have been designed intrinsically to bind target biomolecules, whilst the second approach involves hybrid systems where photoactive moieties are attached to molecules or macromolecules that recognize the defined target.

Established small molecule fluorescence probes for ⁴⁰ biomolecules

Due to their pivotal roles in biological processes, a range of imaging systems for RNA and DNA in live cells has been developed. The Hoechst, SYTO and DRAQ series of stains typify small luminescent molecules commercially developed for these ⁴⁵ applications. They are all taken up by live cells, interact with, and allow for imaging of nucleic acids through two major binding motifs. [9] The central fused rigid tricyclic structure of the anthroquinone derivative DRAQ5 is typical of an intercalating moiety, which inserts between base-pairs. In contrast, the ⁵⁰ bis(benzimidazoles) Hoechst dyes such as H33258 bind to nucleic acids through minor groove binding. A very wide range of cyanine-based luminescence probes has been used to image nucleic acids. Although some cyanine probes, such as the cell-impermeant dyes TO and TOTO, are known to be intercalators, ⁵⁵ the SYTO systems are – like the Hoechst dyes - minor groove binders. [10]

Lipid structures can be imaged through labelling of specific lipid molecules, for example the fluorescent Cholera Toxin B subunit can be attached to GM1 lipids. [11] Lipids can also be ⁶⁰ selectively imaged using small molecules; although a range of probes has been used, the properties of the dye 6-acyl-2-dimethylaminonapthalene, Laurdan, make it particularly suited towards this task as it displays polarity-sensitive solvatochromism. Notably, since a 50 nm blue-shift is observed ⁶⁵ in dye emission on moving from a polar (liquid disordered) to nonpolar (liquid ordered) environment, this phenomenon can be used to probe ratiometrically ordering within the lipid membranes of living cells and vertebrae organisms. [12]
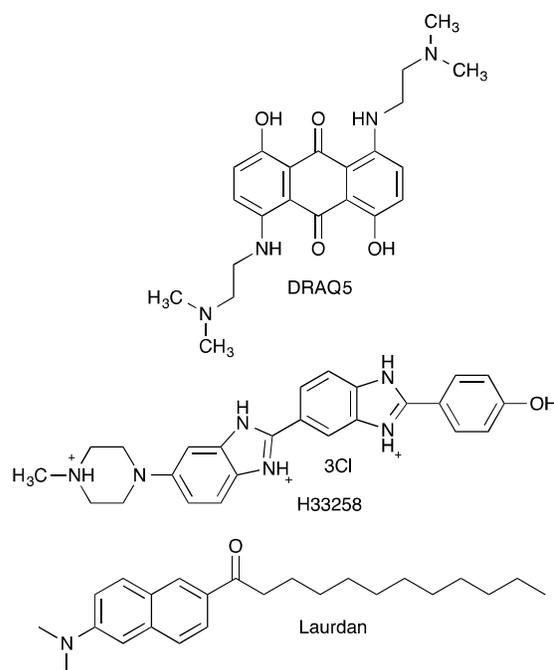
⁷⁰ **Fig. 2** Examples of common, commercially available, small molecules probes for biomolecules. DRAQ5 (top) is a DNA intercalator; whereas H33258 (middle) is a DNA groove binder. Laurdan (bottom) is used to image lipid-rich structures such as membranes.

Compared to nucleic acids and lipids, carbohydrates and ⁷⁵ proteins have a very much greater structural diversity and this means that general small-molecule-based cellular imaging probes for these biomolecules are not widely available; [13] although, as a number of reviews in this special edition illustrate, approaches towards these goals are rapidly making progress.

⁸⁰ Hybrid fluorescence probes methods for biomolecules

In the case of nucleic acid imaging, this second approach encompasses several methods involving *in situ* hybridization. [14] Fluorescence in situ hybridization, (FISH) exploits the ability of complementary oligonucleotide sequences to recognize each ⁸⁵ other. [15] In FISH, a luminescent moiety is attached to a single stranded oligonucleotide that is complementary to a specific target sequence. By using a combination of fluorophores multiple sequences can be targeted; so, for example, FISH has been used to visualize gene transcription simultaneously at multiple sites ⁹⁰ within a single fixed cell nucleus. [16]

