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Using *In-vivo* Fluorescence Imaging in Personalized Cancer Diagnostics and Therapy, an Image and Treat Paradigm

Yasaman Ardeshipour, Ph.D.¹, Victor Chernomordik, Ph.D.¹, Jacek Capala, Ph.D.², Moinuddin Hassan, Ph.D.¹, Rafal Zielinsky, Ph.D.², Gary Griffiths, Ph.D.³, Samuel Achilefu, Ph.D.⁴, Paul Smith, Ph.D.⁵, and Amir Gandjbakhche, Ph.D.^{*,1}

¹NIH/National Institute of Child Health and Human Development, Building 9, 9 Memorial Dr., Bethesda, MD, 20892

²NIH/National Cancer Institute, Building 10-Magnuson Clinical Center, 10 Center Dr, Bethesda, MD, 20892

³NIH/Imaging Probe Development Center, Building 9800, Medical Center Dr., Rockville, MD, 20850

⁴Optical Radiology Lab, Department of Radiology, Washington University, 4525 Scott Avenue, St. Louis, MO 63110

⁵NIH/National Institute of Biomedical Imaging and Bioengineering, Building 13, 3N18A 13 South Dr, Bethesda, MD, 20892

Abstract

The major goal in developing drugs targeting specific tumor receptors, such as Monoclonal AntiBodies (MAB), is to make a drug compound that targets selectively the cancer-causing biomarkers, inhibits their functionality, and/or delivers the toxin specifically to the malignant cells. Recent advances in MABs show that their efficacy depends strongly on characterization of tumor biomarkers. Therefore, one of the main tasks in cancer diagnostics and treatment is to develop non-invasive *in-vivo* imaging techniques for detection of cancer biomarkers and monitoring their down regulation during the treatment. Such methods can potentially result in a new imaging and treatment paradigm for cancer therapy. In this article we have reviewed fluorescence imaging approaches, including those developed in our group, to detect and monitor Human Epidermal Growth Factor 2 (HER2) receptors before and during therapy. Transition of these techniques from the bench to bedside is the ultimate goal of our project. Similar approaches can be used potentially for characterization of other cancer related cell biomarkers.

Keywords

fluorescence imaging; near infrared optical imaging; targeted fluorescent probe; affibody; cancer treatment; cancer diagnostics; Human Epidermal Growth Factor receptor

Corresponding Author: Amir H. Gandjbakhche, Ph.D. gandjbba@mail.nih.gov.

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Introduction

Detection of specific biomarkers in a cancer lesion is one of the most important factors that affects the choice of cancer therapy. Developing drugs targeting specific tumor receptors such as Monoclonal AntiBodies (MAB) has opened an exciting opportunity to selectively target the cancer-causing biomarkers, inactivate specific molecular mechanisms responsible for cell malignancy, and deliver the toxin only to the malignant cells (1-5). Recent advances in MABs show that their efficacy depends strongly on the expression of tumor-specific biomarkers (4). For this reason, development of non-invasive *in-vivo* imaging techniques for detection of cancer biomarkers and monitoring the efficacy of the treatment, especially, at the early stages of therapy is one of the major tasks in cancer diagnostics and treatment.

In clinical studies, the current diagnostic gold standards for specific cancer biomarkers are all based on *ex-vivo* methods, such as immunohistochemistry (IHC), gene amplification based fluorescent *in situ* hybridization (FISH), and enzyme-linked immunosorbent assay (ELISA)(6-8). These methods are invasive and require biopsies from the patients. Inherently, biopsies have a risk of missing the malignant lesion and, during the therapeutic cycle, the number of times that the biopsy can be taken is limited. The current goal is to replace these invasive methods with non-invasive imaging, reduce the time between imaging and diagnosis, and facilitate analysis of therapy progression in the clinic with portable and accessible systems.

In cancer, understanding the pathophysiological status of the tumor is likely to be more important than structural imaging. Considering the different imaging modalities that are available now, it should be noted that MRI, CT and ultrasound (US) are optimal for structural imaging, while PET and optical imaging are better for functional and molecular imaging. In many cases, tumor and normal tissues are similar in appearance and structure, making it hard to discriminate them. Targeted molecular probes can be used to differentiate these regions based on their molecular specifications. They can be useful in finding the tumor margin in clinical surgery or diagnosing the metastatic tumors.

Incorporating advances in high quantum yield Near-InfraRed (NIR) fluorescence dyes (9-10) and the excellent specificity of molecular probes, combined with significant improvements in fluorescence microscopy and macroscopic imaging systems (11-16) make fluorescence imaging a promising candidate for cancer research.

In histopathology and cell biology, labeling the cell surface biomarkers with fluorescent probes helps to identify their role in the origin and progression of diseases (17). Analysis of the affinity of a specific probe or drug molecule targeted to a cancer biomarker is one of the main goals of *in-vitro* fluorescence imaging. These studies play an important role in the early stages of probe and drug development.

