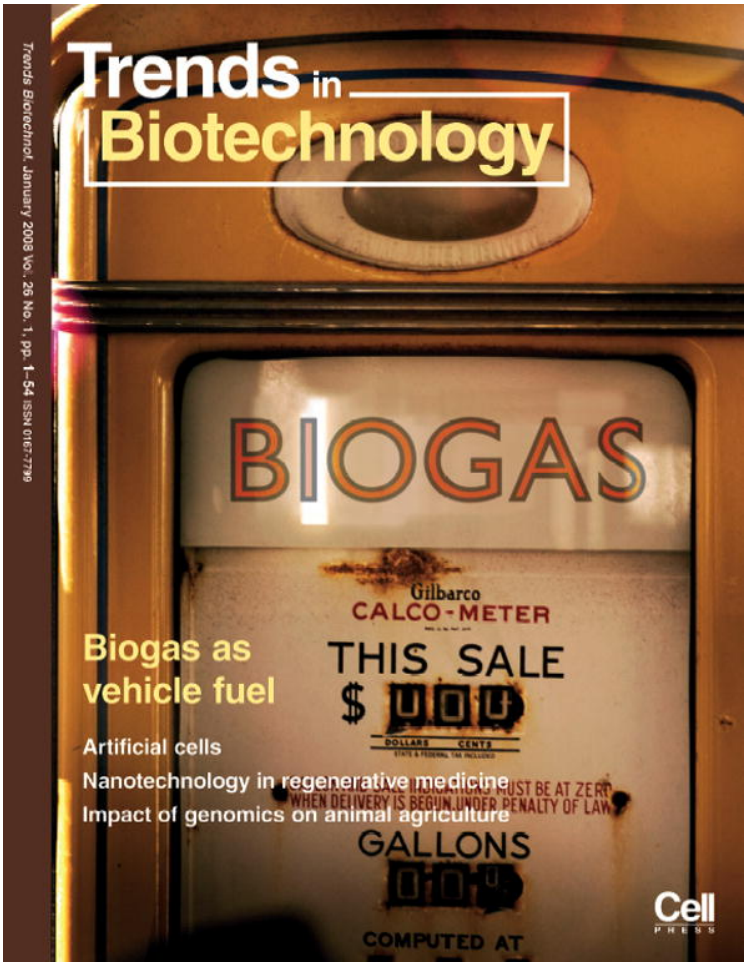

Update

Research Focus

A better fluorescent protein for whole-body imaging

Robert M. Hoffman

AntiCancer, Inc., 7917 Ostrow Street, San Diego, CA 92111-3604, USA
Dept of Surgery, University of California, San Diego, 200 West Arbor Dr., San Diego, CA 92103-8220, USA

Whole-body imaging with fluorescent proteins is a powerful technology with many applications in small animals. Brighter, red-shifted proteins can make whole-body imaging more sensitive owing to reduced absorption by tissues and less scatter. A new protein called Katushka has been isolated. It is the brightest known protein with emission at wavelengths longer than 620 nm. This new protein offers the potential for non-invasive whole-body imaging of numerous cellular and molecular processes in live animals.

Introduction

The discovery, cloning and gene transfer of green-fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has enabled a revolution in cell biology. GFP can be linked genetically with almost any protein, thus providing a permanent and heritable label in live cells to study protein function and location [1]. Many different colors of fluorescent proteins have now been produced in the laboratory or found in nature. With multiple colors, many processes can be visualized simultaneously in cells. Thus, live cells can be permanently labeled with multiple colors for imaging that previously could be performed only on fixed and stained cells. What could only be seen on gels and blots previously can now be visualized in real time in living cells expressing fluorescent proteins.

Whole-body imaging

Our laboratory pioneered *in vivo* imaging with fluorescent proteins [2], including noninvasive whole-body imaging [3]. Whole-body imaging with fluorescent proteins depends in large part on the brightness of the protein. Whole-body imaging with fluorescent proteins can track tumor growth and metastasis (Figure 1), gene expression, angiogenesis and bacterial infection, quantitatively [1]. Non-invasive imaging can be performed even at subcellular resolution (Figure 2), depending on the position of the cells in the animal. Interference by skin autofluorescence is kept to a minimum with the use of proper filters. Simple equipment, such as an LED flashlight with a narrow-band excitation filter in combination with a bandpass emission filter, can be used to whole-body image mice implanted with cells expressing fluorescent proteins [4] (Figure 3).

