Detection and Analysis of Tumor Fluorescence Using a Two-Photon Optical Fiber Probe

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ABSTRACT The utility of a two-photon optical fiber fluorescence probe (TPOFF) for sensing and quantifying tumor fluorescent signals was tested in vivo. Xenograft tumors were developed in athymic mice using MCA207 cells expressing green fluorescent protein (GFP). The TPOFF probe was able to detect ex vivo fluorescence from excised tumors containing as little as 0.3% GFP-expressing cells. TPOFF results were similar to both flow-cytometric analysis of tumor cells after isolation and suspension, and fluorescence determined by microscope images of cryosectioned tumors. TPOFF was then used to measure GFP fluorescence from tumors in live mice. The fiber probe detected fluorescently-labeled Herceptin antibody targeted to HER2-expressing tumors in severe combined immunodeficient mice. Dendrimer nanoparticles targeted by folic acid and having 6-TAMRA as a fluorescent probe were also used to label KB cell tumors in vivo. The fiber probe documented a fourfold increase in tumor fluorescence in animals that received the targeted dendrimer. These results suggest TPOFF can be used as a minimally invasive system for identifying tumor markers and monitoring drug therapy.

INTRODUCTION

The development of noninvasive measures to identify cancer signatures, monitor drug delivery, and evaluate drug-induced effects in tumors has been a goal for enhancing cancer treatment (Nicolette and Miller, 2003). As mechanisms related to tumor development are identified, signatures of these pathways may also have importance in selecting therapeutics. Because the therapeutic index of any cancer drug is partly dependent on the local concentration and duration of exposure to the drug in the tumor (Lankelma, 2002), monitoring the spatial and temporal distribution of cancer drugs within tumors may allow for more effective and precise dosing (Eichler and Muller, 1998). Therefore, there are several reasons why it is important to develop means for in vivo tumor analysis.

One method proposed for tumor analysis in vivo involves the use of fluorescent probes (Weissleder and Ntziachristos, 2003). This approach has the advantage of being more biocompatible than radiation or chemical analysis, and builds on the wide base of technology developed for in vitro analysis using flow cytometry and histochemical staining. However, the use of fluorescent probes for tumor analysis in vivo has technical problems. The most significant issue is the light absorbing and scattering properties of tissues that make it difficult to perform noninvasive fluorescence analysis by spectroscopic techniques (Gan and Gu, 2000; Li et al., 1998; Sefkow et al., 2001; Svanberg, 2002). Although tissue tends to absorb light at ultraviolet-visible wavelengths mainly below 600 nm, scattering still occurs at red and near infrared regions, and other molecules, such as NAD, collagen, and elastin, cause autofluorescence that can interfere with signals (Andersson et al., 1997; Pitts et al., 2001; Svanberg, 2002). Thus, regardless of their wavelength, fluorescent signals from tissues can be retrieved only within several millimeters of a surface. This limits the use of fluorescent markers for most internal tumors, and makes quantitative analysis particularly difficult. Although localized fluorescence from organs has been visualized by whole-body imaging (Mitsiades et al., 2003), such analysis is not quantitative and may require large amounts of fluorescent signals for penetration through tissue barriers.

A fiber optic probe inserted into a tumor through a thin (27-gauge or higher) needle could provide a solution for the delivery and retrieval of light in vivo. Optical fiber-based detection would be minimally invasive since it would not require tissue excision or isolation of cells. The probe could also be inserted using imaging techniques to ensure precise localization within a suspected tissue site. Fiber optic biosensors have previously been employed for in situ quantitation of fluorescent chemicals or biochemical end products (Abel et al., 1996; Baker et al., 1999; Cullum et al., 2000; Mourant et al., 1999; Mulchandani et al., 1999; Tan et al., 1992; Vo-Dinh et al., 1991). However, these methods were based largely on one-photon fluorescence detection. Two-photon fluorescence detection has a number of advantages over a system based on one-photon fluorescence detection. Two-photon fluorescence detection has a spatial resolution of only a few microns owing to its localized nonlinear excitation nature. Two-photon fluorescence detection also allows a broad range of fluorochromes to be excited with a single laser, allowing one to simultaneously measure multiple emitters reporting different analyses (e.g., the presence of a tumor marker and drug). In addition,

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two-photon systems employ near-infrared light for excitation, which minimizes tissue damage, photobleaching, and intrinsic tissue fluorescence. Finally, since the wavelength of the emitted light is significantly shorter than the excitation wavelength, the emission can be easily filtered and separated to obtain a high signal-to-noise ratio.

Although two-photon absorption excitation has been widely applied to microscopic imaging (Denk et al., 1990; Squirrell et al., 1999) and recently to endoscopy based on gradient-index lenses (Jung and Schnitzer, 2003), its use for in vivo biosensing with a flexible fiber optic system has not been explored. We have recently reported the development of a two-photon optical fiber fluorescence (TPOFF) system using a single-mode fiber to deliver femtosecond laser pulses for excitation and to collect emitted fluorescence from cell pellets back through the same fiber (Ye et al., 2002). Whereas other optical systems require separate devices for excitation and collection of fluorescence signals (Lago et al., 1995), this unique configuration using a single optical fiber for both two-photon excitation and collection provides a very compact system for making minimally invasive and highly spatially localized fluorescence measurements in vivo. In the present work, we report in vivo fluorescence detection in tumors possessing varying proportions of green fluorescent protein (GFP)-expressing MCA207 mouse sarcoma cells, based on the TPOFF probe. In addition, we demonstrate the utility of TPOFF to detect a targeted antibody in MCA207 tumors and a targeted fluorescent nanoprobe in human squamous cell (KB) tumors in mice, documenting the presence of two different tumor markers.