In situ hybridization has also been used to construct fluorescence resonance energy transfer, FRET, probes. In this

case, two oligonucleotides designed to hybridize to adjacent regions on a nucleic acid target sequences are labelled with donor and acceptor fluorophores at the 5' and 3' ends respectively. As luminescence is only generated when both probes hybridize to the target, FRET methods enhance signal-to-noise as no output is generated by unbound probes. [17] A related phenomenon is exploited in the nucleotide-based imaging probes known as molecular beacons. In this case a single probe sequence that forms a stem-loop oligonucleotide hairpin is employed. One end of the sequence is appended with a fluorophore and the other with a quencher moiety. In the folded hairpin these two tethered units are held in proximity and luminescence is suppressed. On hybridization to the target sequence the fluorophore-quencher pair is separated and emission is "switched-on." [18] These systems not only offer high signal-to-noise ratios, but – because they are initially hairpin structures that only linearise through a competitive hybridization to a target sequence - they exhibit a higher specificity for perfectly complementary nucleic acid targets compared to conventional linear oligonucleotides. Consequently, molecular beacons are the most widely adopted class of nucleotide-based probes for live-cell imaging, but because DNA is relatively inaccessible for hybridization as it is found as a duplex packed into the nuclear chromatin structure, this technique is predominantly employed in RNA imaging.

The major problem with these nucleotide-based methods is poor cellular penetration: nucleotides are polyanionic hydrophilic macromolecules that do not readily permeate cellular membranes, therefore such systems have to be microinjected into individual cells or delivered using other mechanical or chemically-based transfection techniques. Similar hybrid systems for DNA imaging, for example involving antibody targeting systems or fluorescent proteins, commonly present similar delivery problems.
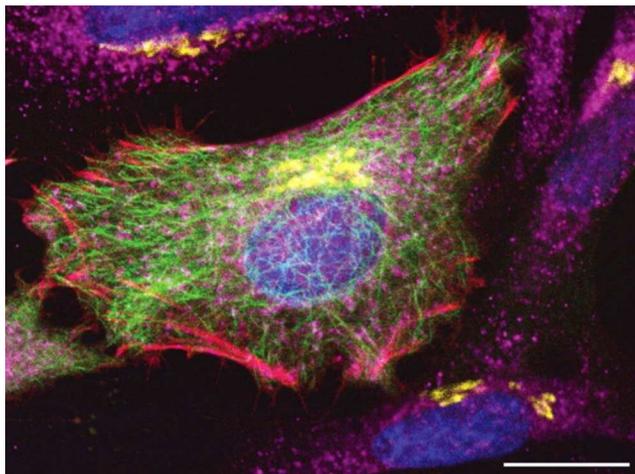
Fig. 3 Fixed HeLa cells stained through the parallel application of a range of targeting methods and fluorophores. HeLa cells transfected with GFP–a-tubulin and tetracysteine–b-actin were stained with ReAsH. After fixation, cells were immunolabeled for the Golgi matrix protein giantin with quantum dots and for the mitochondrial enzyme cytochrome c with Cy5. DNA was stained with Hoechst 33342.Scale bars, 20 μm. Image from Giepmans *et al*,[19] with permission from the American Association for the Advancement of Science.

Protein imaging is dominated by two hybrid approaches. In immunolabelling, small molecule dyes are attached to primary or secondary antibodies. [19] However, again, this technique is largely restricted to permeabilised cells or proteins that are compatible with endocytosis. With the identification of green fluorescence proteins (GFP) and related fluorophores, the second approach - of genetically encoding GFP, or one of its variants, as a fusion to the target protein or gycoprotein within a cell – is the default method of protein labelling. Nevertheless, despite the power of this method, due to the significant size of FPs, there is always the possibility that the fusion may interfere with endogenous protein function.

As illustrated by Figure 3, by using a range of currently available optical probes, some impressively detailed images of intracellular components can be obtained. However, as outlined in the next section, there are still many aspects of cell structure and dynamics that are not so readily imaged. Furthermore, as microscope technologies are developing probes with specific optical properties are also required

Current challenges

The reviews in this special edition will illustrate in more detail the following issues that shows that current research involves both extending the range of biomolecules that can be imaged and the output modalities.

Improving biomolecular targeting

As can be seen from the previous brief - and far from comprehensive – précis, certain classes of biomolecules are poorly served by existing imaging technologies. In particular it is clear that, due to their diversity and their composition from largely aliphatic components, probes for carbohydrates are a current challenge and have not been developed to the extent of other classes of biologically important molecules. Clearly, if these challenges can be met, this is an area with much potential for exciting development. There are a number of other areas where imaging probes are still lacking. For example, although nucleic acid probes are established, optical imaging probes for the wide range of biologically active monomeric nucleotide structures are less well developed.

