Multimodality molecular imaging of disease progression in living subjects

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The enormous advances in our understanding of the progression of diseases at the molecular level have been supplemented by the new field of 'molecular imaging', which provides for *in vivo* visualization of molecular events at the cellular level in living organisms. Molecular imaging is a noninvasive assessment of gene and protein function, protein–protein interaction and/or signal transduction pathways in animal models of human disease and in patients to provide insights into molecular pathogenesis. Five major imaging techniques are currently available to assess the structural and functional alterations *in vivo* in small animals. These are (i) optical bioluminescence and fluorescence imaging techniques, (ii) radionuclide-based positron emission tomography (PET) and single photon emitted computed tomography (SPECT), (iii) X-ray-based computed tomography (CT), (iv) magnetic resonance imaging (MRI) and (v) ultrasound imaging (US). Functional molecular imaging requires an imaging probe that is specific for a given molecular event. In preclinical imaging, involving small animal models, the imaging probe could be an element of a direct ('direct imaging') or an indirect ('indirect imaging') event. Reporter genes are essential for indirect imaging and provide a general integrated platform for many different applications. Applications of multimodality imaging using combinations of bioluminescent, fluorescent and PET reporter genes in unified fusion vectors developed by us for recording events from single live cells to whole animals with high sensitivity and accurate quantification are discussed. Such approaches have immense potential to track progression of metastasis, immune cell trafficking, stem cell therapy, transgenic animals and even molecular interactions in living subjects.

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1. Introduction

Modern biomedicine has experienced tremendous advancement in developing newer therapeutic strategies, better patient care and disease management during the past two decades. This improvement follows our increasing understanding of cellular and molecular events in the various signal transduction pathways and the development of high-throughput technologies. Along with major development of microscopic techniques for live cell imaging at super resolution, macroscopic techniques to monitor molecular events in living organism have also progressed significantly. These noninvasive approaches to analyse structural and functional signatures from small animals are collectively termed as 'molecular imaging techniques' and have become an integral part of modern biomedicine. Currently, molecular imaging techniques can be broadly classified into five categories on the basis of the spectrum and source of energy used for detection (Massoud and Gambhir 2003). These are (i) optical imaging – fluorescence and bioluminescence imaging, (ii) radionuclide imaging – positron emission tomography (PET) and single photon emission computed tomography (SPECT), (iii) X-ray computed tomography imaging (CT), (iv) magnetic resonance imaging (MRI) and (v) ultrasound (US) imaging (Massoud and Gambhir 2003). These are briefly described below.

1.1 *Optical imaging*

The two popular optical imaging modalities (bioluminescence and fluorescence) involve detection of light signatures

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emitted from living animals at visible or near-infrared wavelengths by a sensitive CCD camera. Bioluminescence imaging detects very low levels of light generated due to catalysis of luciferin/coelenterazine substrates by luciferase enzymes and has high sensitivity and low noise. In contrast, fluorescence imaging often encounters high background due to auto-fluorescence and absorption of light signals generated by the excited fluorophores (present in fluorescent proteins) or flurochromes (present in fluorescent dyes, nanoparticles or quantum dots) and is less sensitive (Massoud and Gambhir 2003). Both these modalities possess a spatial resolution of 2–5 mm.

1.2 *Radionuclide imaging*

Like optical imaging modalities, radionuclide imaging, i.e. PET and SPECT imaging, are also functional imaging techniques that detect gamma rays emitted by a radiolabelled (positron emitter or gamma emitter) biomolecule introduced in the body so that it is accumulated in the tissues of interest. In PET, emission of two high-energy gamma rays (511 kev) are simultaneously detected by two detectors situated 180° apart (an event called coincidence) in the scanner, while in SPECT, only one low-energy gamma ray is detected by single detector. To avoid detection of multiple events by the same detector, SPECT requires lead collimeters that decreases the sensitivity (a log fold lower than PET) (Massoud and Gambhir 2003). In contrast to optical imaging, the radionuclide imaging approaches exhibit higher spatial resolution of 1–2 mm with no limit on the depth penetration.

1.3 *MR imaging*

Change in dipolar movement of the hydrogen atoms present in the biomolecules in a strong magnetic field (created by the instrument) results in emission of weak radio waves. This radio wave is detected and amplified by the MR scanner and then used to reconstruct the images. MR imaging provides both functional and anatomical information of the tissue of interest from living animals (Massoud and Gambhir 2003). MR imaging has highest spatial resolution (25–100 μm) among all the modalities.