MATERIALS AND METHODS

Materials

KB cells (a human epidermoid carcinoma cell line) were obtained from ATCC (Rockville, MD). Kevin McDonough at the University of Michigan kindly provided green fluorescent protein (GFP) - and HER2-transfected mouse sarcoma MCA207 cell lines. Herceptin was from the University of Michigan Hospital Pharmacy. Phosphate-buffered saline (PBS), Hanks Balanced Salt Solution (HBSS) and other cell culture reagents were from Gibco BRL (Grand Island, NY). 6-carboxytetramethylrhodamine succinimidyl ester (6TAMRA, 6T) was purchased from Molecular Probes (Eugene, OR). 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide HCl (DPE) was from Pierce (Rockford, IL). The G5-PAMAM dendrimer was synthesized and characterized as described (Tomalia et al., 1985) (M = 26,000; 110 surface NH_2 groups). The athymic nu/nu and SCID mice were purchased from Charles River (Richmond, IN). All other chemicals were from Sigma-Aldrich (St. Louis, MO).

Synthesis of Herceptin-FI conjugate

Herceptin antibody-FI conjugate (HN-FI) was synthesized using standard protocols. Briefly, a dimethylsulfoxide (DMSO) solution of FI in 40-fold molar excess was added to a PBS solution of the antibody (5 mg/ml) and incubated in dark at room temperature. The reaction was then quenched with ammonium chloride (50 mM) and the conjugate was purified by gel filtration on a G-25 Sephadex PD-10 column (Pharmacia, Kirkland, Canada). The conjugate fractions were collected and concentrated.

Synthesis of G5-6T and G5-6T-folic acid (FA) dendrimer conjugates

2.4 μmol of G5 suspended in 1 M $NaHCO_3$ buffer, pH 8, was slowly stirred with 8.2 μmol of 6T for 20 h at 4°C. The mixture was dialyzed in PBS and water in their order, ultrafiltered, and lyophilized to obtain the product G5-6T. For conjugation of FA to G5-6T, 4.3 μmol of FA was initially activated by reaction with 6.5 μmol of DPE in dimethylformamide/DMSO (3:1) for 1.5 h, under N_2. The mixture was then stirred with 0.73 μmol of G5-6T for 2.5 days at 4°C, dialyzed as given above, and the product (G5-6T-FA) was lyophilized. The remaining surface NH_2 groups of G5-6T and G5-6T-FA were acetylated by reacting 0.73 μmol of the synthesized products in two successive steps of 1 h duration with 79.5 μmol of acetic anhydride and 103 μmol of ethylene triamine. This mixture was stirred for 20 h, dialyzed, and ultrafiltered as given above. The product was further purified by size exclusion chromatography, lyophilized, and stored at −20°C. Characterization of the product by ^{1}H-NMR and ultraviolet-visible spectroscopy gave the respective numbers of 6T and FA molecules per dendrimer to be 3 and 4. TPOFF analysis of G5-6T dendrimers with and without FA showed that the optical spectra of these compounds had a maximum emission at 560 nm similar to free 6T.

Tissue culture

MCA207 cells were maintained in DMEM media supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 μm L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, under 5% CO_2. KB cells were maintained in FA-free Roswell Park Memorial Institute-1640, and under the other conditions given above.

Animal model

MCA207 and KB cell xenograft tumors were developed in 7-week-old female athymic nu/nu or SCID mice by subcutaneous inoculation of 5×10^6 cells in the left and right flank area. The cells were rinsed and injected as a 0.1-ml suspension using sterile PBS. Mice were housed under sterile conditions and the tumors were allowed to reach ~0.7–0.8 cm in diameter before analysis. At the end of the study, the mice were anesthetized with isoflurane and the tumors were excised for analysis. The control and GFP-transfected MCA207 tumors were excised and divided into three portions for flow cytometry, imaging, and TPOFF analysis.

Flow cytometric and microscopic analysis

Fresh tumor samples were suspended in Hanks' balanced salt solution (HBSS), crushed, and digested with 1 mg/ml type IV-collagenase for 2 h at room temperature. The digested tissue was passed through a 70-micron filter, rinsed with HBSS and fixed in 2% paraformaldehyde. The fixed cells were rinsed and suspended in PBS containing 0.1% bovine serum albumin for flow-cytometric analysis. For microscopic imaging, the tissue was chilled in Optimum Cutting Temperature solution (Sakura Finetechnical, Tokyo, Japan) by immersing in methylbutane/dry ice mixture (−50°C), and stored at −80°C for cryosectioning. Fifteen-micron sections were thawed, hydrated, and fixed with paraformaldehyde and rinsed. The sections were stained with 4′,6-diamidino-2-phenylindole for 10 min, rinsed, placed on 0.17-μ coverslips and mounted with ProLong (Molecular Probes, Eugene, OR). The images were taken on a Nikon Multiphot (Tokyo, Japan) inverted microscope using a Hamamatsu camera (Shizuoka, Japan). The pixel intensities of the images were measured with NIH Image software (Washington, DC).