In ground-breaking work, the Hamachi group has reported on Zn^{II}-complexes as *in-cellulo* luminescent probes for ATP and related molecules [20]. However, despite their vital biological roles, selective cell probes for specific nucleotides, such as the cyclic nucleotide-based secondary messengers (eg cAMP, cGMP), are not readily available. Indeed, generally speaking, research into optical probes for all signalling molecules is potentially another area for future growth.

In the shorter term, targeting capabilities over available probes can still be improved; nucleic acid provide a case in point. Although, as outlined, general probes for both DNA and RNA are now readily available, substantial proportion of current research in this area is centred on visualizing nucleic acids at the sequence/structure level. This would be a hugely attractive prospect; for example, specific sequences and non-canonical structures, such as quadruplexes, are associated with particular disease states, including cancer. To image such structures in live cells or even *in vivo* will facilitate insights into the detailed dynamic mechanisms of their (dys)functions, and also provide the basis of new convenient optically-based medical diagnostic

methods.

Improving optical properties

The majority of commercial optical probes rely on simple emission intensities to locate target structures. There are disadvantages to this approach that are being addressed through a number of approaches.

Ratiometric sensing of biomolecules

Conventional emission sensing relies on "off-on" switching of luminescence intensity. However. ratiometric probes - which commonly exploit analyte-induce changes in the ratio of emission intensities at two different wavelengths - are hugely more convenient. As the probe response is independent of its concentration, analyte concentrations can be quantitative determined irrespective of probe concentration.

The impact of this concept, first described by Tsien and co-workers in a 1985 paper on Ca^{2+} sensing, [21] is reflected by the fact that their original study is one of the top 50 most cited papers in science with over 20,000 current citations. [22] As a consequence, reports on ratiometric sensors for cations and anions now abound, with many of these systems functioning within live cells. In contrast, *in cellulo* ratiometric sensors for biomolecules are considerably less common.

Two photon Absorption

Commonly, commercial fluorescent dyes used in live cell imaging are photoexcited by relatively high-energy light (350 – 450 nm); such energies are, in themselves, deleterious to cells as they can irreversibly damage DNA and generate cytotoxic reactive oxygen species, ROS. Furthermore, due to the presence of endogenous chromophores, cells and tissues only show maximum transparency in a "biological optical window" of 650 to 1350 nm, meaning that traditional optical microscopy is restricted to depths of 100 μm. For both these reasons, dyes that are photoexcited in the red/infrared region are being sought. In these circumstances depth penetrations of up to a millimetre can be achieved, allowing deep optical imaging of tissues rather than 2-D cell cultures. This can be accomplished using dyes that are photoexcited through two photon absorption, 2PA. [23]

The 2PA process allows access to an excited state using photons of half the energy (or twice the wavelength) of the usual one-photon excitation. Broadly, a dipolar dye with an extended π-delocalized bridge will have enhanced 2PA properties. [24] A second advantage of using 2PA is that it provides enhanced imaging resolution. Compared to a conventional one photon system, emission from a 2PA dye is highly dependent on incident light intensity (quadratic vs linear dependency), therefore all out-of-focus emission is supressed, this also means that dye photobleaching is greatly reduced. [25]

Although the irradiating energy used in these techniques is lower than conventional methods, the overall laser power used can be considerably higher and this may potentially lead to photodamage in itself. This problem can be circumnavigated through the use of pulsed laser sources.

Although a number of readily available probes are already used in this form of microscopy, commercial systems generally have low 2PA cross-sections or poor photostabilities in the required conditions, therefore there is still a need to develop new 2PA probes with enhanced optical and intracellular targeting properties.

Lifetime emission probes

Techniques based on the emission lifetime of a bioprobe offer a number of advantages compared to conventional emission wavelength/intensity-based microscopy. For example, in lifetime imaging microscopy, LIM, the optical output of a probe is independent of probe concentration or incident light intensities, the use of fluorescent and phosphorescent dyes with lifetime well above those of endogenous flourophores negates interference due to autofluorescence from endogenoous fluorophores, additionally - assuming a difference in lifetimes - LIM can be used to distinguish multiple fluorophores with overlapping emission wavelengths. Furthermore, since emission decay is often dependent on physical properties, such as local viscosity, pH, or oxygen concentrations, LIM can provide information on the micro-environment of the probe itself. [26]

Despite these advantages compared to conventional technologies, it is only thanks to the recent increased availability of commercial instruments that research into LIM has begun to develop rapidly in the last decade. Whilst much research in this area still involves improving instrumentation, new LIM-compatible probes for specific biomolecules are also required; in particular, longer lifetime probes are being sought as this allows for increased environmental sensitivity, for example towards oxygen concentration mapping or FRET effects.

