Cell Death and Differentiation (2002) 9, 786–789

www.nature.com/cdd

News and Commentary

In vivo imaging of metastatic cancer with fluorescent proteins

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doi:10.1038/sj.cdd.4401077

Optical imaging of cancers in animal models offers many potential advantages including rapid imaging, lack of contrast agents, radiation and substrates.[1] However, optical imaging has been challenging since tumor cells usually do not have a specific optical quality that clearly distinguishes them from normal tissue. Also, conventional optical imaging has been severely limited by the strong absorbance and scattering of the illuminating light by tissue surrounding the target. As a result, neither the sensitivity nor spatial resolution of current methods are sufficient to image early stage tumor growth or metastasis.[2]

Previous attempts to endow tumors with specific, detectable spatial markers have mostly met with indifferent success. These included labeling with monoclonal antibodies and other high-affinity vector molecules targeted against tumor-associated markers.[3–7] However, results were limited due to achieving only a low tumor/background contrast and to the toxicity of the procedures.

Intravital videomicroscopy (IVVM) is another approach to optical imaging of tumor cells. IVVM allows direct observation of cancer cells.[8] Even in this limited arena, IVVM does not lend itself to following tumor growth, progression, and internal metastasis in a live intact animal due to the invasiveness of the procedure.

A major conceptual advance in optical imaging was to make target tumor the source of light. This renders the incident light scattering much less relevant. One early attempt inserted the luciferase gene into tumors such that they emit light.[9] However, luciferase enzymes transferred to mammalian cells require the exogenous delivery of their luciferin substrate, an essentially impractical requirement in an intact animal. The signal of luciferase is so weak that actual images are not observed, only pseudocolor patterns derived by photon counting. Luciferase-based bioluminescence assays cannot detect single tumor cells and require both elaborate equipment and restraint of the experimental animal (Table 1).[9] Also, it is not known whether luciferase genes can function stably over significant time periods in tumors and in the metastases derived from them.

A more practical approach to tumor luminance is to make the target tissue selectively fluorescent. In one approach, tumor-bearing animals were infused with protease-activated near-infrared fluorescent probes.[10] Tumors with appropriate proteases could activate the probes and be imaged externally. However, the system proved to have severe restrictions. The selectivity was limited since most normal tissues have significant protease activity. In fact, the normal activity in liver is so high as to preclude imaging in this most important of metastatic sites. Also, the short lifetime of the fluorescence probes would appear to rule out real-time growth and efficacy studies. The requirement of appropriate tumor-specific protease activity and requirement of effective tumor delivery of the probes greatly limit the use of this technology.[10]

We have developed stable green fluorescent (GFP)-expressing cancer cells to visualize detection of metastases in exposed or isolated fresh visceral organs and tissues down to the single-cell level.[11–18] Tracking of cancer cells that stably express GFP *in vivo* was found to be more sensitive and rapid than the traditional cumbersome procedures of histopathological examination or immunohistochemistry. In particular, GFP labeling markedly improved the ability to visualize metastases in fresh soft organs and bone.[11–18] A major advantage of GFP-expressing tumor cells is that imaging requires no preparative procedures, substrate, or contrast agents and is, therefore, uniquely suited for visualizing cells and tissues *in vivo* (Table 1).[10–17]

The GFP-based fluorescent optical tumor imaging system presents many powerful features: Only the tumors and metastases contain the heritable GFP gene and are therefore selectively imaged with very high intrinsic contrast to other tissues. GFP expression in the tumor cells is stable over indefinite time periods, allowing the quantitative imaging of tumor growth and metastasis formation as well as their inhibition by agents of all types. The very bright GFP fluorescence enables internal tumors and metastases to be externally observed in critical organs such as colon, liver, bone, brain, pancreas, breast, lymph nodes, prostate, etc. No contrast agents or other compounds or treatment need be administered to the animals, just blue light illumination is necessary.[11–18]

GFP-expressing tumors were sufficiently bright that they often could be viewed through simple video equipment situated external to the animal.[13] However, the sensitivity of tumor visualization was limited by absorption through overlying tissue and worked best for relatively shallow tumors. One application of the dorsal skin chamber in mice used two-photon confocal microscopy to examine tumor gene expression, angiogenesis and physiology in a green fluorescent protein (GFP) expressing tumor.[14] Such studies are however limited to the ectopic primary tumor and can provide only limited information.

