# Green Fluorescent Protein for Metastasis Research

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## 1. Introduction

Our understanding of the cancer metastatic process has advanced considerably in recent years. However, the early stages of tumor progression and micrometastasis formation have been difficult to analyze. These studies are hampered by the inability to identify small numbers of tumor cells against a background of many host cells. The visualization of tumor cell emboli, and micrometastases and their progression over real time during the course of the disease has been difficult to study in current models of metastasis. Previous studies used transfection of tumor cells with the *Escherichia coli* β-galactosidase *(lacZ)* gene to detect micrometastases ***(1,2,33)***. However, detection of *lacZ* requires extensive histological preparation, and therefore it is impossible to detect and visualize tumor cells in viable fresh tissue or the live animal at the microscopic level. The visualization of tumor invasion and micrometastasis formation in viable fresh tissue or the live animal is necessary for a critical understanding of tumor progression and its control.

To enhance the resolution of the visualization of micrometastases in fresh tissue, we have utilized the green fluorescent protein (GFP) gene, cloned from the bioluminescent jellyfish *Aequorea victoria* ***(3)***. GFP has demonstrated its potential for use as a marker for gene expression in a variety of cell types ***(4,5)***. The GFP cDNA encodes a 283 amino acid polypeptide with molecular mass of 27 kDa ***(6,7)***. The monomeric GFP requires no other *Aequorea* proteins, substrates, or cofactors to fluoresce ***(8)***. Recently, GFP gene gain-of-function mutants have been generated by various techniques ***(9–12)***. For example, in the GFP-S65T clone the serine-65 codon is substituted with a threonine codon which results in a single excitation peak at 490 nm ***(9)***. Moreover, to develop

From: *Methods in Molecular Medicine, vol. 58:*
*Metastasis Research Protocols, Vol. 2: Cell Behavior In Vitro and In Vivo*
Edited by: S. A. Brooks and U. Schumacher © Humana Press Inc., Totowa, NJ

higher expression in human and other mammalian cells, a humanized hGFP-S65T clone was isolated *(13)*. The much brighter fluorescence in the mutant clones allows for easy detection of GFP expression in transfected cells *(31)*. We have isolated numerous GFP transfectants of human and animal cancer cells that are stable in vitro and in vivo **(Table 1)** *(17–20,41,42)*. The transfectants are highly fluorescent in vivo in tumors formed from the cells. Using these fluorescent transfectants, orthotopic-transplant animal models *(14–16,41,42)* were utilized for visualizing the metastatic processes in fresh tissue down to the single cell level that heretofore was not possible.

### 1.1. Isolation of Stable High-Level Expression GFP Transfectants of CHO-K1 and ANIP Human Lung Cancer Cells

The dicistronic DHFR-GFP expression-vector transfected CHO-K1 and ANIP cells were able to grow in levels of methotrexate (MTX) up to 1.5 μ*M* and 50 n*M*, respectively. The selected MTX-resistant CHO and ANIP cells had a striking increase in GFP fluorescence compared to the transiently transfected cells. A subclone was isolated CHO-K1 GFP 38 (Clone-38), which proved to be stable in 1.5 μ*M* MTX, possibly due to stable chromosomal integration of the amplified GFP and DHFR genes *(17)*. There was no difference in the cell proliferation rates of parental cells and selected transfectants determined by comparing their doubling times. A subclone of ANIP that expressed the strongest GFP was isolated and termed ANIP 973-GFP-Clone-26 (Clone 26) *(17,18)*.

### 1.2. Stable High-Level Expression of GFP in Tumors in Nude Mice

Three weeks after subcutaneous injection of CHO Clone-38 cells, the mice were killed. All mice had a subcutaneous tumor which ranged in diameter from 13.0 mm to 18.5 mm (mean 15.2 mm ± 2.9). The tumor tissue was strongly fluorescent, thereby demonstrating stable high-level GFP expression in vivo during tumor growth. Extraction experiments showed that GFP expression of the transfectants did not decrease in vivo even in the absence of MTX, as determined by fluorescence spectrometry *(17)*. A >1 cm tumor was formed 5 wk after inoculation of $1 \times 10^7$ ANIP Clone-26 cells on the flank of a nude mouse. This tumor fluoresced very brightly in vivo *(17,18)*.