1.4 *CT imaging*

Images in CT (spatial resolution of 50–100 μm) are obtained when component tissues differentially absorb x-rays passing through the body and are collected by high resolution CCD detectors. CT is essentially an anatomical imaging modality (Massoud and Gambhir 2003).

1.5 *US imaging*

Ultrasound imaging is probably the best example of noninvasive real-time imaging where reflections of high-frequency sound waves (produced from a transducer) are captured by the detectors. Each tissue reflects the sound with a different frequency based on their density, and therefore, creates contrast images at 50–100 μm resolution. This is classically an anatomical imaging modality. However, Doppler imaging and micro-bubbles attached to antibodies specific for blood vessel are now also being used for functional imaging (Massoud and Gambhir 2003).

Two second-generation imaging modalities, Raman spectroscopy and photo acoustics imaging, using non-radioactive ionization and amalgamation of the advantages of ultrasound and optical techniques are currently under development. While Raman spectroscopy attempts to detect endogenous Raman signatures from tissues, photo acoustic imaging, a hybrid biomedical imaging modality employs measurement of the effect of absorbed electromagnetic energy (particularly of light) on matter by means of acoustic detection. Both these techniques attempt to identify changes in specific tissue/biomolecule patterns to provide high specificity. Since optical imaging experiences depth-related limitations, these techniques would be more suitable for superficial imaging of certain cancers such as melanoma, breast, colon, etc.

2. Imaging approaches

The definition of molecular imaging, as provided by the Society of Nuclear Medicine, is ‘the visualization, characterization and measurement of biological processes at the molecular and cellular levels in humans and other living systems’. Based on the probes utilized, molecular imaging can be classified into ‘direct’ and ‘indirect’ imaging categories: direct imaging involves signal generating moieties targeted to endogenous molecules of diseased tissue. On the other hand, indirect imaging utilizes reporter genes to report on activities of artificially introduced genes/promoters in the cells/tissues in living subjects (Ray *et al.* 2001). Direct imaging of target expression is most desirable for drug development but is difficult to achieve as it requires development of specific labelled probe against each endogenous molecule. Efforts are being made to develop probes against direct targets to differentiate normal and diseased tissues required for better disease management. Some well-known examples are radiolabelled Herceptin (monoclonal antibody against Her2Neu receptor, which is often over-

expressed in breast cancer) or Avastin (monoclonal antibody generated against VEGF receptor required for neoangiogenesis) (Dijkers *et al*. 2009; Nayak *et al*. 2010, 2011). Approaches are also being developed to label these targeted probes with nanoparticles or quantum dots for imaging and better delivery. Indirect imaging, on the contrary, is easy to adapt and is widely in use but is clinically less significant since it requires artificial introduction of the reporter genes in the system. Indirect imaging using reporter gene/reporter probe scheme has, however, turned out to be an essential tool for current preclinical imaging research. A variety of reporter genes have been developed for the radionuclide, optical and MR modalities, which can be used to study specific biological processes like promoter activation, transcription, translation, protein–protein interaction, etc., and monitor disease progression as well as therapy. Signal-generating reporter genes are categorized into different groups, viz., optical, PET and SPECT or MR reporter genes, based on their usage for the different imaging techniques (Massoud and Gambhir 2003).

3. Application of fusion reporter strategy

One of the powerful genetic construct for indirect imaging strategy is a multimodality fusion where two or more reporter genes, joined together with short linkers and controlled by a single promoter, gives rise to a single transcript and a single polypeptide (Ray *et al*. 2001). The length of the linker and orientation of each gene are crucial factors to retain the properties of each component protein. Sometimes one or more reporter proteins lose their activity when fused together. However, even if partial functionality