TPOFF measurement through optic fiber

TPOFF detection was performed as previously described (Ye, et al., 2002). Briefly, ultrashort pulses from a Ti:sapphire laser (80-fs pulses at 830 nm) were coupled into a single-mode optical fiber. The fiber was inserted into the

tissue through a 27-gauge needle, with the fiber tip extending to the proximal edge of the needle opening. The two-photon excited fluorescence was collected back through the same fiber. The fluorescence signal was separated from the excitation beam with a dichroic mirror, and further filtered with a short-pass filter (E600SP, Chroma, McHenry, IL) and a spectrometer (250is/sm, Chromex, Albuquerque, NM), then detected with a photon-counting photomultiplier tube. TPOFF counts were taken in multiple internal regions of the tumor, with the tip of the needle cleaned by bath-sonication between each run, and the readings were taken for ~60 s in each region at the rate of one reading per second. TPOFF counts were taken in live mice by anesthetizing the mice by isoflurane inhalation for 1 min, and inserting the needle into the tumor through the skin.

Statistical significance of differences among groups was analyzed by the Student-Newman-Keuls test, with significance calculated at $P < 0.05$ by *t*-test.

RESULTS

Analysis of tumor fluorescence by flow cytometry and microscopic imaging

MCA207 tumor cells were injected subcutaneously to form xenograft tumors in nu/nu mice. The tumors reached an average size of 0.7-cm diameter 10 days after injection of 5×10^6 cells. To obtain tumors with variable GFP content, mice were injected with different proportions of control and GFP-expressing cell lines ranging from 0.3% to 100% GFP-positive cells, while keeping the total number of injected cells the same. The proportion of GFP-positive cells in the tumor was verified by flow cytometry before injection, and microscopic analysis and flow cytometry of cells isolated from the tumor. The overall population of fluorescent cells was proportional to the percentage of GFP-positive cells administered (Fig. 1 *A*). The GFP fluorescence in the tumor interior was somewhat heterogeneous, as demonstrated by microscopic analysis of tissue cryosections (Fig. 1 *B*).

TPOFF fluorescence identification and quantification ex vivo in isolated tumors

TPOFF measurements were performed ex vivo in multiple internal regions of the various GFP-expressing tumors isolated by resection (Fig. 2 *A*). The probe was inserted into different portions of the tumors through a 27-gauge needle and readings were taken continuously during the insertion through the tumor. The results are the average of five readings taken in different parts of each tumor, and demonstrate a dose-dependent increase in fluorescence in tumors developed with increasing proportions of GFP cells. Readings for different regions of each tumor varied with a standard deviation of 10–20%, as expected given the heterogeneous distribution of fluorescence observed in the microscopic images of tissue sections. A comparison of the mean TPOFF readings with flow cytometric values from isolated cells, as well as with microscopic image pixel intensity is shown in Fig. 2 *B*. As the fiber probe excitation area is 4 μ in diameter, pixel intensity analysis was taken at a cross-section width of 10 pixels (~4 μ), to provide a direct comparison. The correlation of the average TPOFF reading versus percent GFP by flow cytometry of isolated cells was very close, giving an R^2 value of 0.99. A similar correlation was observed between flow cytometry and microscopic pixel intensity analysis with an R^2 value of 0.98.

TPOFF tumor fluorescence identification and quantification in vivo

TPOFF measurements were then taken in vivo in tumors in live mice. Tumors ranged from 0 to 50% GFP-expressing cells as verified by flow cytometry performed on an aliquot of cells before implantation. The probe was inserted into the tumor through the skin. A progressive increase in fluorescence was observed with increasing percentages of GFP-expressing cells in the tumor (Fig. 3).

Identification and quantification of targeted tumor fluorescence by TPOFF

The utility of TPOFF probe for quantifying tumor fluorescence was tested in HER2-expressing MCA207 tumors

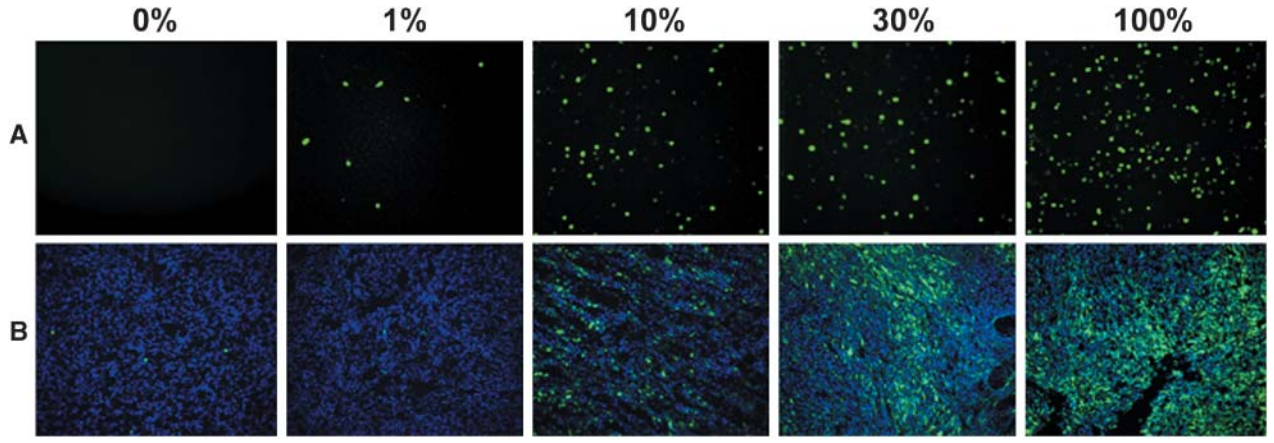

FIGURE 1 Fluorescent microscopic images of nu/nu mice tumors developed with 0–100% GFP-transfected cells. (*A*) Isolated cells; (*B*) cryosections.