### 1.3. GFP-Expressing Macro- and Micrometastases in Nude Mice

Six nude mice were implanted with 1-$mm^3$ cubes of CHO-K1 Clone-38 tumor into the ovary by surgical orthotopic implantation (SOI) *(32)* and were killed at 4 wk *(17)*. All mice had tumors in the ovaries. The tumor had also seeded throughout the peritoneal cavity, including the colon, cecum, small intestine, spleen, and peritoneal wall. The primary tumor and peritoneal metastases were strongly fluorescent. Numerous micrometastases were

**Table 1**
**GFP Transformed Cell Lines Stable In Vitro and In Vivo**

| Tumor type | Cell lines | Tumor type | Cell lines |
|---|---|---|---|
| Prostate | PC-3 | Renal, human | SN-12 |
| | DU-145 | | A-498 |
| | LNCaP | | |
| | | Brain | 324 |
| Melanoma, mouse | B16F10 | | U-251 |
| | B10FO | | U-87 |
| Melanoma, human | LOXMIVI | Ovarian, human | CHO-K1 |
| | SK-MEL-5 | | OVCAR-8 |
| | UACC257 | | OVCAR-3 |
| | | | OVCAR-5 |
| Lung cancer, human | H460 | | RGMI no. 186 |
| | HOP62 | | |
| | EKVX | Larynx | HEP2 (CCL23) |
| | A549/ATCC | | |
| | ANIP973 | CNS | SNB-19 |
| | Lewis Lung | | SNB-75 |
| Colon cancer, human | HCT116 | Tongue | SCC-25 |
| | COLO205 | | |
| | SW620 | Pancreas | BX PC-3 |
| | LS180 | | MIA-PACA 2 |
| | HCT15 | | PACA-1 |
| | KM12 | | |
| | HT29 | Bladder | HTB-9 |
| | WIDR | | RT-4 |
| Breast cancer, human | MDA-MB-435 | Fibrosarcoma | HT Z1080 |
| | MDA-MB-231 | | |
| | MDA-MB-468 | Stomach | NUGC-4 |
| | MCF-7 | | |

detected by fluorescence on the lungs of all mice. Multiple micrometastasis were also detected by fluorescence on the liver, kidney, contralateral ovary, adrenal gland, para-aortic lymph node, and pleural membrane at the single-cell level. Single-cell micrometastases could not be detected by standard histological techniques. Even these multiple-cell small colonies were difficult to detect by hematoxylin and eosin staining, but they could be detected and visualized clearly by GFP fluorescence. Some colonies were observed under confocal microscopy. As these colonies developed, the density of tumor cells was markedly decreased in the center of the colonies.

### 1.4. Patterns of Lung Tumor Metastases after Surgical Orthotopic Implantation (SOI) Visualized by GFP Expression

Primary tumor grew in the operated left lung in all mice after SOI of GFP-transfected ANIP Clone-26. GFP expression allowed visualization of the advancing margin of the tumor spreading in the ipsilateral lung. All animals explored had evidence of chest wall invasion and local and regional spread. Metastatic contralateral tumors involved the mediastinum, contralateral pleural cavity, and the contralateral visceral pleura. While the ipsilateral tumor had a continuous and advancing margin, the contralateral tumor seems to have been formed by multiple seeding events. These observations were made possible by GFP fluorescence of the fresh tumor tissue *(18,19)*. When non-GFP-transfected ANIP was compared with GFP-transformed ANIP for metastatic capability similar results were seen *(18)*. Contralateral hilar lymph nodes were also involved as well as cervical lymph nodes shown by GFP expression. A cervical lymph node metastasis was brightly visualized by GFP in fresh tissue *(18,19)*.