Whole-body imaging is more effective when the fluorescent protein emits at longer wavelengths that are absorbed less by tissues and by physiological molecules, such as hemoglobin. Longer wavelength light is also less scattered [1].

Corresponding author: Hoffman, R.M. (all@anticancer.com).

Red-fluorescent proteins

Red-emitting fluorescent proteins (RFPs) were first described in the late 1990s. The first such protein was isolated and cloned from the coral *Discosoma* sp. that was obtained from an aquarium shop in Moscow [5] and termed DsRed. After extensive modification by mutagenesis, a bright red protein was eventually isolated, termed DsRed2, with an emission wavelength peak of 588. DsRed2 can be used for whole-body imaging and has been used to non-invasively follow cancer metastasis in real time [6] in nude mice (Figure 1). DsRed2 has also been used to whole-body image tumors growing in transgenic GFP nude mice, allowing for the color coding of cancer- and host cells [7].

In 2004, a report appeared [8] that described a series of red-shifted proteins obtained by mutating DsRed. These proteins, termed mCherry, mRaspberry, mPlum and mTomato, had emission maxima as long as 649 nm. However, these mutants have low quantum yields, thereby reducing their brightness.

Katushka

In the September 2007 issue of *Nature Methods*, a bright, red-shifted fluorescent protein was described by Shcherbo *et al.* [9]. This protein was cloned and developed in the laboratory of Sergei and Konstantin Lukyanov at the Shemyakin-Ovchinnikov Institute of Biorganic Chemistry in Moscow [9]. This protein, named Katushka [a derivative of a derivative of a derivative of the Russian female name Yekaterina (Katherine)], originated from the sea anemone *Entacmaea quadricolor*. With the use of degenerate primers, approximately 100 000 clones were generated by mutagenesis from the initial clone from *E. quadricolor*, termed TurboRFP. The clones were screened for high brightness in the far-red part of the spectrum. A bright, red-shifted variant was isolated with an excitation peak at 588 mm and an emission peak at 635 nm, both of which are relatively nonabsorbed by tissues and hemoglobin. After four cycles of random mutagenesis and further selection for bright, far-red-shifted proteins, Katushka was isolated. Katushka has many favorable properties in addition to its absorption and emission peaks, including a rapid maturation time of 20 min. Importantly, an extinction coefficient of 65 000 M^{-1} cm^{-1} and quantum yield of 0.34 make Katushka the brightest fluorescent protein with an emission maximum beyond 620 nm. In cells, Katushka has no visible aggregates or other toxic effects.

Transgenic *Xenopus laevis* frogs were generated with Katushka expressed in muscle cells driven by the cardiac