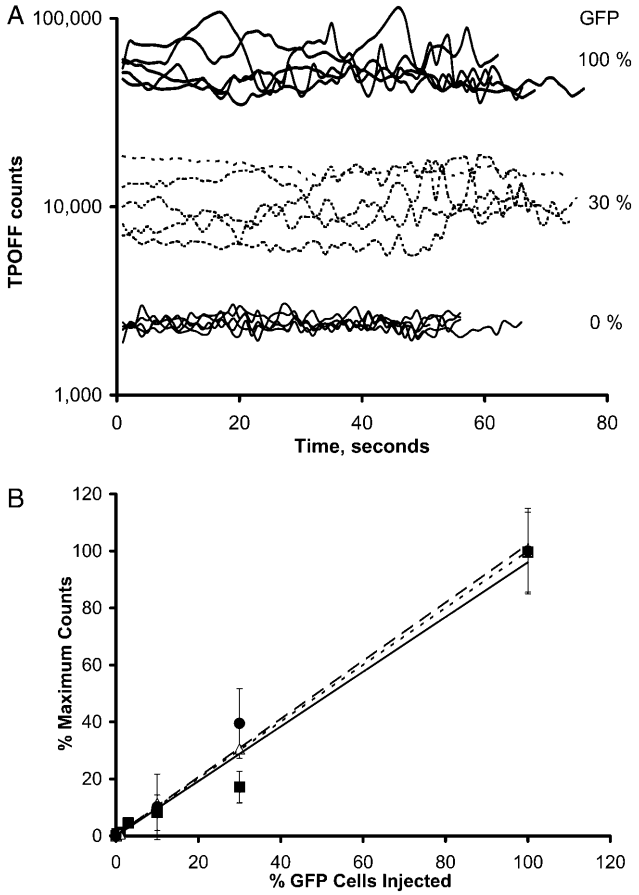

FIGURE 2 TPOFF counts of isolated tumors. TPOFF counts were taken in distinct internal regions of each tumor by repeated insertion of the probe for 60–70 s. The needle was vertically moved into the tumor and one reading was recorded per second. (*A*) Five individual tracings of TPOFF counts from three tumors developed with 0, 30, and 100% GFP cells. Similar data were obtained in four different sets of tumors. (*B*) Comparison of TPOFF counts to flow cytometric and microscopic data. The values are expressed as percentage of the maximum fluorescence obtained for each method. For each method there is a linear relationship between average counts and percent GFP-transfected cells. The TPOFF counts (*square symbols*, *solid line*) shown are the mean obtained for five different internal regions of the tumors. The flow cytometric FL1 fluorescence (*triangle symbols*, *short-dashed line*) is the mean obtained for 10,000 cells. The microscopic image pixel intensities (*circle symbols*, *long-dashed line*) shown are the mean of five different areas 10 pixels wide × 640 pixels long.

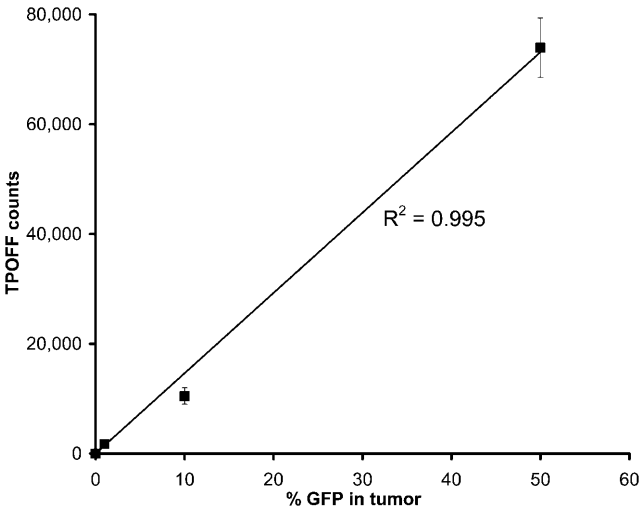

FIGURE 3 TPOFF counts of tumors in live nu/nu mice. The tumors were developed with 0–50% GFP-transfected cells. TPOFF counts were taken at four distinct internal regions of each tumor by repeated insertion of the probe through the skin of the anesthetized mouse. Shown are the mean ± SE of the TPOFF counts, showing the linear relationship between percent GFP-transfected cells and TPOFF counts.

using systemically administered fluorescent labeled antibody HN-FI as the targeting agent (Molina et al., 2001). Free FI or control antibody administered intravenously failed to associate with either control or HER2-expressing tumors. However, significantly higher HN-FI fluorescence was detected in the HER2-expressing tumor as compared to control tumors at 4 h, as demonstrated by both TPOFF probe and flow cytometry, in isolated tumors ex vivo (Fig. 4) and in live mice in vivo (Fig. 5). Although the TPOFF data showed some accumulation of HN-FI in the control tumor, flow cytometric analysis of isolated cells failed to show any corresponding increase in fluorescence (compare Fig. 5, *A* and *B*). This is probably due to the TPOFF detection of bound HN-FI in tumor macrophages or other blood components, which are separated from tumor cells when selected for flow cytometric analysis.