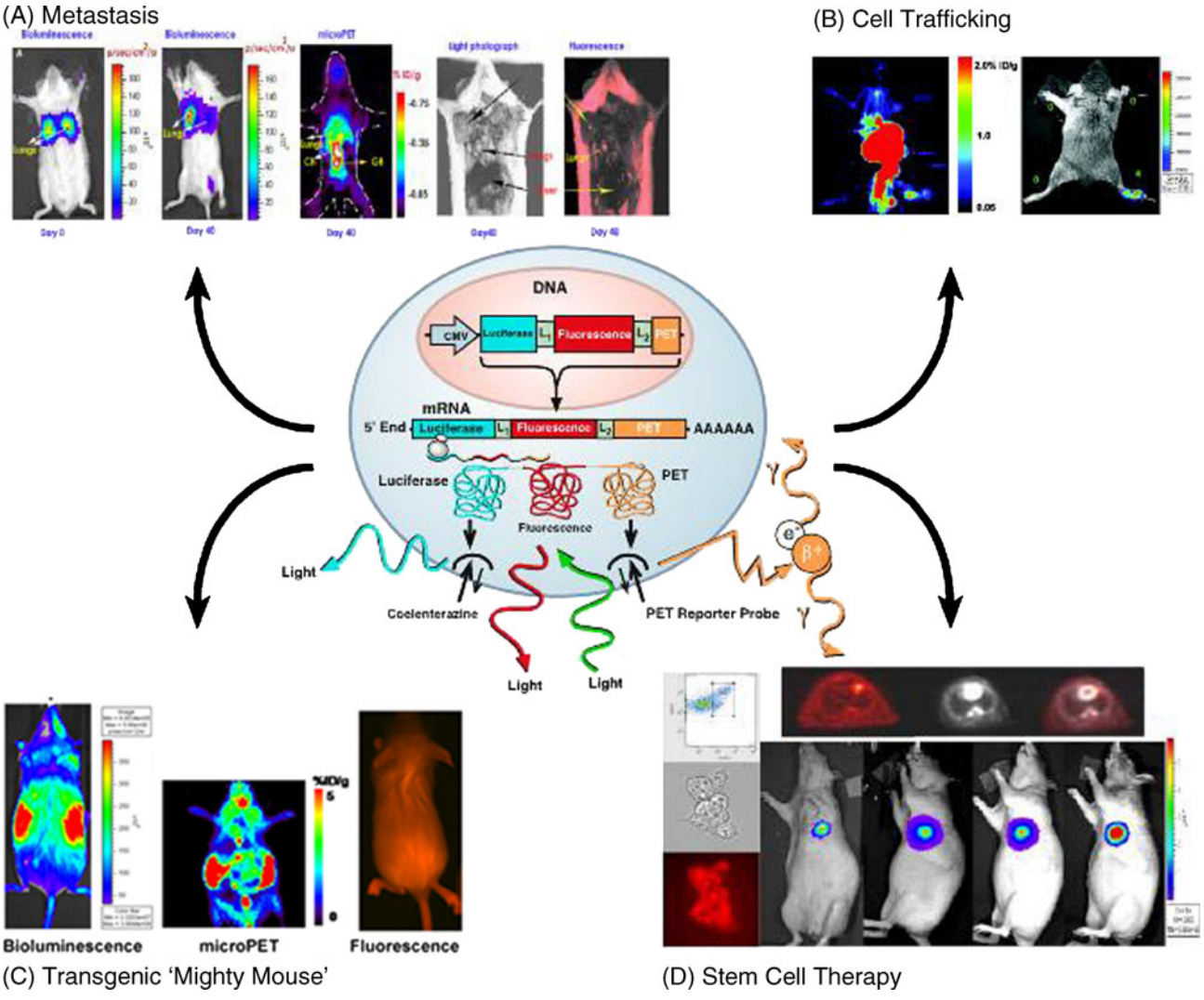

Figure 1. For caption *see* page 502.

can be retained, the fusion reporter vector is the best multimodality vector since decoupling (as seen in the dual promoter based approach) or differential attenuation of gene expression in different cell types (as observed in the IRES based approach) is unlikely to occur (Wang *et al*. 2005). Composition and length of the linkers, and orientation of the polypeptides, are critical parameters that influence the construction of a successful fusion protein. We (Ray *et al*. 2003) were the first to build an optical-PET reporter fusion reporter and demonstrate validation in cell culture and tumour-xenograft-bearing mice by bioluminescence and microPET imaging. We explored various linkers of different lengths and composition and found that a linker of 20 a.a was optimal to retain sufficient activities for both reporters. This bi-fusion reporter could be used to image signals from small living animals but not from live cells (Ray *et al*. 2003). Therefore, we generated a small library of triple fusion reporters where the third partner is a fluorescent protein to facilitate imaging of molecular events in single live cell as well as living animals with high sensitivity and quantification (Ray *et al*. 2004). The ability to noninvasively obtain molecular information in systems ranging from a single live cell to the multicellular environment of an animal holds tremendous potential for studying cancer metastasis, efficacy of drug therapy and many other applications in a preclinical setting. Using various combinations of reporter genes, we showed that *CMV-hrl-mrfp1-ttk* fusion reporter could retain maximum activity of all three reporters when compared with individual ones (Ray *et al*. 2004). Among these fusions, both *hrl-mrfp1-ttk* & *fl-mrfp1-ttk* have been used in various applications by us and other investigators. As shown by us, *hrl-mrfp1-ttk* vector can be used to follow metastasis for a period of 40 days and revealed that while tTK protein provides precise quantification of metastatic tumours, the hRL protein helps to detect a very small lesion that was beyond detection limit of the PET imaging. *In vivo* fluorescence imaging could not be used for detecting these metastatic lesions due to low sensitivity (autoflourescence due to fur and scattering of light); however, tumours were visible in sacrificed and dissected mice (Ray *et al*. 2004) (figure 1A).

