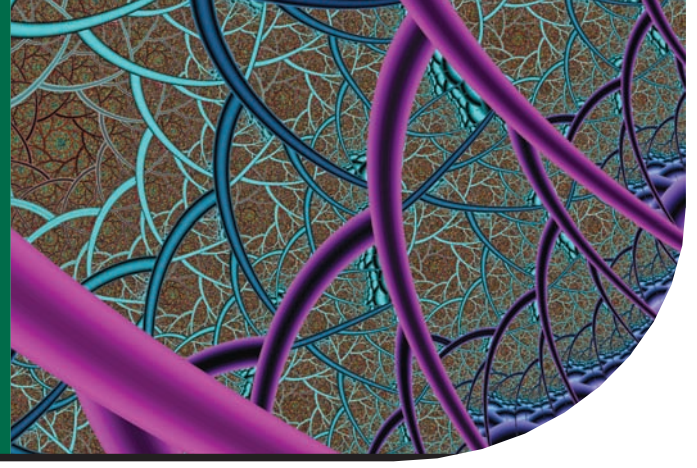

Fluorescence Imaging of Cancer in Live Animals: Time to Get Excited?

Without question, modern medicine is founded on the ability to perform pre-clinical research on diseases and their respective drug treatments. Many human disorders like cancer and infection have corresponding models in mice, providing a convenient route for pre-clinical drug and biochemical studies to take place. However, the research of these models can be cumbersome, and require large numbers of animals for study. Recent advances in small animal fluorescence imaging have dramatically increased the pace of such research, and alleviated many of the common difficulties associated with it. The tools are simple but powerful. With a given fluorescent probe or genetic reporter, a researcher is empowered to perform longitudinal imaging studies on individual cohorts of animals. This dramatically reduces the overall use of animals, while improving the statistical analysis of the data that are collected from a given experiment. The technology is safe, facile, and relatively inexpensive, and thus facilitates in vivo research at universities which may not have the imaging expertise and monetary resources of an associated medical school. Here we describe model systems in cancer that incorporate fluorescent imaging strategies, and how these methods are improving and enhancing research in this critical field.

> ***The increasing use of fluorescence for pre clinical imaging of tumours, combined with the inherent safety, ease of use, and relatively low cost of the technology is spurring the development of agents and instruments for clinical application, and is indeed something about which to get excited.***

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Fluorescence imaging of living specimens may be undertaken at a range of magnification. The core concept at each level is to use filtered light to excite fluorochromes within the animal, with the emitted light captured by a charge coupled device (CCD) with appropriate emission filter. An animal may be imaged at the macroscopic level to detect fluorescent signals emanating from all tissues. This is typically referred to as "whole animal fluorescence imaging" and has the advantage of providing a rapid snapshot of events taking place within the context of the whole body. Next, fluorescence imaging may also be performed at the single cell level within animals. This is referred to as 'intravital microscopy (IVM)' and is a powerful tool for directly visualising individual cells and their processes in living animals. IVM imparts a unique advantage to fluorescence based methods over other optical modalities like bioluminescence, which do not have the spatial resolution to image at the cell and sub-cellular level. The use of optical wavelength light makes the technology safe and easy to use, thus enabling a multitude of applications for cancer imaging.

Dramatic progress in molecular biology has facilitated the fluorescence imaging of a vast array of cells and tissues in living animals. Much of this progress has centered on the use of genetic reporters like green fluorescent protein (GFP) [1] or beta-galactosidase [2]. In the case of GFP, several red-shifted variants have been developed to permit imaging at greater tissue depths [3]. The importance of these proteins cannot be understated as the 2008 Nobel Prize for Chemistry was awarded to Osamu Shimomura, Martin Chalfie, and Roger Tsien for their work in this field. When these proteins are stably expressed in mammalian cell lines, they will fluoresce and thus permit their detection during whole animal or intravital imaging.

A number of examples demonstrate this strategy in the literature. Professor Robert Hoffman and colleagues have demonstrated the use of GFP-expressing tumours in several models using both intravital and whole animal fluorescence methods. Recently, they used viral particles encoding GFP to seek out and illuminate prostate tumour metastases in the abdomen [4]. After the tumour metastases were detected, the researchers used the image information to guide the surgical removal of the tumours (*Figure 1*). Indeed, this example demonstrates the utility of fluorescence for pre-clinical research, and also how this technology may translate into clinical applications. Other prominent examples include the use of other colours of fluorescent protein, like red fluorescent protein (RFP) to monitor prostate tumour progression for several weeks in mice [5], or td Tomato to do the same in animal models of breast cancer [6]. The ability to monitor cancer cell dynamics in vivo provides incredibly useful data for the understanding of tumour pathogenesis and ultimately drug efficacy. They allow the colour-coding of cancer cells growing in vivo in mice, and may one day provide a route to identifying growing tumours in humans to facilitate their removal.