G5 dendrimers with FI and FA as the respective detecting and targeting agents (G5-FI-FA) have been shown to target and internalize into KB cells in vitro due to binding to the high-affinity folate receptor, and show saturable receptor binding kinetics obtained by flow cytometry and TPOFF (Quintana et al., 2002; Ye et al., 2002). KB cell tumors in SCID mice that expressed high-affinity FA-receptor (FAR) were targeted by folate-conjugated G5 dendrimers bearing the fluorescent dye 6-TAMRA (6T). The TPOFF counts obtained for the tumors were converted to pmols of dendrimer from standard curves of TPOFF readings generated for different dendrimer concentrations. As shown in Fig. 6 *A*, there was an eightfold increase in the levels of G5-6T-FA in KB cell tumors as compared to the nontargeted G5-6T dendrimer, whereas the liver and spleen failed to show any uptake of the dendrimers. There was a threefold increase in the fluorescence of G5-6T-FA dendrimers in FAR-positive KB cell tumors as compared to the FAR-negative MCA207 tumors (Fig. 6 *B*).

As shown in Fig. 6, in the FAR-positive KB cells, G5-6T-FA reached average levels of ~2.5 μM. As there are 3–4 FA per dendrimer, the average intracellular FA equivalent is 7.5–10 μM. These values compare well with radioactive FA binding studies that show KB cell binds ~1–10 μM of FA in the presence of extracellular concentrations of 5–100 nM of free FA (personal communication). In vitro TPOFF data using fluorescein isothiocyanate-conjugated dendrimer

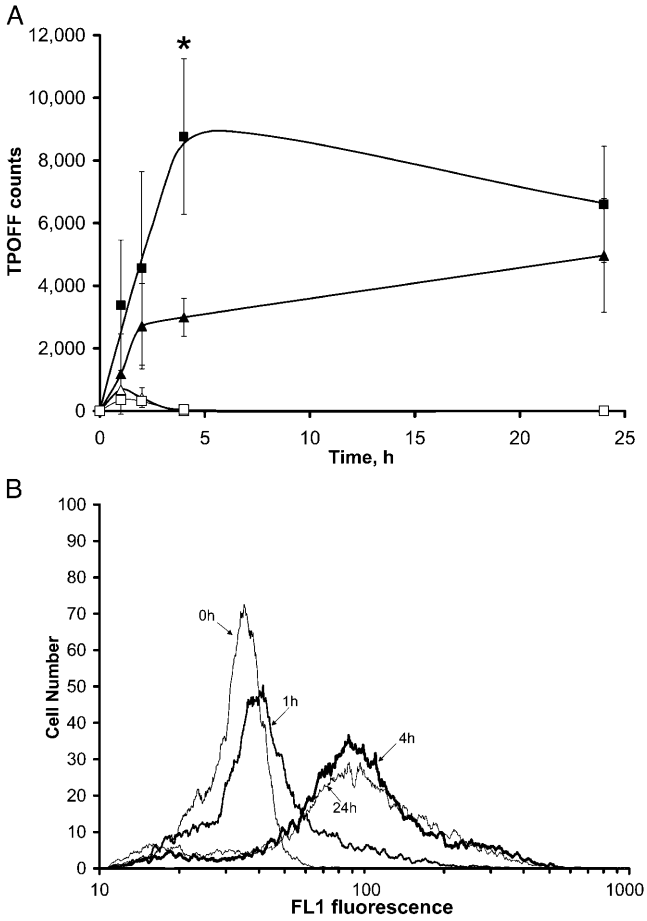

FIGURE 4 Ex vivo TPOFF counts of mouse tumors developed with control MCA207 cells and HER2-transfected MCA207 cells. Control- and HER2-tumors were developed on the left and right flank areas, respectively, of SCID mice. The mice were intravenously injected with Herceptin-FI (HN-FI, 1.7 nmol) or FI (4 nmol) and the tumors were excised at different time intervals for fluorescence measurements. (*A*) Mean ± SE of TPOFF counts taken for 30–60 s in 8–10 different internal regions of the tumor. (Δ, ▲) Control tumor; (□, ■) HER2 tumor; (Δ, □) FI-injected; (▲, ■) HN-FI-injected. $*p = <0.05$ versus control tumor injected with HN-FI. (*B*) Flow cytometric data of isolated cells from tumors collected at different time points from the HN-FI injected mice.

