USE OF GFP FOR *IN VIVO* IMAGING: CONCEPTS AND MISCONCEPTIONS

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ABSTRACT

Although GFP and fluorescent proteins are used extensively for in vivo imaging, there are many misconceptions about GFP imaging especially compared to luciferase. GFP is not toxic, indeed, transgenic animals with GFP expressed in every cell (1) live as long as non-transgenic animals. Cancer cells with GFP are as aggressive and malignant as the cells without GFP (2-4). Cell lines can be made very bright with fluorescent proteins with no toxicity. The in vivo signal from fluorescent proteins is at least 1,000 times greater than luciferase (5). GFP is so bright that a single molecule of GFP can be seen in a bacterium (6). GFP can be observed through the skin on deep organs (7). Skin autofluorescence presents no problem for in vivo GFP imaging with proper filters (8). Fur can be rapidly clipped removing this autofluorescence (9). GFP is readily quantified by the image area which correlates to tumor volume (10). There are now numerous clones of GFP, RFP, YFP and proteins that change color (11) that can be used in vivo.

Keywords: green fluorescent protein, mice, imaging, non-invasive, autofluorescence

1. INTRODUCTION

Naturally fluorescent proteins have revolutionized biology by enabling what was formerly invisible to be seen clearly in the living state. These proteins have allowed us to visualize, in real time, important aspects of cancer in living animals, including tumour cell mobility, invasion, metastasis and angiogenesis. These multicoloured proteins have allowed the colour-coding of cancer cells growing *in vivo* and enabled the distinction of host from tumour with single-cell resolution. Visualization of many aspects of cancer progression *in vivo* has been accomplished noninvasively as well as invasively with fluorescent proteins (2).

To image and follow the natural course or impediment of tumor progression and metastasis, high specificity and sensitivity, a strong signal, and high resolution are necessary. The *GFP* gene, cloned from the bioluminescent jellyfish *Aequorea victoria* (12), was chosen to satisfy these conditions because it has great potential for use as a cellular marker (13). GFP cDNA encodes a 283-amino acid monomeric polypeptide with *M*r 27,000 (14, 15) that requires no other *A. victoria* proteins, substrates, or cofactors to fluoresce (16). Gain-of-function bright mutants expressing the *GFP* gene have been generated by various techniques (17, 18) and have been humanized for high expression and signal (19). Red fluorescent proteins (RFP) from the *Discosoma* coral and other organisms have similar features as well as the advantage of longer-wavelength emission (20).

2. MISCONCEPTIONS

Despite the numerous published reports on the efficiency of imaging with fluorescent proteins, there are comments in the literature denying these facts. Some examples are quoted below:

". . . fluorescence imaging still suffers from pitfalls such as the inability to quantify photon output, high autofluorescence in the blue-green window resulting in low signal-to-noise ratios, fluorophore photo-bleaching, and high levels of photon attenuation and scattering in living tissues" (21).

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"Photon attenuation, however, is strongly nonlinear as a function of depth and of the optical heterogeneity of tissue, which obscures signal quantification. Planar imaging is further complicated by the inability to resolve depth and by tissue scattering, which limits spatial resolution. For these reasons, although planar methods are useful, they do not harness the true potential of the optical imaging technologies" (22).

"Yang et al. (Proc. Natl. Acad. Sci. USA 97, 1206-1211, 2000), for example, demonstrated that the use of a highly sensitive color CCD camera can detect green fluorescent proteins expressed by tumors implanted superficially in living animals" (22).

"However, it (planar GFP imaging in vivo) also has important limitations, such as the single projection viewing, the restricted penetration depth of a few millimeters and the nonlinear relationship between the signal strength and the depth and the tissue optical properties. These features limit the applicability of the method primarily to superficial observations and may lead to erroneous interpretation of the data collected if the nonlinear effects are not explicitly corrected or accounted for" (22).

"Although GFP imaging of surface tumors is feasible and experimentally useful, deep-seated tumors and organ structures have to be accessed surgically for observation" (23).

". . . fluorescent techniques have tremendous utility in the study (of) cells in culture, excised tissues, small transparent organisms, or biological processes that occur at superficial tissue sites in mammals" (24).

"A drawback of GFP is its low emission wavelength (~510 nm), which overlaps with the autofluorescence of many tissues" (23).

"In fluorescent imaging, the excitation light also will excite naturally occurring fluors in the tissue (autofluorescence) in addition to the fluorescent tag. This is the source of noise in FLI (fluorescence imaging), and it can be significant, especially at shorter wavelengths of light (300 to 600 nm)" (24).