One possible disadvantage of LIM is that *any* electron or energy transfer process can competitively deactivate the emissive state, so imaging of specific targets can be affected by the presence of common species such as the aforementioned oxygen, as well as specific endogenous fluorophores.

Breaking the diffraction limit

Until relatively recent, all forms of optically based microscopy suffered from a spatial resolution barrier. In 1835 Airey described circular distortions caused by closely spaced points - now known as Airey discs - that occur due to light diffraction at the lens aperture. Around 40 years later, Abbé mathematically defined the diffraction limit (d) for imaging at a given wavelength (λ) of light in a medium of refractive index (n) as $d = \lambda/2n(\sin\theta)$, where θ is the angle defined by the cone of focussed light. This relationship means that in conventional optical microscopy, spatial resolution – the largest distance at which the image of two point-like objects seems to merge - is restricted approximately to half the wavelength of the imaging light. [27] Therefore, shorter wavelength light produces better resolution than longer wavelength light. However, as explained above, high-energy light is deleterious to cells. So, in practise, resolution is normally restricted to features above 200 – 250 nm. However, in the last decade a number of techniques, collectively known as super-resolution microscopy, SRM, have emerged that allow this diffraction barrier to be broken.

Super resolution can be achieved by exploiting the intrinsic emission properties of specific probes and/or through sophisticated data-processing algorithms. These concepts are illustrated by examples

In a technique such as structured illumination microscopy (SIM), probes that are photostable throughout image collection are required, so many existing conventional optical probes are suited to these technologies. In SIM a periodic illumination pattern is projected onto a sample and then a set of images are recorded after translation and rotation of this mask pattern. Super

resolution is then achieved through a post-collection mathematical data analysis. [28] Using specific illumination sequences, 2-D resolutions down to 50 nm can be obtained, furthermore image collection speeds are also suitable for dynamic live cell imaging. [29] In contrast to the image processing approach of SIM, most other SRM technologies are also reliant on stochastic emission from single probe molecules. Perhaps the best know approach exploiting this concept is stochastic optical reconstruction microscopy, STORM.

Techniques such as STORM, are dependant on luminophores that can switch between emissive ON and non-emissive OFF states during image collection. In the STORM experiment at any given moment only a small fraction of probes are in the ON state, thus emission from individual molecules is highly likely to be spaced out further than the resolution limit. The positions of these outputs are then precisely defined by fitting to a point-spread function. Through collection of a stacked series of images, a combined map - typically incorporating the position of 10^4 - 10^7 single probe molecules in the ON state as "pixels" - a final pointillistic super-resolution image is constructed. [30]
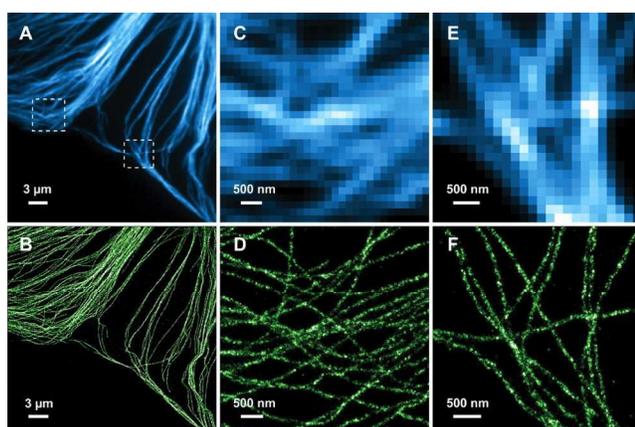
Fig. 4 A comparison of conventional and STORM imaging. (**A**) Conventional immunofluorescence image of microtubules in a large area of a BS-C-1 cell. (**B**) STORM image of the same area. (**C** and **E**) Conventional and (**D** and **F**) STORM images corresponding to the boxed regions in (**A**). Image adapted from Bates *et al*, [31] with permission from the American Association for the Advancement of Science.

Due to its specific requirements, STORM is driving the development of new molecular probes. Probes with high quantum yields but short lifetimes are needed to supply ON/OFF blinking. This was initially accomplished through the use of photo- or redox switched systems. More recently dSTORM (direct STORM), in which bright probes switch to dark charge-transfer or triplet states, has facilitated the use of conventional dyes in this super-resolution technique. [32] However this technique can potentially yield reactive oxygen species that will enhance cellular photodamage

Although SRM has developed at a fierce rate in the last decade, challenges still remain; for example, resolution in three-dimension needs to be improved, whilst the development of 2PA-STORM probes will allow live super-resolution imaging over longer time frames and at greater depth.