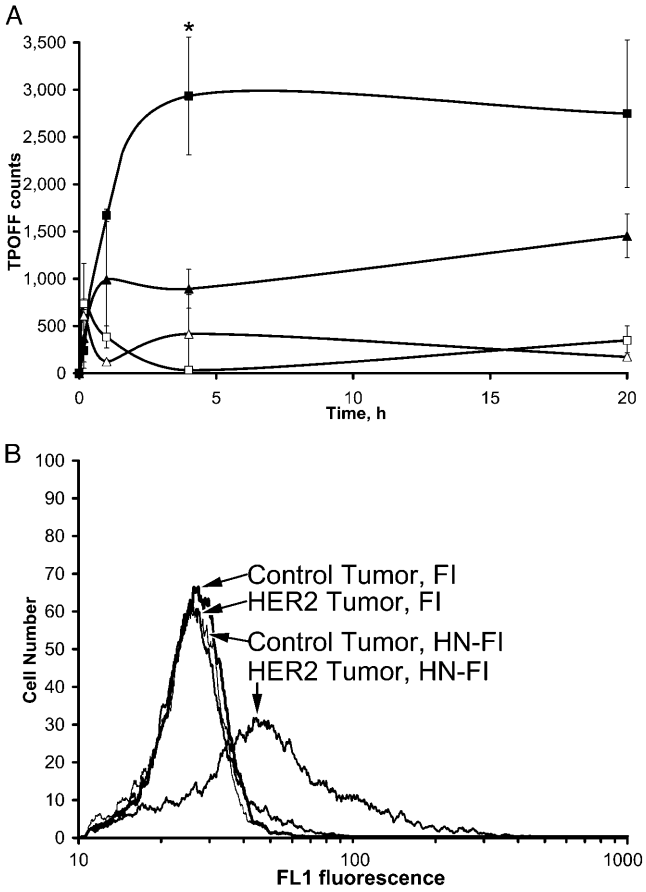

FIGURE 5 In vivo TPOFF counts of live mouse tumors developed with control and HER2-transfected MCA207 cells. The mice were intravenously injected with Herceptin-FI (HN-FI, 0.65 nmol) or FI (2 nmol), anesthetized at the time points shown and TPOFF counts were taken. (*A*) Mean ± SE of TPOFF counts. (△, ▲) control tumor; (□, ■) HER2 tumor; (△, □) FI-injected; (▲ ■) HN-FI-injected. $*p = <0.05$ versus control tumor injected with HN-FI. (*B*) Flow cytometric data for isolated cells from tumors collected from euthanized mice following the in situ TPOFF measurements.

showed that at saturation, KB cell pellet binds ~0.57 μM of the dendrimer G5-FI-FA (Ye et al., 2002). As 6T is less susceptible to photobleaching compared to FI, the lower levels of FI-conjugated dendrimer on KB cells observed in vitro is probably due to rapid bleaching of FI in cell pellet, which may not be detected in standard FI solution due to diffusion.

DISCUSSION

The development of optical techniques for quantifying tissue fluorescence is crucial for several aspects of cancer treatment, including the surveillance of drug activity, the detection of specific tumor biomarkers, the monitoring of tumor biochemical abnormalities and drug-induced tumor cell apoptosis. Currently available ex vivo methods for the analysis of fluorescently labeled molecules in tumors require invasive procedures such as surgery or biopsy. Our results demonstrate the utility of a technique that allows detection of fluorescence deep inside tumors, using two-photon excitation through a fiber optic probe inserted via a 27-gauge needle. Using the TPOFF probe, tumors containing as little as 0.3% GFP cells could be identified, with sensitivity comparable to flow cytometry on isolated cells. The ability to demonstrate a small fraction of fluorescent cells heterogeneously distributed in a tumor suggests the potential of this method to detect fluorescently tagged cancer signatures in tumors or the ability to monitor the uptake of fluorescently labeled drugs in tumors.

In contrast to microscopy, fluorescence detected by TPOFF can be quantified to provide concentrations of dye

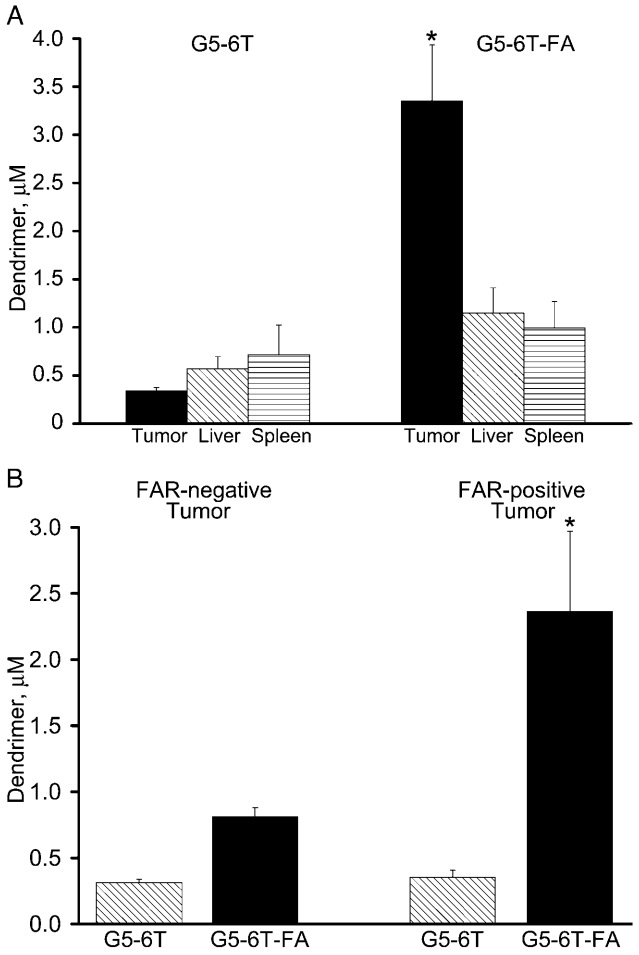

FIGURE 6 Tumor targeting of FA-conjugated dendrimer. (*A*) KB cell tumors were developed in SCID mice. The mice were injected with 10 nmol of the dendrimers G5-6T or G5-6T-FA, and after 15 h, TPOFF counts were taken in the tissues shown. $*p = <0.05$ versus G5-6T control and G5-6T-FA in liver and spleen. (*B*) An independent experiment showing uptake of dendrimers in FAR-positive KB, and FAR-negative MCA207 tumors. Other conditions are as given above. $*p = <0.05$ versus KB tumor injected with G5-6T and FAR-negative tumor injected with either G5-6T or G5-6T-FA.