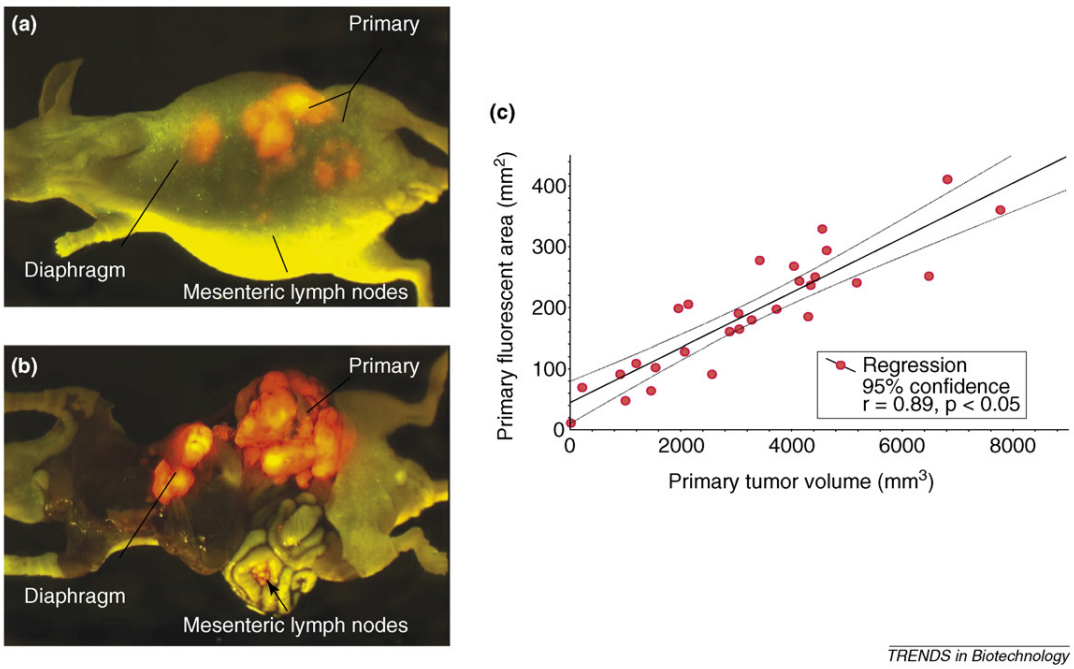

Figure 1. External versus internal quantitative imaging [6]. **(a)** External and **(b)** open images of a single, representative mouse at autopsy on day 17 after surgical orthotopic implantation (SOI). Extensive locoregional and metastatic growth is visualized by exciting DsRed2 selectively that is expressed in the tumors. A strong correlation between the fluorescence visualized externally and that obtained after laparotomy is evident. **(c)**. Red-fluorescent area quantified using external fluorescence imaging correlated strongly with tumor volume measured directly. At autopsy, measurement of externally visualized fluorescent area and direct measurements of the primary tumor of each mouse were obtained. Significant correlation ($r = 0.89$, $p = 0.05$) was observed between these values.

actin promoter. DsRed-Express and mPlum-expressing transgenic *X. laevis* frogs were generated for comparison in *X. laevis* embryos. A strong fluorescence signal could be detected noninvasively from the heart rudiment as well as from somites with Katushka. However, in DSRed-Express or mPlum-expressing frogs, the heart could not be imaged, demonstrating the potential of Katushka for whole-body imaging. Low toxicity was demonstrated by 6-month-old frogs expressing Katushka, which looked totally normal with a body size similar to non-transgenic counterparts.

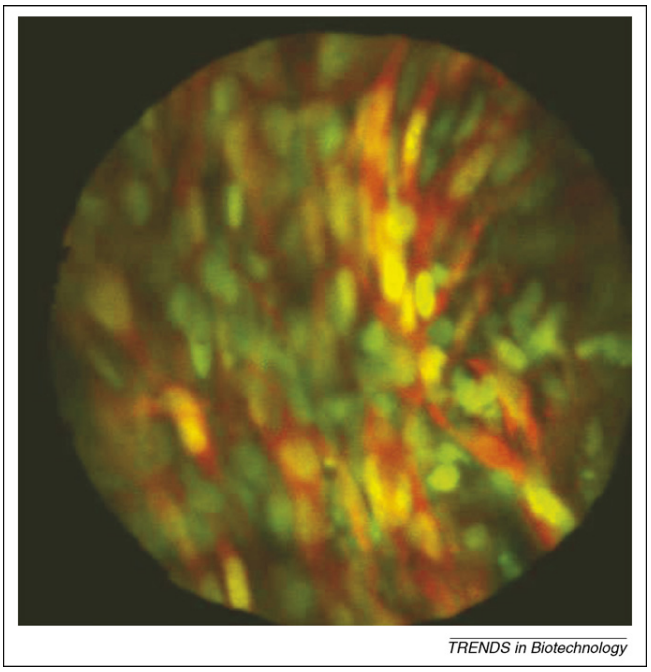

Figure 2. Noninvasive, subcellular imaging of dual-color mouse-mammary cancer cells and GFP stromal cells in the footpad of a live GFP nude mouse [10]. Dual-color mouse mammary tumor 060562 (MMT) cells were injected in the footpad of GFP transgenic nude mice. Whole-body images were acquired with the Olympus IV100 laser scanning microscope using the 3-mm stick objective 14 days after cell injection. Non-invasive image of dual-color MMT cells in the footpad of a live GFP mouse. Note the numerous spindle-shaped dual-color MMT cells interdispersed among the GFP host cells.