### 1.5. GFP-Expressing Metastases after Intravenous Injection in Nude Mice

CHO-K1 Clone-38 GFP transfectants injected via the tail vein were detected and visualized in the peritoneal wall vessels to the single-cell level *(17)*. These cells formed emboli in the capillaries of the lung, liver, kidney, spleen, ovary, adrenal gland, thyroid gland, and brain.

A total of $1.0 \times 10^7$ ANIP Clone-26 cells were injected in the nude mice in the tail vein. The mice were killed at 4 and 8 wk. In both groups, numerous micrometastatic colonies were detected in the whole lung tissue by GFP expression *(19)*. Even 8 wk after injection, most of the colonies were not obviously further developed compared with mice killed at 4 wk *(19)*. Numerous small colonies that ranged in number down to fewer than 10 cells were detected at the lung surface in both groups. Brain metastases were visualized in both groups *(19)*. After 8 wk, a mouse had systemic metastases in the brain, the submandibular gland, the whole lung, the pancreas, the bilateral adrenal glands, the peritoneum, and the pulmonary hilum lymph nodes *(19)*. All metastases were detected by GFP expression in fresh tissue. We visualized actively colonizing as well as dormant tumor cells in the lung. Many tumor cells in the lung have remained as small, but live, colonies more than 8 wk after intravenous injection *(19)*. Dormant micrometastasis is one of the most important steps to understand in tumor progression *(34)*. In recent studies, the mechanism of this important phenomenon was studied with regard to angiogenesis and other chemical regulators of tumor colonization *(34)*. However, these experimental models did not allow direct observation of the dormant colonies in fresh live tissue as it occurs over time as do the present studies.

### 1.6. Isolation of Stable High-Level Expression GFP Transductants of H460 Human Lung Cancer Cells

GFP-Neo$^r$-retroviral-vector transduced cells were able to grow in vitro at levels of G418 up to 800 μg/mL. The selected G418-resistant H460-GFP cells had bright GFP fluorescence. There was no difference in the cell proliferation rates of parental cells and the GFP tranductants as determined by comparing their doubling times in vitro *(41)*.

### 1.7. Stable High-Level Expression of GFP in H460 Tumors in Nude Mice

Three weeks after subcutaneous injection of H460-GFP cells, the mice were killed. All three mice had a subcutaneous tumor, which ranged in diameter from 1.5 cm to 2.1 cm. The tumor tissue was strongly GFP fluorescent, thereby demonstrating stable high-level GFP expression in vivo during subcutaneous tumor growth. Lung metastases were found, but no metastases were found in systemic organs in the subcutaneous tumor model of H460-GFP *(41)*.

### 1.8. GFP-Expressing Lung and Bone Metastases of H460–GFP in Nude Mice

Eight nude mice were implanted in the left lung by SOI with 1-mm$^3$ cubes of H460-GFP tumor tissue derived from the H460-GFP subcutaneous tumor *(41)*. The implanted mice were killed at 3–4 wk at the time of significant decline in performance status. All mice had tumors in the left lung weighing from 0.985 g to 2.105 g (mean = 1.84 ± 0.4). All tumors (8/8) metastasized to the contralateral lung and chest wall. Seven of eight tumors metastasized to the skeletal system . It was determined that the vertebrae were the most involved skeletal site of metastasis, as seven of eight mice had vertebral metastasis. Three of seven mice had skull metastases visualized by GFP. Metastasis could also be visualized in the tibia and femur marrow by GFP fluorescence. The tumor lodged in the bone marrow and seemed to begin to involve the bone as well.

All of the experimental animals were found with contralateral lung metastases *(41)*. Extensive and widespread skeletal metastasis, visualized by GFP expression, were found in approx 90% of the animals explored. Thus, the H460-GFP SOI model revealed the extensive skeletal metastasizing potential of lung cancer. Such a high incidence of skeletal metastasis could not have been previously visualized before the development of the GFP-SOI model described here which provided the necessary tools.