in tumors, which in turn can be related to absolute concentrations of cancer molecules or drugs within tumors. This is demonstrated by the ability to determine the intracellular folate concentration in tumor cells by TPOFF using the dendrimer nanoprobe. Also, because two-photon excitation and collection back through a single-mode fiber results in a probed volume of $\sim 10\ \mu m^3$, the physical structure and heterogeneity of a tumor or drug distribution within a tumor can be assessed. This is important when evaluating therapeutic options or the response of a tumor to therapy, an analysis that cannot be assessed using flow cytometry on isolated cells. Thus, TPOFF measurements have advantages over ex vivo techniques.

Further development of the TPOFF detection system could make it a useful tool to simultaneously monitor multiple tumor activities without the need for biopsy and other analytical procedures. Our current work suggests TPOFF probe has the potential for monitoring drug distribution in tumors or identifying cancer signatures that can be fluorescently tagged through ligand binding. TPOFF probe might also be able to detect cellular events such as tumor therapy response or oncogene activity using fluorescence resonance energy transfer (FRET)-based probes. Gene expression could also be analyzed through a fluorescently conjugated specific antisense mRNA or FRET reagent (Tsuji et al., 2000). In addition, monitoring might be coupled to therapy if a photocleavable nanoparticle-therapeutic complex is targeted to cells and activated by the TPOFF to release drug, as directed by the presence of tumor signature fluorescence signal (Selbo et al., 2002). Thus, multiple cancer therapeutic activities can be achieved through the use of the TPOFF system.

In this study, the data collection was performed by physically placing the probe via a needle in tumor regions and moving the needle manually during TPOFF data acquisition. Development of an automatic system that would enable rapid placement of the fiber at different spatial locations in a predetermined tumor volume could enhance the quality of information obtained from this analysis. It could also shorten the time the probe is inserted in the tumor. One could envision the development of MEMS systems for this type of manipulation.

There are limitations to the use of this fiber probe for tumor analysis. First, the TPOFF probe only verifies that a cellular mass is a tumor. Actual detection of the in situ presence and position of the suspected tumor will have to first be gathered through other techniques, such as MRI, CT scan, or optical imaging, prior to fiber probe analysis (Tearney et al., 1997; Weissleder, 2002). However, these techniques can then be employed to direct the fiber insertion for verification that the mass is a tumor through signature identification. Secondly, the signal levels have to be higher than the intrinsic fluorescence of a given tissue. This can be overcome, as shown in our studies, by using dyes whose two-photon excitation and emission wavelengths are different from the major autofluorescent wavelengths of the tissue. This still may be a problem when employing FRET reagents (Periasamy, 2001) or techniques where fluorescence energy is low. However this is a problem inherent to fluorescent probes, whether used in our technique or in functional fluorescent imaging.

These studies demonstrate that TPOFF can evaluate tumor fluorescence in vivo in deep tissue with similar sensitivity to flow cytometry or tissue histopathology. This approach has advantages over both in vitro approaches, as it is more quantitative than histochemistry and can analyze tumor heterogeneity better than flow cytometry. Given that the probe can be inserted into the tissue using a 27-gauge needle, the system may prove valuable as a minimally invasive method of diagnosing and monitoring tumor tissue.

REFERENCES

Abel, A. P., M. G. Weller, G. L. Duveneck, M. Ehrat, and H. M. Widmer. 1996. Fiber-optic evanescent wave biosensor for the detection of oligonucleotides. *Anal. Chem.* 68:2905–2912.

Andersson, H., T. Baechi, M. Hoechl, and C. Richter. 1997. Autofluorescence of living cells. *J. Microsc.* 191:1–7.

Baker, S. L. R., Y. Zhao, M. A. Marletta, and R. Kopelman. 1999. Cellular applications of a sensitive and selective fiber-optic nitric oxide biosensor based on a dye-labeled heme domain of soluble guanylate cyclase. *Anal. Chem.* 71:2071–2075.

Cullum, B. M., G. D. Griffin, G. H. Miller, and T. Vo-Dinh. 2000. Intracellular measurements in mammary carcinoma cells using fiber-optic nanosensors. *Anal. Biochem.* 277:25–32.

Denk, W., J. H. Strickler, and W. W. Webb. 1990. Two-photon laser scanning fluorescence microscopy. *Science.* 248:73–75.

Eichler, G. H., and M. Muller. 1998. Drug distribution: the forgotten relative in clinical pharmacokinetics. *Clin. Pharmacol.* 34:95–99.

Gan, X., and M. Gu. 2000. Spatial distribution of single-photon and two-photon fluorescence light in scattering media: Monte Carlo simulation. *Appl. Opt.* 39:1575–1579.

Jung, J. C., and M. J. Schnitzer. 2003. Multiphoton endoscopy. *Optics Lett.* 28:902–904.