Subsequent mutagenesis experiments generated a monomeric version of Katushka, which is a dimer. The monomer, highly suitable for fusions with other proteins, is termed mKate. Both Katushka and mKate demonstrate high photostability.

Method of choice for whole-body imaging

The features of fluorescent-protein-based imaging, such as a strong and stable signal, enable noninvasive whole-body imaging down to the subcellular level [10] (Figure 2). These properties make fluorescent-protein-based imaging (especially with red-shifted fluorescent proteins) far superior to luciferase-based imaging. Luciferase-based imaging, with its weak signal [11], which precludes image acquisition and enables only photon counting with pseudocolor-generated images, has limited applications [12]. For example, cellular imaging *in vivo* is not presently possible with luciferase. The dependence on circulating luciferin makes the signal from luciferase imaging unstable [12]. The one possible advantage of luciferase-based imaging is that no excitation light is necessary. However, far-red absorbing proteins, such as Katushka, greatly reduce any problems with excitation, even in deep tissues, as shown by Shcherbo *et al.* [9].

Proteins, such as Katushka, as well as photo-activatible- [13] and photoconvertible-fluorescent

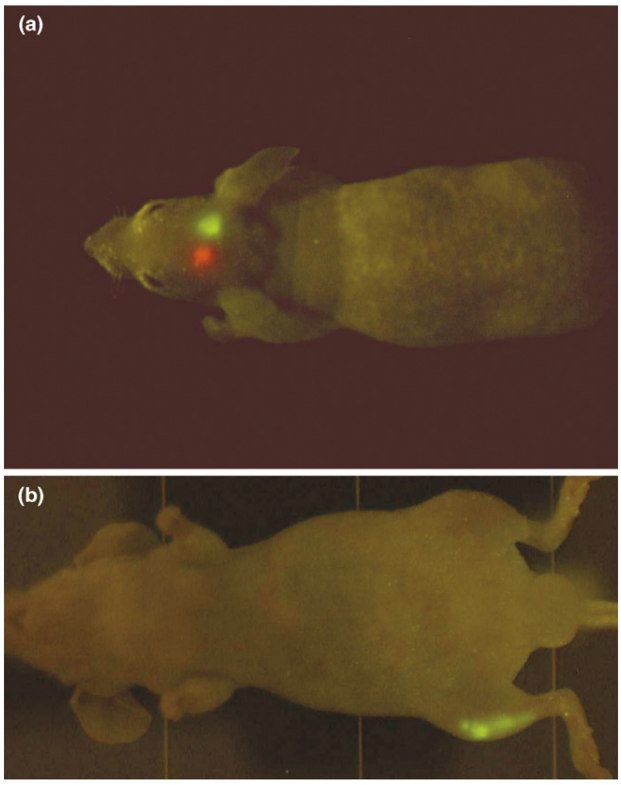

Figure 3. Whole-body imaging of GFP and RFP tumors in a nude mouse [4]. **(a)** GFP- and RFP-expressing tumors implanted in the brain in a single nude mouse. The excitation light was produced with a simple blue-LED flashlight equipped with an excitation filter with a central peak of 470 nm. The image was acquired with a Hamamatsu charge-coupled device (CCD) camera. **(b)** GFP-expressing tumor implanted into the tibia of the right hind leg of a nude mouse imaged with the blue-LED flash light, as in (a).

proteins [14], provide powerful tools for future whole-body imaging experiments. Imaging instrumentation, such as those with variable magnification [15] or scanning lasers [10] and multiphoton microscopy [16], make fluorescent proteins tools of choice for whole-body imaging. Whole-body imaging with fluorescent proteins can now reach the subcellular level using cells labeled in the nucleus with GFP and RFP in the cytoplasm [10]. However, there are misconceptions in the literature suggesting that fluorescent-protein-based imaging is inferior to luciferase [17–20]. The results described here should clarify this subject greatly.