"The wavelengths of light that are needed to excite many of the fluors that have been developed for use in microscopy and cytometry are in the blue and green region of the visible spectrum. Light of these wavelengths has limited penetration through mammalian tissues because of absorption, which is, in large part, caused by hemoglobin" (24).

"Noise in fluorescent imaging arises from the autofluorescence of tissue (this is not the case with reporter genes that encode bioluminescent proteins, because there is little, or essentially no, background autoluminescence), and once a reporter gene is expressed, the signal depends on the absorbing and scattering properties of the tissue, which are greater with shorter wavelengths of light" (24).

"At present, the primary disadvantage of fluorescence, compared to bioluminescence, is the presence of background signal from auto-fluorescence of innate biological molecules" (25).

3. CONCEPTS

However, in contrast to the above published statements, we describe below specific examples demonstrating that *in vivo* imaging with fluorescent proteins is far superior to *in vivo* imaging with luciferase.

A triple fusion reporter vector containing a luciferase gene, red fluorescence protein gene and a thymidine kinase gene was found to highly express all three genes. Mice implanted with 293T cells expressing the vector were imaged with a highly sensitive cooled charge-coupled device camera compatible with both luciferase and fluorescence imaging. When the quantitative data in this report are analyzed, it can be seen that the intensity of photon emission from RFP expression in the tumor cells in mice is at least 1,000 times greater than that of luciferase (5).

Indeed, fluorescent proteins are so bright that a single molecule of GFP can be detected as bacteria. A fusion protein of a fast-maturing yellow fluorescent protein (YFP) and a membrane-targeting peptide was expressed under a repressed condition. The membrane-localized YFP can be detected with single-molecule sensitivity. It was found that the protein

molecules are produced in bursts, with each burst originating from a stochastically transcribed single messenger RNA molecule, and that protein copy numbers in the bursts follow a geometric distribution (6).

In 2000, we initially demonstrated that we could noninvasively image, in real time, GFP-expressing tumors growing and metastasizing in live mice. The whole-body optical imaging system is external and noninvasive. Noninvasive GFP imaging enables unprecedented continuous visual monitoring of malignant growth and spread within intact animals. We have established human and rodent tumors that stably express very high levels of the *Aequorea victoria* GFP and transplanted these to appropriate animals. B16F0-GFP mouse melanoma cells were injected into the tail vein or portal vein of 6-week-old C57BLy6 and nude mice. Whole-body optical images showed metastatic lesions in the brain, liver, and bone of B16F0-GFP that were used for real time, quantitative measurement of tumor growth in each of these organs. The AC3488-GFP human colon cancer was surgically implanted orthotopically into nude mice. Whole-body optical images showed, in real time, growth of the primary colon tumor and its metastatic lesions in the liver and skeleton. Imaging was with either a trans-illuminated epifluorescence microscope or a fluorescence light box and thermoelectrically cooled color charge-coupled device camera (7).

A blue LED flashlight (LDP LLC, Woodcliff Lake, NJ, USA; www.maxmax.com/OpticalProducts.htm) with an excitation filter (midpoint wavelength peak of 470 nm) and an emission D470/40 filter (Chroma Technology, Brattleboro, VT, USA) for viewing was used for whole-body imaging of mice with GFP and RFP-expressing tumors growing in or on internal organs. The images were readily seen by the naked eye with no anesthesia, substrate, or restraint of the animal needed. Even more striking was that the intensity of the whole-body image is 70% of the open image. Although some information is lost with whole-body imaging due to light scattering, a remarkable amount of information is obtained, even with such simple instrumentation. No skin autofluorescence was found due to the use of the proper excitation filter (8).

There have also been reports that GFP is toxic (26, 27). In this light, it should be noted that transgenic mice have been produced which express GFP in essentially every cell. These mice live as long as wild-type mice (1), demonstrating no toxicity from GFP. The transgenic mouse lines were produced with a GFP/cDNA under the control of a chicken beta-actin promoter and cytomegalovirus enhancer. All of the tissues from these transgenic lines, with the exception of erythrocytes and hair, were green under excitation light (1).

In addition, the *in vivo* metastatic behavior of cancer cells with and without GFP have been compared: MDA-MB-435 human breast cancer cells engineered with GFP were injected into the cardiac left ventricle of athymic mice. Femurs were analyzed by fluorescence microscopy, immunohistochemistry, real-time PCR, flow cytometry, and histomorphometry at times ranging from 1 hour to 6 weeks. Single cells were found in distal metaphyses at 1 hour post-injection and remained as single cells up to 72 hours. At 1 week, numerous foci (2-10 cells) were observed, mostly adjacent to osteoblast-like cells. By 2 weeks, fewer but larger foci (> or =50 cells) were seen. Most bones had a single large mass at 4 weeks (originating from a colony or coalescing foci) which extended into the diaphysis by 4 to 6 weeks (3). GFP-tagging permitted detection of single MDA-MB-435 cells and microscopic metastases in bone at early time points following arrival and at stages of proliferation prior to coalescence of individual metastases (4).

