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Imaging Transgenic Nude Mice Expressing Spectrally-distinct Fluorescent Reporters Emitting From Blue to Far Red Light With One Instrument With Single-nanometer-tuning of Laser-excitation Fluorescence

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Abstract. *Background/Aim: Transgenic nude mice expressing green fluorescent protein (GFP), red fluorescent protein (RFP), or cyan fluorescent protein (CFP) were previously developed by our laboratory, AntiCancer Inc. In the present study, we demonstrate imaging of the GFP, RFP, or CFP nude mice with single-nanometer-tuning laser fluorescence excitation with a single instrument. Materials and Methods: Female transgenic C57/B6 nude GFP, RFP, and CFP mice aged six weeks were used. The images were obtained using the UVP Biospectrum Advanced system (Analytik Jena US LLC) with excitation at 480 nm and peak emission at 513 nm for GFP; 520 nm and 605 nm, respectively, for RFP; and 405 nm and 480 nm, respectively, for CFP. Results: For each color transgenic fluorescent mouse, images without background could be obtained individually with the UVP Biospectrum Advanced system. Conclusion: Using a single instrument, brilliant and well-defined images of GFP, RFP, and CFP mice were obtained with single-nanometer-tuning laser fluorescence excitation. This imaging system will be used in future studies to analyze cancer cells in the colored mice that are spectrally distinct in order to determine how stromal cells and cancer interact in the tumor microenvironment.*

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Transgenic green fluorescent protein (GFP), red fluorescent protein (RFP), or cyan fluorescent protein (CFP) nude mice were previously developed at our laboratory, AntiCancer Inc. (1-3). Fluorescent-protein-imaging *in vivo,* pioneered by our laboratory, is used to investigate tumor growth and progression at the macro, cellular, and subcellular levels *in vivo* (4-8). Using spectrally-distant fluorescent proteins, cancer and stromal cells could be distinguished by multi-color imaging in the tumor microenvironment (TME) (1, 3, 4, 9-13).

In the present study, we demonstrate imaging of transgenic nude mice with spectrally-distinct fluorescent reporters expressing GFP, RFP, or CFP, with single-nanometer-tuning laser fluorescence excitation using one instrument.

Materials and Methods

Mice. Transgenic C57/B6 nude mice expressing GFP, RFP, or CFP aged six weeks (AntiCancer, Inc., San Diego, CA, USA) were used (Figure 1). The cytomegalovirus enhancer and the chicken β-actin promoter regulate the expression of the fluorescent protein genes in these transgenic nude mice (1-3). All mice were bred at AntiCancer Inc. in a barrier facility with a high efficiency particulate air (HEPA)-filtered rack, and kept in standard settings with 12-h light/dark cycles. The present protocol was approved by the AntiCancer Inc. Institutional Animal Care and Use Committee following the National Institutes of Health Guide for the Care and Use of Animals.

Imaging. The present study used a UVP Biospectrum Advanced system (Analytik Jena US LLC) (Figure 2) for imaging with single-nanometer-wavelength tuning of laser-excitation fluorescence, at 480 nm and peak emission at 513 nm for GFP; 520 nm and 605 nm,

respectively, for RFP; and 405 nm and 480 nm, respectively, for CFP. Imaging was performed using an anesthetic solution of 50% ketamine (100 mg/ml), 38% xylazine (100 mg/ml), and 12% acepromazine maleate (10 mg/ml) injected intramuscularly.

Results

Bright-field images of a transgenic CFP nude mouse; an RFP transgenic nude mouse and a GFP transgenic nude mouse are shown in Figure 1. The expression of RFP is so strong in the transgenic RFP nude mouse that it appears red even in bright light. Merged very bright images of transgenic GFP, RFP, and CFP nude mice were obtained with single-nanometer-tuning fluorescence laser excitation (Figure 3).

Discussion

The present study described a single instrument (Analytik Jena UVP Biospectrum Advanced system) with single-nanometer-tuning laser fluorescence excitation, that could image brightly and clearly transgenic mice expressing fluorescent reporters emitting over a broad range of wavelengths from 480 nm to 605 nm with excitement ranging from 405 nm to 520 nm.

The application of fluorescent proteins to *in vivo* imaging is routinely used to monitor the growth and progression of tumors at the macro, cellular, and subcellular levels (4-8).

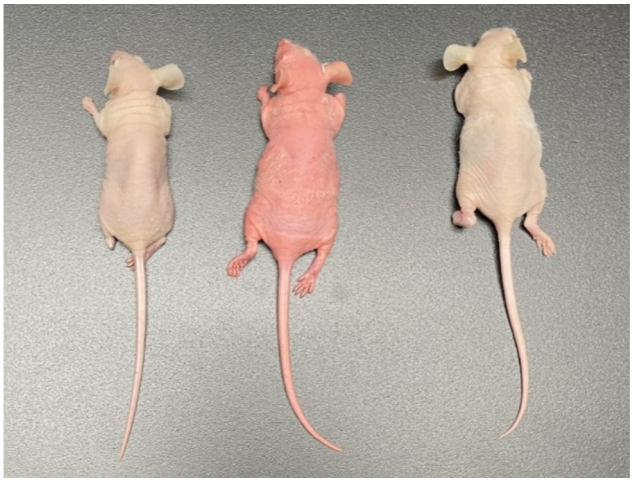

Figure 1. *Brightfield images of transgenic nude mice (Left: Cyan fluorescent protein nude mouse. Center: Red fluorescent protein nude mouse. Right: Green fluorescent protein nude mouse).*

In vivo imaging with fluorescent proteins was pioneered by our laboratory (7-9).