These vectors have tremendous application in stem cell therapy and immune cell therapy. In an elegant study, Cao *et al*. (2006) transplanted embryonic stem cells stably expressing *Ubi-fl-mrfp1-ttk* fusion (ESC-TF) in infracted heart of living rats and monitored the survival and expansion of ESC-TF by bioluminescence and microPET imaging (Cao *et al*. 2006). This study revealed the power of molecular imaging to track the side effects of ESCs in therapeutic applications, like the inherent potential of teratoma formation (figure 1D).

In another study, Yaghoubi *et al*. (2007) have also demonstrated that T-cell hybridomas expressing *hrl-mrfp-ttk* fusion reporter proteins migrate to inflamed paws of

Figure 1. Examples of multimodality imaging of molecular events using fluorescence microscopy, bioluminescence, fluorescence and microPET modalities. Schematic diagram of a multimodality triple fusion vector carrying a fluorescence (monomeric red fluorescent protein or enhanced green fluorescent protein), a bioluminescence (firefly luciferase or renilla luciferase) and a PET (HSV1-sr39 thymidine kinase or HSV1-thymidine kinase) reporter gene connected by two small peptide linkers is shown in the middle. Transcription of this fusion vector yields a single mRNA and subsequent translation leads to a single polypeptide that is capable of retaining partial, if not full activities, of all the three proteins. (**A**) Multimodality imaging of metastasis of A375M cells stably expressing the *hrl-mrfp-ttk* fusion reporter gene in living mice: 7×10^5 A375M cells stably expressing the triple fusion were injected via tail-vein in a SCID mouse and imaged for bioluminescence signal following tail-vein injection of coelenterazine at day 0 and day 40. Prominent bioluminescence signals were found from the region of both lungs ($1.3–1.5\times10^5$ max (p/sec/cm^2/sr)) at day 0 and from the left lung (2×10^5 max (p/sec/cm^2/sr)) at day 40. Following a bioluminescence scan, the mouse was imaged in microPET using 18F-FHBG. A strong signal (~0.78%ID/g) was present from the chest region (Ch) with lower signal (0.35%ID/g) from the lung region. The stronger PET signal was found to be from a metastatic tumour present deep inside the body as evident from the fluorescence photograph. Note the gallbladder (GB) retains FHBG, and therefore, a background signal from the GB is also seen in the microPET images. The next panel shows the light photograph of the same SCID mouse after sacrifice and organ exposure, while the leftmost panel is a whole body fluorescence imaging of the same SCID mouse showing fluorescing metastatic tumours in lung and chest regions that correspond with the bioluminescence and PET images (reprinted from Ray *et al*. 2004) with permission). (**B**) Multimodality imaging of immune cell trafficking in collagen-induced arthritis mouse. 26×10^6 A2-TFR cells (joint targeting T-cell hybridomas transduced with a Lentivirus carrying the hRluc-mRFP-HSV1-tsr39tk triple fusion gene) were injected into the tail-vein of collagen-induced arthritic mouse after inflammation had developed in the paws. The mouse was imaged for coelenterazine activity (right panel) and [^{18}F]FHBG distribution (left panel) using the Xenogen CCD camera and the Concorde MicroPET R4, respectively, the day after injection of the cells (reprinted from Yaghoubi *et al*. 2007 with permission). (**C**) Multimodality imaging of a transgenic mouse constitutively expressing the *fl2-tdt-ttk* fusion reporter by optical bioluminescence, fluorescence and microPET: Cells of the transgenic mouse contain the tri-fusion gene, which is driven by the chicken β-actin promoter. Ubiquitous expression of the tri-fusion protein allows imaging of every single cell from the transgenic mouse using bioluminescent, fluorescent, and PET imaging techniques. Injection of D-luciferin and 18F-FHBG is required for bioluminescent (left panel) and PET imaging (middle panel), respectively. Fluorescent imaging (right panel) requires the activation of the tri-fusion protein using a light source. Both fluorescent and bioluminescent images are captured with an *in vivo* optical imager and PET images are obtained by using a small animal microPET scanner. (**D**) Multimodality imaging of murine embryonic stem cells (mESCs) in nude rat myocardium. After lentiviral transduction of mESCs with the *fl-mrfp1-ttk* fusion reporter, about 28% of mESC cells were positive for RFP on FACS and the cell morphology was unchanged under bright field and fluorescence microscopy (left vertical panel). The upper panel shows the microPET images of transplanted cells scanned with [^{18}F]FHBG (left), [^{18}F]FDG (middle), or fusion image (right) in rat myocardium (green arrow is left ventricle free wall). Noninvasive bioluminescence imaging of mESCs in rat heart at day 2, 7, 14, and 21 (from left to right in lower panel) was performed by injecting D-luciferin intra-peritoneally (reprinted from Cao *et al*. 2006 with permission).