### 1.9. Isolation of Stable High-Level Expression GFP Transductants of Human Resistance PC-3 Prostate Carcinoma Cells

The GFP- and neomycin-retroviral-vector transduced PC-3 cells were able to grow in levels of G418 up to 1000 μg/mL. The selected G418-resistant cells

had a striking bright GFP fluorescence *(42)*. There was no difference in the cell proliferation rates of parental cells and selected transductants determined by comparing their growth rate in monolayer culture.

### 1.10. Stable High-Level Expression of GFP in PC-3 Tumors Growing Subcutaneously in Nude Mice

Six weeks after subcutaneous injection of PC-3 cells, the mice were killed. The tumor tissue was strongly fluorescent, thereby demonstrating stable high-level GFP expression in vivo during tumor growth *(42)*. Except for the lung and inguinal and iliac lymph nodes, no obvious GFP fluorescent metastases were found in systemic organs in the subcutaneous tumor model.

### 1.11. Bone and Visceral Metastasis Visualized by GFP after Orthotopic Tumor Progression of PC-3

Five of five mice developed strongly fluorescent orthotopic tumors after SOI in nude mice. Three of five tumors metastasized to the skeletal system. The skeletal metastasis included the skull, rib, pelvis, femur, and tibia. All the tumors metastasized to the lung, pleural membrane, and kidney. Four of five tumors metastasized to liver and two of five tumors metastasized to the adrenal gland. In two mice, individual cancer cells or small colonies could be seen in the brain and in one mouse a few cells could be seen in the spinal cord by GFP fluorescence *(42)*.

### 1.12. Fluorescence Optical Tumor Imaging (FOTI)

High-level GFP expression has enabled the real-time external noninvasive whole-body fluorescence optical tumor imaging (FOTI) of the primary tumor and regional and distant metastases in their normal target organs such as brain, bone, lymph node, and liver in live animals *(44)*. The primary tumor and metastasis can be followed in real time in the intact animals by FOTI. A Princeton Instrument charge-coupled device (CCD) thermoelectrically cooled camera is used to collect the optical images from a fluorescence Leica dissecting microscope with a mercury lamp. The images are analyzed and processed with Image Pro-Plus software.

### 1.13. In Vivo Videomicroscopy to Follow Steps of Metastasis

We took advantage of stable GFP-transfected cells for monitoring and quantifying sequential steps in the metastatic process *(43)*. Using CHO-K1 cells that stably express GFP, the visualization of sequential steps in metastasis within mouse liver, from initial arrest of cells in the microvasculature to the growth and angiogenesis of metastases were quantification by intravital videomicroscopy *(35,36)*. Individual, nondividing cells, as well as micro- and macrometastases could clearly be detected and quantified, as could fine cellular details such as pseudopodial

projections, even after extended periods of in vivo growth. The GFP-fluorescent tumor cells had preferential growth and survival of micrometastases near the liver surface. Furthermore, we observed a small population of single cells that persisted over the 11-d observation period, which may represent dormant cells with potential for subsequent proliferation. This study demonstrates the advantages of GFP-expressing cells, coupled with real-time high resolution videomicroscopy, for long-term in vivo studies to visualize and quantify sequential steps of the metastatic process.

### 1.14. Lung Colony Growth by GFP-Transfected Lung Tumor Cells in Histoculture

ANIP Clone-26-seeded mouse lungs were removed from the mice and then histocultured on collagen-sponge-gels *(20–29)*. Tumor colonies grew and spread rapidly in the lung tissue over time in histoculture *(37–39)*. The progressive colonization of normal lung tissue by the lung tumor cells in individual cultures was visualized at multiple time points. After 6 d in histoculture, the tumor colonies were still classifiable as microcolonies. However, by d 14, very extensive growth of the colonies had occurred with three different areas of GFP-labeled malignant cells being visible. By d 24 of histoculture, the tumor colonies had grown significantly, reaching sizes of 750 μm in diameter and involving approximately one-half of the histocultured mouse lung. By 52 d of histoculture, tumor cells had involved the lung even more extensively and appeared to form multiple layers and histologically suggestive structures on the histocultured lung. Also, by d 52, GFP-expressing satellite tumor colonies formed in the sponge-gel distant from the primary colonies in the lung tissue *(20,29)*.