There are also reports that GFP is immunogenic (28-30). However, studies of syngenic mice with GFP tumors show the tumors can be highly malignant, suggesting that any antigenicity of GFP did not affect malignancy (9).

Despite the published statements listed above that imaging with fluorescent proteins is non-quantitative, it is not the case. A highly fluorescent RFP-expressing pancreatic cancer model was orthotopically established in nude mice with the MIA-PaCa-2 human pancreatic cancer cell line. Rapid tumor growth and widespread metastases developed in the mice within two weeks, leading to a median survival of 21 days. The noninvasive imaging of the RFP-expressing tumor and metastases was compared to tumor volume measurements with high correlation. Thus, noninvasive, sequential imaging with fluorescent permits quantification of tumor growth and dissemination (10).

4. THE FUTURE

4.1 New fluorescent proteins

New fluorescent proteins have been isolated, some that are far red shifted, which will make noninvasive imaging even more powerful.

Labas et al. have isolated 11 new fluorescent protein genes, among which there are three red-emitters. Phylogenetic analysis has shown that GFP-like proteins from representatives of subclass Zoantharia fall into at least four distinct clades, each clade containing proteins of more than one emission color. This suggests multiple recent events of color conversion. Combining this result with previous mutagenesis and structural data, Labas et al. propose that (i) different chromophore structures are alternative products synthesized within a similar autocatalytic environment, and (ii) the phylogenetic pattern and color diversity in reef Anthozoa is a result of a balance between selection for GFP-like proteins of particular colors and mutation pressure driving the color conversions. These data suggest many types of fluorescent proteins will be discovered in the near future (11).

Fluorescent proteins with distinguishable colors from yellow-orange to red-orange have now been developed by mutagenesis (31).

A far-red fluorescent protein, named Katushka, which is seven- to ten-fold brighter compared to proteins with similar emission has been developed. Katushka appears to be highly resistant to photo-bleaching. Katushka was shown to be superior for whole-body imaging by direct comparison with other red and far-red fluorescent proteins (32).

4.2 New imaging systems

Recently the Olympus OV100 whole-mouse imaging system, with a sensitive CCD camera and four objective lenses, parcentered and parfocal, enabling imaging from macrocellular to subcellular, has been developed. With this instrument, we have observed the nuclear and cytoplasmic behavior of cancer cells in real time in blood vessels as they moved by various means or adhered to the vessel surface in the abdominal skin flap. During extravasation, real-time dual-color imaging showed that cytoplasmic processes of the cancer cells exited the vessels first, with nuclei following along the cytoplasmic projections. Both cytoplasm and nuclei underwent deformation during extravasation. Different cancer cell lines seemed to strongly vary in their ability to extravasate. With the dual-color cancer cells and the highly sensitive whole-mouse imaging system described here, the subcellular dynamics of cancer metastasis can now be observed in live mice in real time (33).

4.3 Fluorescent proteins can enable non-invasive imaging at the subcellular level.

To noninvasively image cancer cell/stromal cell interaction in the tumor microenvironment and drug response at the cellular level in live animals in real time, we developed a new imageable three-color animal model. The model consists of GFP–expressing mice transplanted with dual-color cancer cells labeled with GFP in the nucleus and red fluorescent protein in the cytoplasm. The Olympus IV100 Laser Scanning Microscope, with ultra-narrow microscope objectives (‘‘stick objectives’’), is used for three-color whole-body imaging of the two-color cancer cells interacting with the GFP-expressing stromal cells. In this model, drug response of both cancer and stromal cells in the intact live animal is also imaged in real time. Various in vivo phenomena of tumor-host interaction and cellular dynamics were imaged, including mitotic and apoptotic tumor cells, stromal cells interacting with the tumor cells, tumor vasculature, and tumor blood flow. This new model system enables the first cellular and subcellular images of unperturbed tumors in the live intact animal. New visible real-time targets for novel anticancer agents are provided in this model, including the color-coded interacting cancer and stromal cells, tumor vasculature, and blood flow. This imageable model should lead to many new insights of in vivo cancer cell biology and to novel drug discovery. These data therefore demonstrate that single cells expressing fluorescent proteins can be imaged noninvasively (34).

Fluorescent protein imaging will enable further understanding of the critical steps of metastasis and provide visible targets for antimetastasis drug development. In conclusion, new red-shifted proteins and superior imaging equipment will enable fluorescent imaging technology to develop the new field of *in vivo* cell biology.

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