Outlook

As Neils Bohr is reputed to have stated; "*Prediction is very difficult, especially about the future.*" Nevertheless, it does seem clear, that several aspects of this research are ripe for further development. Therefore - paradigm-changing breakthroughs aside - I offer some personal, far from complete, suggestions for current areas that provide promise for the future.

A general trend in optical microscopy is increased specificity and resolution; already many cell types, and sub-cellular compartments can be selectively imaged and in some cases specifc proteins and nucleic acid strucures - and sub-structures - have already been targeted. In the future, this work will be extended so that these molecules as well as carbohydrates and glycoproteins can be imaged within a defined compartment.

The photophysical and chemical properties of metal complexes make them well suited to several of the emerging applications discussed above. For example, due to their distinctive coordination geometries, and the diversity of their excited states, d^8, and d^6-metal centre[33] as well as lanthanide complexes[34,35] are forming the basis of an increasing number of optical probes for biomolecules and biomacromolecules.

These species quite often display emission from triplet states (formally phosphorescence) and thus have large Stokes shifts and long lived luminescence. In conventional emission-based optical microscopy these properties are useful for enhancing signal/noise ratios through time-gating image collection, but it also makes them appropriate starting points for the development of live-time probes for LIM. Indeed, given that metal complexes for conventional time-gated confocal microscopy and LIM are already becoming commercially available, it seems research in this area is ripe for further development. The dipolar nature of metal-to-ligand charge-transfer, MLCT, excited states in d-metal based also suggests that they have great potential for 2PA microscopy as well: certainly MLCT has been successfully exploited in systems displaying other nonlinear optic effects.

In recent years, nanoparticles - particularly quantum dots (QDs) and gold nanoparticles (AuNPs) - have attracted increasing attention as potential bioprobes [36] and bioimaging agents. [37] QDs are resistant to photobleaching, display high quantum yields and absorbances, and have tunable sharp emission energies, which can potentially be exploited in multiplexing.

Despite these appealing properties, significant stumbling blocks to the routine use of QDs as optical probes for biomolecules remain, in particular - due to their size - uptake by living cells is restricted and there are concerns about their toxicity. Furthermore while the attachment of macromolecular targeting moieties - such as antibodies - to QDs is now routine, specific targeting has not always been accomplished. In particular, the use of anchored small molecules target specific receptors is still much less developed.

AuNPs possess many of advantages of QDs however and although their optical properties are currently not so versatile, approaches to address this issue are beginning to emerge. Nevertheless, there is no doubt that - as these challenges are met - nanoparticles will provide new tools for optical microscopy.

One emerging area that also seems set to grow in importance is the identification and synthesis of multimodal imaging probes. As more and more technologies for imaging at the cellular and subcellular scale are developed, the possibility of visualizing

living systems with multiple/complementary technologies is becoming increasingly achievable. This approach, will not only provide "orthogonal" evidence of targeting to specific biomolecules, but also provide dynamic information at a range of sensitivities, tissue depths, and resolutions.

Finally, although the term theranostics was coined over 10 years ago [38] to describe molecular systems that could simultaneously provide diagnostic imaging and therapy - and has since been much discussed in the introductory paragraph of many papers - up until recently, many new probes that have been put forward as examples of this concept are just luminescent analogues of extant therapeutics and therapeutic leads that do not provide real diagnostic insight. However, even in the shorter term, this is an achievable target for a number of therapeutic regimes. For example, photodynamic therapy fundamentally requires photo-excitable molecules as sensitizers, which ideally localize in therapeutic targets. It requires virtually no re-engineering to create theranostic systems from this treatment method; although for reasons of penetration depth 2PA systems will be required. In the longer term, technologies for several developing therapeutic areas - such as vectors for gene delivery and systems that target cell death – offer great potential for real theranostics. This is an area where the potential versatility of functionalized nanoparticles offer great promise.

Given that future commercial demands for new biological and medical imaging agents is predicted to increase considerably, it seems the explosion of research interest in this area over the last decade of so will continue well into the foreseeable future. The reviews in this special issue provide authoritative snapshots of the state-of-the-art in this multidisciplinary research subject.

Notes and references

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† Electronic Supplementary Information (ESI) available: [details of any ‡ Footnotes should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

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