The tumor host-organ chimeric histoculture system we have developed with GFP-fluorescing tumor cells can significantly advance the ability to understand colonization of normal tissue, which may be the governing step of cancer metastasis *(40)*.

## 2. Methods

### 2.1. DNA Manipulations and Expression Vector Constructions

The dicistronic expression vector *(pED-mtxr)* was obtained from Genetics Institute (Cambridge, MA) *(14)*. The expression vector containing the codon-optimized *hGFP-S65T* gene was purchased from CLONTECH Laboratories, Inc. (Palo Alto, CA). To construct the *hGFP-S65T* containing expression vector, *phGFP-S65T* is digested with *Hin*dIII, blunted at the end. The entire hGFP coding region is then excised with *Xba*I. The pED-mtxr vector is digested with *Pst*I, blunted at the end, and further digested with *Xba*I. The *hGFP-S65T* cDNA fragment is then unidirectionally subcloned into *pED-mtxr (17–20)*.

### 2.2. Cell Culture, Transfection, Selection

CHO-K1 cells are cultured in Dulbecco's modified Eagle medium (DMEM) (GIBCO) containing 10% fetal calf serum (FCS) (Gemini Bio-products, Calabasas, CA), 2 m*M* L-gulutamine, and 100 μ*M* nonessential amino acids (Irvine Scientific, Santa Ana, CA). ANIP cells are cultured in RPMI 1640 (GIBCO) containing 10% FCS (Gemini Bio-products, Calabasas, CA), 2 m*M* L-glutamine, and 100 μ*M* nonessential amino acid (Irvine Scientific, Santa Ana, CA) *(17–20)*.

For transfection, near-confluent CHO-K1 or ANIP cells are incubated with a precipitated mixture of LipofectAMINE™ reagent (GIBCO), and saturating amounts of plasmids for 6 h before being replenished with fresh medium *(17–20)*.

CHO-K1 cells and ANIP are harvested by trypsin–EDTA 48 h post-transfection, and subcultured at a ratio of 1:15 into selective medium that contained 1.5 μ*M* MTX. Cells with stably integrated plasmids are selected by growing transiently transfected cells in the MTX-containing medium. Clones are isolated with cloning cylinders (Bel-Art Products, Pequannock, NJ) by trypsin–EDTA. They are amplified and transferred with conventional culture methods. CHO Clone-38 and ANIP Clone-26 were chosen because of their high-intensity GFP fluorescence and stability *(17–20)*.

### 2.3. Retroviral DNA Expression Vector

The RetroXpress vector pLEIN is purchased from CLONTECH Laboratories, Inc. (Palo Alto, CA). The pLEIN vector expresses enhanced green fluorescent protein *(EGFP)* and the neomycin resistance ($neo^r$) genes on the same bicistronic message that contains an IRES site *(41,42)*.

### 2.4. Cell Culture, Retroviral Production, Transduction, and Subcloning

PT67, an NIH3T3-derived packaging cell line, expressing the 10 Al viral envelope, is purchased from CLONTECH Laboratories, Inc. PT67 cells are cultured in DME (Irvine Scientific, Santa Anna, CA) supplemented with 10% heat-inactivated FBS (Gemini Bio-products, Calabasas, CA). For vector production, packaging cells (PT67), at 70% confluence, are incubated with a precipitated mixture of DOTAP™ reagent (Boehringer Mannheim), and saturating amounts of pLEIN plasmid for 18 h. Fresh medium is replenished at this time. The cells are examined by fluorescence microscopy after 48 h. For selection of GFP transductants, the cells are cultured in the presence of 500 μg/mL–2000 μg/mL of G418 (Life Technologies, Grand Island, NY) for 7 d *(41,42)*.