Much of this absorption is due to scattering in the skin. We have found that the simple expedient of introducing a minimally invasive, reversible skin flap can increase sensitivity of detection, often by an order of magnitude or

more.[19] This makes possible direct observation of tumor growth and metastasis as well as tumor angiogenesis and gene expression. The images revealed the microscopic stages of tumor growth and metastatic seeding, often down to the single cell level, as well as later stages of tumor spread and angiogenesis. Single tumor cells, expressing GFP, seeded on the brain image through a scalp skin-flap. Lung tumor micro-foci representing a few cells are viewed through a skin-flap over the chest wall while contralateral micrometastases were imaged through the corresponding skin-flap. Pancreatic tumors and their angiogenic microvessels were imaged via a peritoneal wall skin-flap. A skin-flap over the liver allowed imaging of physiologically relevant micrometastases originating in an orthotopically implanted tumor. Single tumor cells on the liver arising from intraportal injection were also detectable. Possible future technical developments are suggested by the image, via a lower-abdominal skin-flap, of an invasive prostate tumor expressing both red and green fluorescent proteins in separate colonies. The improvement in sensitivity afforded by the skin flap window now permits imaging on essentially any internal organ at least for mouse sized animals.[19]

Angiogenesis has become one of the most intense areas of cancer research since studies attest to its crucial role in tumor growth and metastasis. Such data are central to understanding phenomena such as interference of the primary tumor of metastatic growth and, most importantly, the effect of chemotherapeutic agents on angiogenic vessels. However, obtaining precise data relating angiogenesis to tumor growth and metastasis is difficult as long as animal sacrifice is required for each measurement. The fluorescent orthotopic metastatic model affords, for the first time, a relatively noninvasive method of visualizing angiogenic vessel induction, in both space and time, for primary and metastatic tumors.[20] The microvessels were also directly visualized through a skin-flap window on the peritoneal wall.[19] The vessel development can be closely followed and quantified in real time, allowing precise answers to the relation of angiogenesis and cancer progression. The technique also affords rapid evaluation of drugs which may affect development of these vessels. Of special interest is the very low morbidity associated with the technique which allows tumors to be followed for long periods of time.

Materials and Methods

Expression vectors

The pLEIN and pLNCX$_2$ vectors were purchased from CLONTECH Laboratories, Inc. (Palo Alto, CA, USA). The pLEIN vector expresses enhanced green fluorescent protein (EGFP) and the neomycin resistance gene on the same bicistronic message which contains an internal ribosome expression site (IRES). The pLNCX$_2$ vector contains the neomycin resistance gene for antibiotic selection in eukaryotic cells. The red fluorescent protein gene (RFP), (DsRed2, CLONTECH Laboratories, Inc., Palo Alto, CA, USA), was inserted in the pLNCX$_2$ vector at the Bg*l*I and *Not*I sites.[19]

GFP and RFP vector production

For retroviral transduction, PT67, an NIH3T3-derived packaging cell line, expressing the 10 Al viral envelope, was purchased from CLONTECH Laboratories, Inc. PT67 cells were cultured in DME (Irvine Scientific, Santa Ana, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gemini Bio-products, Calabasas, CA, USA). For vector production, packaging cells (PT67), at 70% confluence, were incubated with a precipitated mixture of DOTAP^{TM} reagent (Boehringer Mannheim), and saturating amounts of pLEIN or pLNCX$_2$-DsRed2 plasmid for 18 h. Fresh medium was replenished at this time. The cells were examined by fluorescence microscopy 48 h post-transfection. For selection, the cells were cultured in the presence of 500 μg/ml–2000 μg/ml of G418 increased in a step-wise manner (Life Technologies, Grand Island, NY, USA) for 7 days.[19]