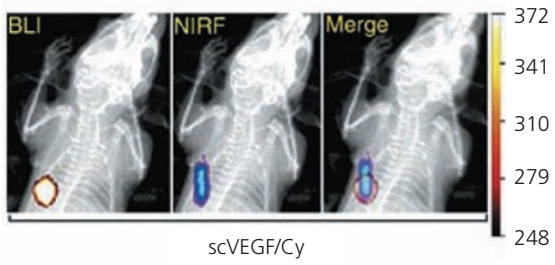

Figure 2. Multimodal imaging of a VEGF-Cy5.5 molecular probe in tumour tissue. Reprinted with permission from Nature Medicine.

Synthetic fluorescent probes provide a convenient solution to detecting receptor or other biomacromolecule expression on tumour cells.

This strategy is also appropriate when it is impossible to alter the genome of a tissue or organism, or when researchers wish to avoid artifacts of clonal selection or random genomic insertion of exogenous DNA. The fluorescently labeled probe is injected intravenously and circulates throughout the blood and into the different tissues of the body. Special affinity groups attract the probe to cancer cells to achieve high-contrast imaging. Probes have been synthesised that rely on many different methods of targeting cancerous cells. Some current imaging agents now include fluorescent conjugates of peptides [7,8,9], proteins [10], sugars [11,12], and other biologically relevant molecules (hepsin, folate) [13,14]. One prominent example utilised a near infrared fluorescent conjugate of vascular endothelial growth factor (VEGF) to detect tumours [10]. In this case, Professor Joseph M. Backer and colleagues conjugated VEGF to a near infrared carbocyanine fluorophore (Cy5.5) to detect tumours that overexpress the VEGF receptor (strain 4T1) in mice. These researchers used bioluminescent cancer cells (*Figure 2*, left panel) to delineate the tumour, and subsequently injected the probe (*Figure 2*, centre panel). The right panel of *Figure 2* shows co-registration of the fluorescence signal with that of the bioluminescence. With this important result, the Backer group validated their tumour affinity group, and subsequently labelled VEGF with a radioisotope to permit nuclear imaging. In this example, the fluorescent method confirmed tumour affinity in a facile manner, and expedited the development of a radiolabelled probe for deep tissue imaging that may be translatable to human medical imaging and treatment.

Probes that utilise the activity of endogenous cellular proteases are known as 'smart' probes, and present a new frontier for imaging of cancer tissue and their relevant biochemical pathways. Proteases are central to diseases, including cancer. This strategy employs probes that are self-quenched in their intact state, and thus have a very low level of fluorescence. When the probe reaches a specific protease at a tumour site, it is cleaved to produces a product with strong fluorescent signal. One example used a smart probe cleavable by cathepsin-B in conjunction with endoscopic fluorescence imaging of colonic adenocarcinoma in a murine model [15].

Figure 1. Fluorescence guided surgical removal of GFP expressing tumours. Image reprinted with permission from PNAS [4].

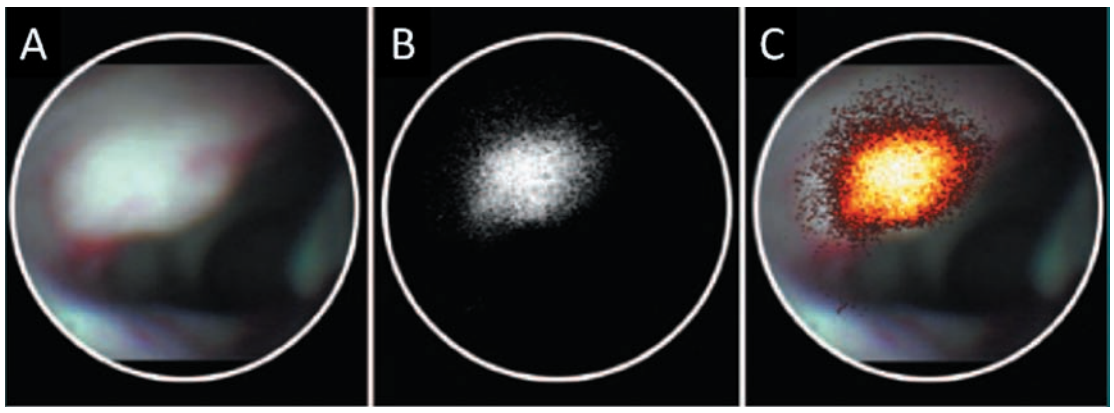

Figure 3: Fluorescence endoscopic imaging of colonic adenocarcinoma using an activatable "smart" probe. Image reprinted with permission from Radiology.