Perspectives

Fluorescent proteins have enabled the new field of *in vivo* cell biology to develop [21]. The future is highly promising with proteins, such as Katushka and mKate, for non-invasive dynamic imaging of numerous cellular processes that occur even in deep tissues in animals. Previously, such processes could only be studied biochemically. Transgenic mice engineered with bright proteins (Figure 4) might also offer many new possibilities for whole-body imaging. Future human use is also possible, for example, using specifically engineered viruses to label existing tumors

Figure 4. Nude mouse that expresses RFP ubiquitously.

in vivo for diagnostic and surgical-navigation applications [22].

References

1 Hoffman, R.M. (2005) The multiple uses of fluorescent proteins to visualize cancer *in vivo*. *Nat. Rev. Cancer* 5, 796–806
2 Chishima, T. *et al.* (1997) Cancer invasion and micrometastasis visualized in live tissue by green fluorescent protein expression. *Cancer Res.* 57, 2042–2047
3 Yang, M. *et al.* (2000) Whole-body optical imaging of green fluorescent protein-expressing tumors and metastases. *Proc. Natl. Acad. Sci. U. S. A.* 97, 1206–1211
4 Yang, M. *et al.* (2005) Facile whole-body imaging of internal fluorescent tumors in mice with an LED flashlight. *Biotechniques* 39, 170–172
5 Matz, M.V. *et al.* (1999) Fluorescent proteins from nonbioluminescent *Anthozoa* species. *Nat. Biotechnol.* 17, 969–973
6 Katz, M.H. *et al.* (2003) A novel red fluorescent protein orthotopic pancreatic cancer model for the preclinical evaluation of chemotherapeutics. *J. Surg. Res.* 113, 151–160
7 Yang, M. *et al.* (2003) Dual-color fluorescence imaging distinguishes tumor cells from induced host angiogenic vessels and stromal cells. *Proc. Natl. Acad. Sci. U. S. A.* 100, 14259–14262
8 Shaner, N.C. *et al.* (2004) Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein. *Nat. Biotechnol.* 22, 1567–1572
9 Shcherbo, D. *et al.* (2007) Bright far-red fluorescent protein for whole-body imaging. *Nat. Methods* 4, 741–746
10 Yang, M. *et al.* (2007) Whole-body subcellular multicolor imaging of tumor-host interaction and drug response in real time. *Cancer Res.* 67, 5195–5200
11 Ray, P. *et al.* (2004) Imaging tri-fusion multimodality reporter gene expression in living subjects. *Cancer Res.* 64, 1323–1330
12 Hoffman, R.M. and Yang, M. (2006) Whole-body imaging with fluorescent proteins. *Nat. Protocols* 1, 1429–1438
13 Lukyanov, K.A. *et al.* (2005) Innovation: photoactivatable fluorescent proteins. *Nat. Rev. Mol. Cell Biol.* 6, 885–891
14 Ando, R. *et al.* (2002) An optical marker based on the UV-induced green-to-red photoconversion of a fluorescent protein. *Proc. Natl. Acad. Sci. U. S. A.* 99, 12651–12656
15 Yamauchi, K. *et al.* (2006) Development of real-time subcellular dynamic multicolor imaging of cancer cell trafficking in live mice with a variable-magnification whole-mouse imaging system. *Cancer Res.* 66, 4208–4214
16 Condeelis, J. and Segall, J.E. (2003) Intravital imaging of cell movement in tumours. *Nat. Rev. Cancer* 3, 921–930
17 Gross, S. and Piwnica-Worms, D. (2005) Spying on cancer: molecular imaging *in vivo* with genetically encoded reporters. *Cancer Cell* 7, 5–15

18 Weissleder, R. and Ntziachristos, V. (2003) Shedding light onto live molecular targets. *Nat. Med.* 9, 123–128
19 Ntziachristos, V. *et al.* (2005) Looking and listening to light: the evolution of whole-body photonic imaging. *Nat. Biotechnol.* 23, 313–320
20 Zhao, H. *et al.* (2005) Emission spectra of bioluminescent reporters and interaction with mammalian tissue determine the sensitivity of detection *in vivo*. *J. Biomed. Opt.* 10, 41210
21 Hoffman, R.M. (2004) *In vivo* imaging with fluorescent proteins: The new cell biology. *Acta Histochem.* 106, 77–87
22 Kishimoto, H. *et al.* (2006) *In vivo* imaging of lymph node metastasis with telomerase-specific replication-selective adenovirus. *Nat. Med.* 12, 1213–1219