Imaging the interaction of cancer and stromal cells in the TME using spectrally-distinct fluorescent proteins has been demonstrated (1, 3, 4, 9-13). Bouvet *et al*. observed the tumor-host interaction in liver metastasis using transgenic

Figure 2. *External and internal view of the Analytik Jena UVP Biospectrum Advanced system. Left; External view. Right: Internal view). Scale bar: 10 cm.*

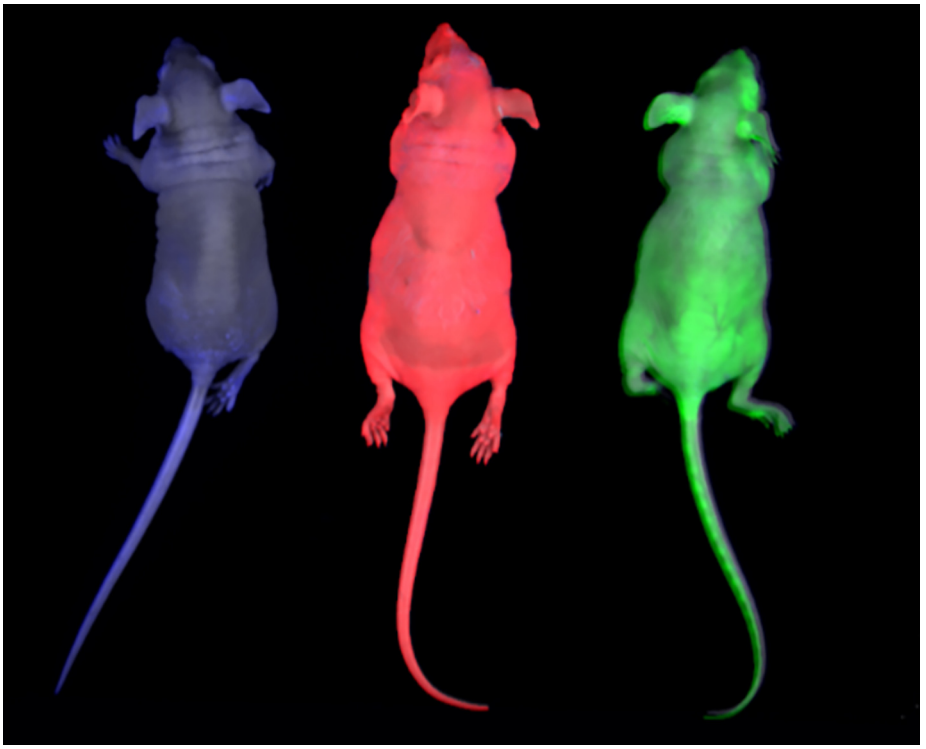

Figure 3. *Merged images of cyan fluorescent protein (CFP), red fluorescent protein (RFP), and green fluorescent protein (GFP) nude mice. Excitation at 405 nm and peak emission at 480 nm for the CFP nude mouse; 520 nm excitation and 605 nm peak emission for the RFP nude mouse; 480 nm excitation and 513 nm peak emission for the GFP nude mouse; Exposure time: 40 ms.*

nude mice expressing GFP and human colon-cancer cell lines expressing RFP (12). Suetsugu *et al*. demonstrated pancreatic-cancer-patient tumors established in NOD/SCID mice that were subsequently passaged orthotopically in transgenic nude mice expressing RFP, acquired stroma expressing RFP. Further passage to transgenic nude mice expressing GFP and CFP resulted in the tumors acquiring GFP and CFP stroma, respectively. Multicolored stroma are useful in analyzing the role of host stroma in the initiation and progression of metastasis (13).

Recent studies have used single-nanometer-tuning laser excitation to image orthotopic tumors expressing fluorescent reporters without background (14-16).

In the present study, multi-spectral *in vivo* imaging with single-nanometer-tuning (14-16) was performed on nude mice expressing transgenic fluorescent protein with emission ranging from 480 to 605 nm.

Conclusion

The very narrow-bandwidth-tuning laser excitation enabled the acquisition of very bright and well-defined images of GFP, RFP, and CFP transgenic mice with a single instrument. Further studies will use this imaging system to study spectrally-distinct cancer cells in each colored mice to determine the interaction of cancer and stromal cells in the TME.

Conflicts of Interest

AW, NC, and SG are employees of Analytik Jena. JR is an employee of Anticancer Inc. KM, SM, MS, BMK, and RMH are non-salaried associates of AntiCancer Inc. AntiCancer Inc. uses mouse models of cancer for contract research.

Authors' Contributions

KM, SG, AW, and NC performed experiments. KM and RMH wrote this article. JR provided the transgenic nude mice, SM, MS, and BMK, critically reviewed this article.

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