In this case, researchers used a variation of IVM in which an endoscope was used to excite the probe and detect fluorescence. The images in *Figure 2* clearly delineate and highlight the tumour in the colon of the mouse, thus facilitating its detection. Other noteworthy examples include fluorescent smart probes that target caspases [16], and matrix metalloproteinases [17]. In each case, these smart probes represent compounds with pre-clinical utility for monitoring protease activity in given tissue, and overall permitting cancer cell detection. Further, as noted with the imaging example in the colon, these probes also maintain translational potential as fluorescence imaging applications become clinically adopted. There are some drawbacks to using fluorescence methods to image cancer cells. Perhaps the largest is that it has yet to provide a concrete source of quantitative data relative to PET and SPECT imaging modalities. A change in signal from a fluorescent reporter might not correlate with tumour properties like size or vitality, but rather the concentration or environment of the fluorophore itself.. Further, fluorescent probes can only be imaged at relatively shallow depths beneath the skin due to tissue scattering and absorbance of light.

While fluorescence imaging suffers from limited spatial resolution and depth sensitivity, fluorescence tomographic instrumentation and methods have been recently developed to partially mitigate these issues.

Nevertheless, as Ballou and co-workers have demonstrated, most tumours include many different microenvironments with heterogeneity in perfusion, oxygenation, permeability, interstitial pressure, and focal necrosis [18], each of which can have deleterious effects on probe uptake. Thus, interpretation of data involving probes or genetic reporters must still be regarded semi-quantative, and other experimental methods must be included to fully test and confirm a given hypothesis.

Fluorescence methods in living animals provide a convenient route to glean valuable information about pre-clinical models of cancer.

Here we showed one example in which a cancer affinity group was validated by using its corresponding fluorescent conjugate for in vivo imaging studies. The small size of mice usually circumvents any limitations that would be presented by deep tissue, yet translational applications for fluorescence imaging of humans in shallow tissue still have importance. Here we have shown that fluorescence endoscopy of the colon as well as fluorescence guided surgery are within the practical realm of application. The increasing use of fluorescence for pre-clinical imaging of tumours, combined with the inherent safety, ease of use, and relatively low cost of the technology is spurring the development of agents and instruments for clinical application, and is indeed something about which to get excited.

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Advancing the Study of Highly Dynamic Processes within Cells

Much of our understanding of the structural organisation of the living cell has come about through recent advances in fluorescence labelling of target molecules and laser scanning microscopy. With the release of DirectFRAP from **Carl Zeiss**, scientists can now make similar strides in probing the dynamics of membrane transport and the movement of molecules within the living cell.

FRAP, FLIP, photoactivation, conversion of Dendra, on-off switching of Dronpa and other photomanipulation techniques, use a combination of intense pulses of laser light and widefield epi-fluorescence observation to measure the movement of fluorescent markers within the cell. Fitted to the Carl Zeiss Axio Observer microscope, DirectFRAP overcomes the dynamic compromises inherent in previous systems by eliminating the link between laser intensity and the size of the ROI, allowing simultaneous photo-manipulation across the entire area and first image acquisition in as little as two milliseconds. The precise millisecond control of the laser pulses is achieved by acousto-optic tuneable filters (AOTFs) and the system is notable for its brilliant image formation at high acquisition rates and a wide observation field in fast experiments.

Flexible diaphragm options enable a high level of flexibility during experiments and DirectFRAP has been designed to be used in combination with other Carl Zeiss imaging systems, such as the Laser TIRF 3 or Cell Observer SD (Spinning Disc). These system combinations permit the observation of processes in a single Z plane and are ideal for the examination of the smallest cell structures. The same lasers can be used simultaneously for DirectFRAP and Laser TIRF 3 or Cell Observer SD. With all systems, laser pulse control and data acquisition is performed by the ZEISS AxioVision software.

Circle no. 184

Advanced Fluorescence Microscopy Made Easy

Olympus has introduced the all-in-one FSX100 fluorescence microscope system to enable even the most inexperienced users to create high-end research images.

By coupling high quality microscopy and imaging components with precision automation and advanced software, the Olympus FSX100 presents simplified workflows so that users can obtain high quality images and image series by simply loading their sample, defining their observation mode and regions of interest (ROI) and then capturing their images.

The advanced UIS2 optics used in the FSX100 include an SAPO 40x (NA 0.95) objective and 0.4x-2.0x optical zoom, providing an overall magnification range of 17x-80x. The three pre-installed fluorescence filter cubes cover a broad range of dyes with fluorescence illumination provided by an easy to fit, pre-centred metal-halide burner with a 2,000-hour lifespan. A highly stable LED illumination system is used to provide consistent brightfield and phase contrast illumination with a 16,000-hour lifespan.

All components are motorised and controlled via the interactive software, ensuring that functions such as focusing, exposure, fluorescence wavelength selection and even cover slip thickness correction are automated. As a result the user does not have to touch the microscope at all and can perform advanced imaging processes with ease. Due the compact and self-contained design of the FSX100, it can be placed on the bench at the point of discovery, or easily transported to wherever it is needed, making microscopy very flexible. Furthermore, the stage is completely enclosed and therefore provides full darkroom facilities, ensuring maximum imaging sensitivity.

The highly intuitive software guides users through the whole imaging process. An overview image of the sample is generated automatically and user can capture the automatically optimised images or manually adjust settings.

Circle no. 185