0167-7799/$ – see front matter
doi:10.1016/j.tibtech.2007.10.006 Available online 26 November 2007

Research Focus

Novel tomato flavours introduced by plastidial terpenoid pathway engineering

Beat Mollet[1], Peter Niederberger[1] and Vincent Pétiard[2]

[1] Nestlé Research Center, Nestec Ltd., Vers-chez-les-Blanc, CH-1000 Lausanne 26, Switzerland
[2] Nature Source Genetics, 95 Brown Road, Box 1055, Ithaca, NY 14850, USA

Until recently breeding efforts centred on high-yield production while sacrificing flavour and taste quality traits of mass produced food products, such as tomatoes. The recent publication of Davidovich-Rikanati *et al.* demonstrates the technical feasibility of the genetical engineering of pathways in tomato plants to modify their fruit flavour profile in a proof-of-concept approach. The reported work ranks among an increasing number of reported successful modifications of edible plants with a focus on the benefits to end-consumers.

Introduction

Tomatoes are among the most common vegetables industrially produced and distributed on all continents. Even though tomatoes are used in convenience-food products, they are nevertheless perceived as healthy and tasty vegetables. The health-beneficial properties of tomatoes are attributed high levels of compounds such as vitamins (A and C) and antioxidants, in particular the carotenoid lycopene. Nutritional studies have shown evidence that lycopene reduced the risk of many chronic diseases, such as cancer, cardiovascular and neurodegenerative diseases [1–4]. The benefits of lycopene are associated with its potent antioxidant properties and its ability to scavenge free radicals [5,6].

Worldwide production volumes of tomatoes are huge, either as a mass-product grown on an agro-industrial scale or as a high-quality product grown locally. Thus, according to statistics of the Food and Agriculture Organization of the United Nations, 126 million tons of tomatoes were produced in 2005, of which 20% were commercialised as fresh tomatoes. Consumer choice is driven by a range of individual preferences: organoleptic quality (taste, aroma and colour), size and shape, origin of production, agricultural production conditions or simply convenience and price. A commonly deplored deficiency of mass-produced tomatoes is a lack of aroma and taste [7]. The fact that for industrial mass production, tomatoes are ripened during transportation and storage has logistic and commercial benefits, but is disadvantageous to the taste and health qualities that the consumer is looking for. For a long time, most vegetable seed companies focused their breeding efforts on high production yield and good transportation properties to meet low price production of fresh and processing tomatoes. However, more recently, major tomato seed companies have started to combine output traits that aim to increase the quality of the final fruits with input traits that were directed to agricultural properties. Interestingly, an exclusive deal has recently been announced between Unilever (one of the main tomato processors) and Nunhems (the Bayer Crop Sciences vegetable seed company) for the development of new exclusive varieties that, in addition to good agronomic performance traits, have improved end-product qualities, including taste, nutritional value and health benefits (http://www.bayerchina.com.cn/news/news_en_4.html). Agricultural properties, such as high production yield and low-cost production, can hardly be compromised in the mass-production tomato segment; indeed, agricultural properties define the varieties that growers will select and produce, and hence ultimately also drive the tomato seed business. Efficient and targeted technologies that can bring back quality attributes (e.g. taste and flavour) without loosing the required agricultural properties would certainly be desirable. It is in the light of these observations that the recent publication of Davidovich-Rikanati *et al.* in Nature Biotechnology [8] needs to be looked at.

Metabolic pathway engineering

In that publication, Davidovich-Rikanati *et al.* have studied the diversion of the early plastidial terpenoid pathway by expressing the geraniol synthase gene from *Ocimum basilicum* (basil) placed under the control of a tomato-ripening-specific promoter sequence. Various monoterpenes that contribute to fruity and floral scents were found to accumulate in the resulting transgenic tomatoes; those tomatoes were rated mostly positively by an untrained tasting panel. This work helps further our understanding

Corresponding author: Mollet, B. (beat.mollet@rdls.nestle.com).