GFP and RFP gene transduction of tumor cells lines

For GFP gene transduction, 20–40% confluent human and animal tumor cells were incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67 cells and RPMI 1640 or other culture media (GIBCO) containing 10% fetal bovine serum (FBS) (Gemini Bio-products, Calabasas, CA, USA) for 72 h. Fresh medium was replenished at this time. Tumor cells were harvested with trypsin/EDTA and subcultured at a ratio of 1:15 into selective medium which contained 50 μg/ml of G418. The level of G418 was increased to 800 μg/ml in a step-wise manner. Clones expressing GFP were isolated with cloning cylinders (Bel-Art Products, Pequannock, NJ, USA) by trypsin/EDTA and were amplified and transferred by conventional culture methods in the absence of selective agent.[19]

Table 1 Comparison of optical imaging methods for tumor growth and metastasis

Method	Min. no. of cells imageable *in vitro*	Ref.	Min. no. of cells imageable *in vivo*	Ref.	Need for substrate	Ref.	Need for anesthesia	Ref.	Method of visualization	Ref.
GFP	1	Chishima *et al*., Cancer Res, 1997[11]	1	Chishima *et al*., Cancer Res, 1997[11] Yang *et al*., PNAS, 2002[19]	No	Chishima *et al*., Cancer Res, 1997[11] Yang *et al*., PNAS, 2002[19]	No	Yang *et al*., PNAS, 2000[13]	Direct imaging	Yang *et al*., PNAS, 2000[13]
Luciferase	300	Dusich *et al*., Proc AACR, 2002[22]	3000	Vooijs *et al*., Cancer Res, 2002[21]	Yes	Vooijs *et al*., Cancer Res, 2002[21]	Yes	Vooijs *et al*., Cancer Res, 2002[21]	Photon counting	Vooijs *et al*., Cancer Res, 2002[21]