### 2.5. Retroviral Transduction of Tumor Cells

For GFP gene transduction, 20%-confluent cancer cells are incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67 cells and RPMI

1640 (GIBCO) containing 10% FBS (Gemini Bio-products, Calabasas, CA) for 72 h. Fresh medium is replenished at this time. Cells are harvested with trypsin–EDTA 72 h post-infection, and subcultured at a ratio of 1:15 into selective medium that contains 200 μg/mL of G418. The level of G418 is increased to 800–1000 μg/mL gradually. Clones expressing GFP are isolated with cloning cylinders (Bel-Art Products, Pequannock, NJ) with trypsin–EDTA and are amplified and transferred by conventional culture methods *(41,42)*.

### 2.6. Doubling Time of Stable GFP Clones

Parental cells and GFP transductants are seeded at $2.0 \times 10^5$ in 60 mm culture dishes. The cells are harvested and counted every 24 h using a hemocytometer (Reichert Scientific Instruments, Buffalo, NY). The doubling time is calculated from the cell growth curve over 6 d *(17–20,41,42)*.

### 2.7. Subcutaneous Tumor Growth

Three 6-wk-old BALB/c *nu/nu* female mice are injected subcutaneously with a single dose of $10^6$–$10^7$ GFP transductants. Cells are first harvested by trypsinization and washed 3× with cold serum-containing medium, then kept on ice. Cells are injected in a total volume of 0.4 mL within 40 min of harvesting. The nude mice are sacrificed to harvest the tumor fragments 3 wk after tumor cells injection *(17–20,41,42)*.

### 2.8. Surgical Orthotopic Implantation (SOI) of CHO-K1 GFP in Nude Mice

Tumor fragments (1 $mm^3$) derived from the nude mouse subcutaneous CHO-K1 GFP tumor were implanted by SOI on the ovarian serosa in six nude mice *(17,30)*. The mice are anesthetized by isofluran inhalation. An incision is made through the left lower abdominal pararectal line and peritoneum. The left ovary is exposed and part of the serosal membrane is scraped with a forceps. Four 1-$mm^3$ tumor pieces are fixed on the scraped site of the serosal surface with an 8-0 nylon suture (Look, Norwell, MA). The ovary is then returned into the peritoneal cavity, and the abdominal wall and the skin are closed with 6-0 silk sutures. Four weeks later, the mice are killed and the lungs and the other organs are removed. All procedures of the operation described above are performed with a ×7 magnification microscope (Olympus).

### 2.9. Surgical Orthotopic Implantation of ANIP-GFP or H460-GFP Human Lung Cancer in Nude Mice

Tumor fragments (1 $mm^3$) derived from the ANIP-GFP or H460-GFP subcutaneous tumor growing in nude mouse are implanted by SOI on the left lung in eight nude mice *(18,41)*. The mice are anesthetized by isofluran inhala-

tion. The animals are put in a position of right lateral decubitus, with forelimbs restrained. A 0.8-cm transverse incision of skin is made in the left chest wall. Chest muscles are separated by sharp dissection and costal and intercostal muscles are exposed. A 0.4–0.5-cm intercostal incision between the third and fourth rib on the chest wall is made and the chest wall is opened. The left lung is taken up by a forceps and tumor fragments are sewn promptly into the upper lung promptly by one suture. The lung is then returned into the chest cavity. The incision in the chest wall is closed by a 6-0 surgical suture. The closed condition of the chest wall is examined immediately and, if a leak existed, it is closed by additional sutures. After closing the chest wall, an intrathoracic puncture is made by using a 3-mL syringe and 25G $^1/_2$ needle to withdraw the remaining air in the chest cavity. After the withdrawal of air, a completely inflated lung can be seen through the thin chest wall of the mouse. Then the skin and chest muscle are closed with a 6-0 surgical suture in one layer. All procedures of the operation described are performed with a ×7 magnification microscope (Olympus).