Lago, A., A. T. Obeidat, A. E. Kaplan, J. B. Khurgin, and P. L. Shkolnikov. 1995. Two-photon-induced fluorescence of biological markers based on optical fibers. *Optics Lett.* 20:2054–2056.

Lankelma, J. 2002. Tissue transport of anti-cancer drugs. *Curr. Pharm. Des.* 8:1987–1993.

Li, X., B. Chance, and A. G. Yodh. 1998. Fluorescent heterogeneities in turbid media: limits for detection, characterization, and comparison with absorption. *Appl. Opt.* 37:6833–6844.

Mitsiades, C. S., N. S. Mitsiades, T. T. Bronson, D. Chauhan, N. Munshi, S. P. Treon, C. A. Maxwell, L. Pilarski, T. Hideshima, R. M. Hoffman, and K. C. Anderson. 2003. Fluorescence imaging of multiple myeloma cells in a clinically relevant SCID/NOD in vivo model: biologic and clinical implications. *Cancer Res.* 63:6689–6696.

Molina, M. A., J. Codony-Servat, J. Albanell, F. Rojo, J. Arribas, and J. Baselga. 2001. Trastuzumab (Herceptin), a humanized anti-Her2 receptor monoclonal antibody, inhibits basal and activated Her2 ectodomain cleavage in breast cancer cells. *Cancer Res.* 61:4744–4749.

Mourant, J. R., T. M. Johnson, G. Los, and I. J. Bigio. 1999. Non-invasive measurement of chemotherapy drug concentrations in tissue: preliminary demonstrations of *in vivo* measurements. *Phys. Med. Biol.* 44:1397–1417.

Mulchandani, A., S. Pan, and W. Chen. 1999. Fiber-optic enzyme biosensor for direct determination of organophosphate nerve agents. *Biotechnol. Prog.* 15:130–134.

Nicolette, C. A., and G. A. Miller. 2003. The identification of clinically relevant markers and therapeutic targets. *Drug Discov. Today.* 8:31–38.

Periasamy, A. 2001. Fluorescence resonance energy transfer microscopy: a mini review. *J. Biomed. Optics.* 6:287–291.

Pitts, J. D., R. D. Sloboda, K. H. Dragnev, E. Dmitrovsky, and M.-A. Mycek. 2001. Autofluorescence characteristics of immortalized and carcinogen-transformed human bronchial epithelial cells. *J. Biomed. Optics.* 6:31–40.

Quintana, A., E. Raczka, L. Piehler, I. Lee, A. Myc, I. Majoros, A. Patri, T. Thomas, J. Mulé, and J. Baker, Jr. 2002. Design and function of a dendrimer-based therapeutic nanodevice targeted to tumor cells through the folate receptor. *Pharma. Res.* 19:1310–1316.

Sefkow, A., M. Bree, and M.-A. Mycek. 2001. Method for measuring cellular optical absorption and scattering evaluated using dilute cell suspension phantoms. *Appl. Spectroscopy.* 55:1495–1501.

Selbo, P. K., A. Hogset, L. Prasmickaite, and K. Berg. 2002. Photochemical internalization: a novel drug delivery system. *Tumour Biol.* 23:103–112.

Squirrell, J. M., D. L. Wokosin, G. White, and B. D. Bavister. 1999. Long-term two-photon fluorescence imaging of mammalian embryos without compromising viability. *Nat. Biotechnol.* 17:763–767.

Svanberg, S. 2002. Tissue diagnostics using lasers. *In* Lasers in Medicine. R. W. Waynant, editor. CRC Press, Boca Raton, FL. 135–169.

Tan, W., Z.-Y. Shi, and R. Kopelman. 1992. Development of submicron chemical fiber optic sensors. *Science.* 258:778–781.

Tearney, G. J., M. E. Brezinski, B. E. Bouma, S. A. Boppart, C. Pitris, J. F. Southern, and J. G. Fujimoto. 1997. *In vivo* endoscopic optical biopsy with optical coherence tomography. *Science.* 276:2037–2039.

Tomalia, D. A., H. Baker, J. Dewald, M. Hall, G. Kallos, S. Martin, J. Roeck, J. Ryder, and P. Smith. 1985. A new class of polymers: starburst-dendritic macromolecules. *Polym. J.* 17:117–132.

Tsuji, A., H. Koshimoto, Y. Sato, M. Hirano, Y. Sei-Iida, S. Kondo, and K. Ishibashi. 2000. Direct observation of specific messenger RNA in a single living cell under a fluorescence microscope. *Biophys. J.* 78:3260–3274.

Vo-Dinh, T., J. P. Alarie, R. W. Johnson, M. J. Sepaniak, and R. M. Santella. 1991. Evaluation of the fiberoptic antibody-based fluorimmunosensor for DNA adducts in human placenta samples. *Clin. Chem.* 37:532–535.

Weissleder, R. 2002. Scaling down imaging: molecular mapping of cancer in mice. *Nature Rev.* 2:1–8.

Weissleder, R., and V. Ntziachristos. 2003. Shedding light onto live molecular targets. *Nat. Med.* 9:123–128.

Ye, J. Y., M. T. Myaing, T. B. Norris, T. P. Thomas, and J. R. Baker, Jr. 2002. Biosensing based on two-photon fluorescence measurements through optical fibers. *Optics Lett.* 27:1412–1414.