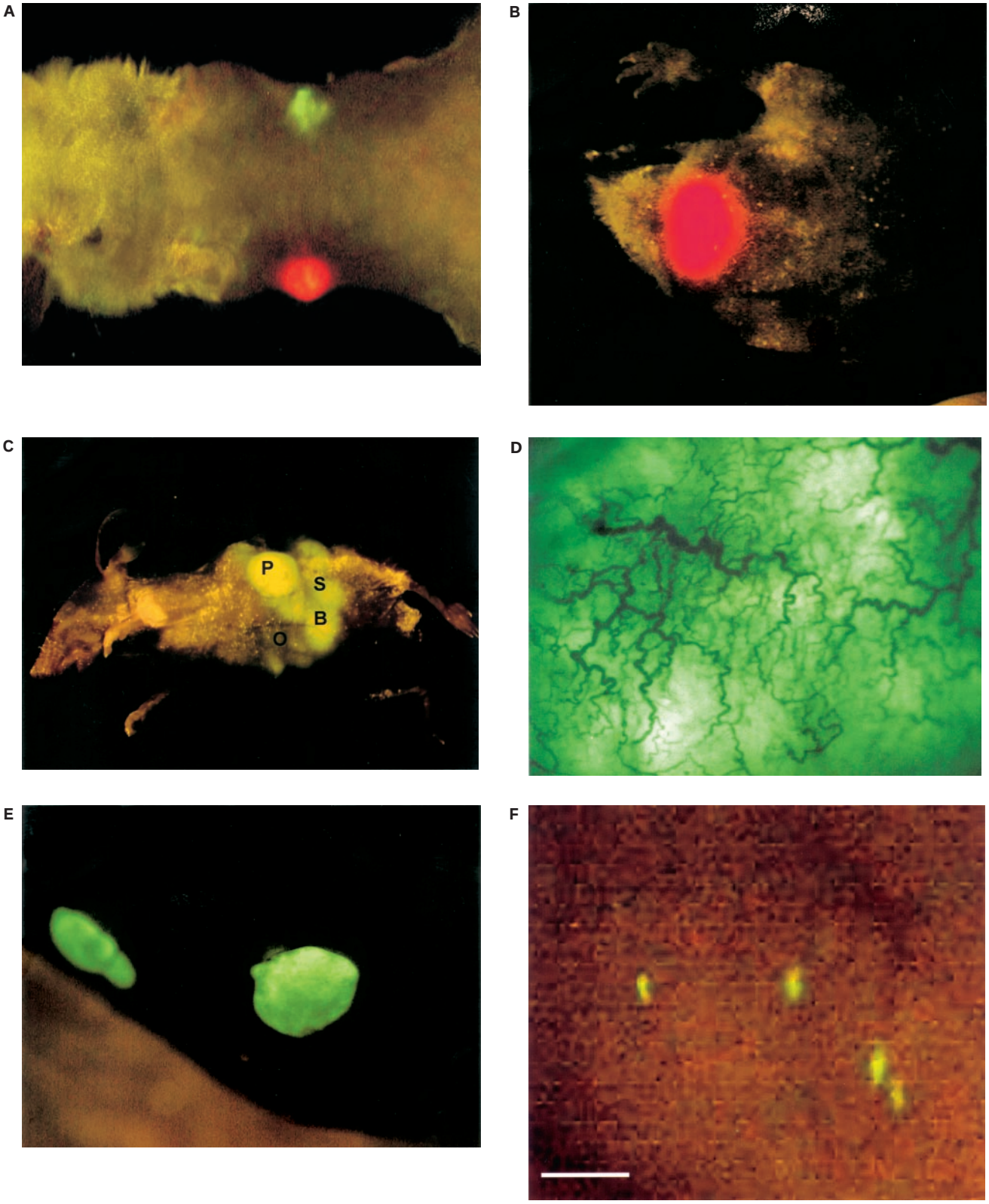

Figure 1 (**A**) Whole-body dual color imaging of the MX-1-GFP human breast tumor implanted on the left mammary fat pad and MX-1-RFP tumor imaged on the right mammary fat pad. (**B**) Whole-body image of U87 glioma RFP tumor implanted in the brain. (**C**) Whole-body images of the BxPC-3-GFP primary tumor (P) and omental (O), bowel (B), and spleen (S) metastases.[12] (**D**) Whole-body image of subcutaneously-growing PC-3 prostate tumor angiogenesis. (**E**) Intravital image of liver metastasis of colon cancer HCT-116. (**F**) External direct view of single cell metastases in the liver of colon cancer 205 imaged through skin-flap window.[19] Bar=100 μm

Fluorescence imaging

A Leica fluorescence stereo microscope model LZ12 equipped with a mercury 50 W lamp power supply was used. To visualize both GFP and RFP fluorescence at the same time excitation was produced through a D425/60 band pass filter, 470 DCXR dichroic mirror and emitted fluorescence was collected through a long pass filter GG475 (Chroma Technology, Brattleboro, VT, USA). Macroimaging was carried out in a light box (Lightools Research, Encinitas, CA, USA). Fluorescence excitation of both GFP and RFP tumors was produced through an interference filter 440+/−20 nm using slit fiber optics for animal illumination. Fluorescence was observed through a 520 nm long pass filter. Images from the microscope and light box were captured on a Hamamatsu C5810 3-chip cool color CCR camera (Hamamatsu Photonics Systems, Bridgewater, NJ, USA).[19]

Images were processed for contrast and brightness and analyzed with the use of Image Pro Plus 4.0 software (Media Cybernetics, Silver Springs, Maryland, USA). High resolution images of 1024 × 724 pixels were captured directly on an IBM PC or continuously through video output on a high resolution Sony VCR model SLV-R1000 (Sony Corp., Tokyo, Japan).[19]

Prospectives

The whole-body and external direct imaging via a skin flap window of GFP- and RFP-labeled tumor cells affords extraordinarily high resolution and dual color imaging with a small investment in a light source and detection equipment. The system described here is capable of multicolor fluorescent and affords unique approach to the study of the critical factors in cancer progression, making the future study of cancer *in vivo* much brighter[23,24] (Figure 1).

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