### 2.10. SOI of PC-3-GFP Cells

Two tumor fragments (1 $mm^3$) from a high GFP-fluorescent subcutaneous tumor from a single animal are implanted by SOI in the dorsolateral lobe of the prostate in five nude mice *(42)*. After proper exposure of the bladder and prostate following a lower midline abdominal incision, the capsule of the prostate is opened and the two tumor fragments are inserted into the capsule. The capsule is then closed with an 8-0 surgical suture. The incision in the abdominal wall is closed with a 6-0 surgical suture in one layer *(42)*. The animals are kept under isoflurane anesthesia during surgery. All procedures of the operation described above are performed with a ×7 magnification microscope (Olympus).

### 2.11. Analysis of the Metastases

Mice are killed when their performance status begins to decline and the systemic organs are removed. The orthotopic primary tumor and all major organs as well as the whole skeleton are explored. The fresh samples are sliced at approx 1 mm thickness and observed directly under fluorescence microscopy. The samples are also processed for histological examination for fluorescence in frozen sections. The slides are then rinsed with phosphate-buffered saline (PBS) and then fixed for 10 min at 4°C in 2% formaldehyde plus 0.2% glutaraldehyde in PBS. The slides are washed with PBS and stained with hematoxylin and eosin using standard techniques.

### 2.12. Stability of GFP Expression

The subcutaneous GFP tumors from the nude mice are minced for in vitro culture. Cells are subcloned in cell culture medium in the absence of selective

pressure. A total of $10^7$ parental cells are harvested. Cell extracts are prepared by lysis in 0.1% IGEPAL CA-630 (Sigma) with 1 m*M* EDTA in PBS *(17)*. The cell extracts are diluted 1:10 with PBS. GFP fluorescence is measured with a fluorescence photometer (Hitachi F-2000; excitation 490 nm, emission 515 nm).

### 2.13. Microscopy

Light and fluorescence microscopy are carried out using a Nikon microscope equipped with a xenon lamp power supply. A Leica stereo fluorescence microscope model LZ12 equipped with a mercury lamp power supply was also used. Both microscopes have a GFP filter set (Chroma Technology, Brattleboro, VT). An MRC-600 confocal imaging system (Bio-Rad) mounted on a Nikon microscope with an argon laser is also used. Photomicrographs are processed for brightness and contrast with Image Pro Plus, Version 3.0, software (Media Cybernetics, Silver Spring, MD).

### 2.14. Intravenous Injection

Nude mice are injected in the tail vein with a single dose of $1 \times 10^7$ GFP cancer cells. Cells are first harvested by trypsinization and washed 3× with cold serum-containing medium, then kept on ice. Cells are injected in a total volume of 0.8 mL of serum free medium within 40 min of harvesting. After various times, the mice are killed and fresh visceral organs are analyzed by fluorescence microscopy.

### 2.15. Tumor Progression in Histoculture

Whole lung tissues seeded with ANIP-GFP clone 26 cells are aseptically removed from the nude mice. The lung tissues are divided into pieces of approx 2–3 mm in diameter, which are then placed on prehydrated collagen spongegels (Upjohn Co., Kalamazoo, MI). The gels are floated in 24-well plates at the air–water interface in RPMI 1640 containing 20% FCS (Gemini Bio-products, Calabasas, CA), 2 m*M* L-glutamine, and penicillin. The histocultures are incubated at 37°C in a humidified atmosphere containing 95% air and 5% $CO_2$. The lung tumor colony growth in the histocultured host lung tissue is repeatedly observed in the same cultures with fluorescence photomicroscopy of the GFP expression at d 6, 14, 24, and 52 of histoculture *(20,29)*.

Using the methods developed described in this review, GFP fluorescence will facilitate the understanding of tumor growth and progression including seeding and target organ colonization that should provide new insights into metastatic mechanisms and treatment of metastatic disease. These developments with tumor cells that stably express GFP in vivo and in vitro provide invaluable new tools for understanding the most important steps in tumor host–organ interaction, tumor progression, and metastasis.

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