collagen-induced arthritic (CIA) mice. Quantified data from optical images demonstrate that the higher the degree of inflammation, the greater the number of T-cell hybridomas trafficking into the paws. However, increasing the number of cells injected intravenously did not result in greater number of cells trafficking to the paws (figure 1B).

Transgenic animals are proving to be vital for developing new treatments and cures for diseases. We have created a transgenic mouse 'mighty mouse', constitutively expressing the *fl2-tdt-ttk* fusion reporter under a chicken β-actin promoter. These transgenic mice are viable and show variable expression of all the three reporters in different tissues (higher expression in muscle, heart and pancreas and lower in liver and intestine). This mighty mouse strain will be a potential source of multiple investigations including stem cell biology and organ transplant (figure 1C).

Finally, a multimodality reporter fusion can be developed using protein-specific cleavage site that will act as a sensor for activation of the particular protein. Ability of any drug molecule leading to activation of the reporter proteins can be imaged indirectly by increase in the signal of multiple signatures. Ray *et al.* (2008) developed such a multimodality sensor that comprised a bioluminescent, a fluorescent and a PET reporter gene linked together by a four-amino-acid spacer (DEVD), a classical caspase 3 cleavage site. Activated caspase 3, a central effecter caspase of many apoptotic pathways, is known (Ray *et al*. 2008) to cleave cellular proteins containing DEVD (such as poly-ADP ribose phosphorylase or PARP, lamins, etc.), leading to the final destruction of the cells. Activation of caspase 3 by two protein kinase C inhibitors can be imaged with this sensor from living cells to living animals using FACS, *in vivo* bioluminescence and microPET modalities (Ray *et al*. 2008). This unique sensor could act as an important screening tool for *in vitro* and *in vivo* testing of caspase activator/inhibitors.

At Advanced Centre for Treatment, Research and Education in Cancer, India, molecular imaging techniques in various research applications are being actively pursued. We are aiming to monitor development of chemoresistance of ovarian carcinoma in *in vitro* as well as *in vivo* animal models. Amplification and mutation is PI3KCA (the kinase domain of phospho inositide kinase or PIK3) gene has been found to be associated with progression of chemoresistance in ovarian carcinoma. We have developed a PI3KCA1 sensor (PI3KCA1 promoter driving *fl2-tdt* fusion) and are currently investigating modulation of this promoter by luciferase assay and bioluminescence imaging (unpublished data).

4. Concluding remarks

Noninvasive molecular imaging has opened new possibilities to visualize gene and protein functions, protein–protein interactions and signal transduction pathways in animal models of human diseases to obtain insights into molecular pathogenesis of a disease. Multimodality fusion reporter vectors combining bioluminescence, PET and fluorescence imaging are contributing to a much better understanding of complex molecular pathways from single living cell with higher sensitivity and from living subjects with higher sensitivity and specificity in a tomographic and quantitative manner. This will facilitate rapid translation of approaches developed in cells to preclinical models and clinical applications.

Molecular imaging is still evolving. Although various molecular and cellular events are being visualized from a living organism at the cellular level, many events are yet to be imaged noninvasively by the current methodologies. Changes in intracellular pH, electrical impulses by nerve cells and reactive oxygen species generation are some examples of molecular events that still require dedicated probes and imaging techniques to be detected in the context of intact cells anywhere from the living subjects. Thus developments of newer approaches using 'direct' and 'indirect' imaging methods, along with newer generation of imaging modalities will be important to characterize more complex interactions that occur in living subjects and to enhance the contribution of 'molecular imaging' to many facets of biological and biomedical research.

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