In contrast to *in-vitro* and *ex-vivo* experiments that deal with cell cultures and tissue samples, *in-vivo* preclinical studies facilitate investigation of different phases of a disease in a more realistic setting, i.e., in a live animal. Common methodologies in preclinical studies require sacrificing the animals at different stages of disease or treatment to study the lesion after excision of the organ. These methods require sacrificing many animals to obtain sufficient and reliable statistical results. Fluorescence imaging can be used as an *in-vivo* imaging technique to study the same phenomenon without removing the tumor or sacrificing the animal. In general, fluorescence imaging, compared to other imaging techniques, does not need ionizing radiation probes, and thus its cost is much lower than CT and MRI and can be implemented in a portable device.

In this paper, we review the fluorescence imaging methods including those that have been developed and used in our group to detect and monitor specific cancer biomarker expression *in-vitro* and *in-vivo* for diagnostics and therapy. Here we focus our study on the HER2 receptor, a cancer biomarker that is highly expressed in about 30% of the breast cancer cases (18-20). Overexpression of this receptor is correlated with poor prognosis and resistance to specific chemotherapy (21). To optimize the treatment procedure, it is important to identify the level of expression of the HER2 receptors during the diagnostic process and to monitor it over the course of treatment.

In order to image the HER2 receptors, we used HER2 specific affibody molecules as a targeting agent (22,23). Affibody molecules are highly water soluble and about 20 times smaller than antibodies and 4 times smaller than antibody fragments (24-27). Due to their small size, they have better conjugation to HER2 receptors and shorter washout time from the body and normal tissues. To track these probes, affibody molecules were conjugated to NIR fluorescent dyes.

Currently, most of the *in-vivo* fluorescence studies are based on mapping the fluorescence intensity. The drawback of this approach is the sensitivity of the fluorescence intensity to the fluctuations of the excitation light, distance of the probe from the tumor, and other parameters of the system. To overcome this problem and quantify the specific receptors of the tumor before and during the therapy, we introduced an algorithm based on the compartmental ligand-receptor model. This algorithm uses the dynamic of the normalized fluorescence intensity (uptake) in the tumor compared to the normal tissues at the contralateral site (28). The results were compared with ELISA, a standard *ex-vivo* method that is commonly used to quantify cancer biomarkers.

The other measurable parameter in fluorescence imaging is the fluorescence lifetime. It can provide useful clinical information, because fluorescence lifetime is potentially sensitive to local biochemical environment, e.g., temperature and pH, or molecular interactions (29,30). On the other hand, its value does not depend on the concentration of the fluorophores or the intensity of the excitation light (31). Potential applications of *in-vivo* fluorescence lifetime in cancer diagnosis and investigation of the early-phase treatment response in the clinic are as follows: first, *in-vivo* monitoring of the environmental differences (e.g. pH) in the tumor compared to normal tissues (32-34); second, *in-vivo* monitoring of the internalization of a specific drug into malignant and disease cells by using a fluorescent probe with a pH sensitive lifetime; third, developing a fluorescent probe that is sensitive to molecular interactions and capable of revealing the binding of a specific drug molecule to a specific disease/cancer receptor.

Fluorescent imaging Techniques

Fluorescence intensity imaging (CW)

The most common fluorescence imaging system is based on continuous wave (CW) fluorescence imaging. This method uses a CW light source to provide the excitation light. The intensity of the reflected or transmitted fluorescence signal is detected by a CCD camera or a PMT. Implementation of this method is less expensive and easier than other fluorescence imaging techniques that we will discuss later. The disadvantage of this method is that it only captures the intensity information of the fluorescence signals. As noted earlier, this approach is sensitive to the fluctuations of the excitation light, distance of the probe from the tumor, and parameters of the system.

In this paper, the results were obtained using the epi-illumination fluorescence imaging technique. This method captures the reflected fluorescence signal from the tissue. Owing to

the diffusion of excitation and emission light in the tissue, this method can image the fluorescence activities at depths ranging from millimeters to a few centimeters, based on the excitation and emission wavelengths of the fluorophore.

One of the methods that can help to reduce the uncertainties in the analysis of raw CW fluorescence imaging and improve its quantitative value is normalization of the fluorescence intensity to the background signal. This approach decreases the sensitivity of the fluorescence signal to the system parameters, such as intensity of excitation light and gain of the detector modules, as well as background tissue properties. It has been shown in refs. (35-36) that data analysis for fluorophores, deeply embedded in tissue and highly heterogeneous media works better, if the normalized ratio of fluorescence emission signal to the unfiltered diffused signal, coming from the excitation light, is used instead of the ratio of fluorescence to background signal. The main reason is that the unfiltered diffused signal contains more information of the heterogeneity and optical properties of the background tissues than just the background fluorescence signal, measured before injection of the fluorescence dye.

Applications of fluorescence to *in vivo* imaging are not limited to the above mentioned technique. It should be noted that fluorescence has been recently used for functional studies in small animal models, applying multiphoton fluorescence to microscopy to elucidate fundamental problems of physiology. If the fluorophore absorbs several photons simultaneously, the emitted photon will have several times higher energy compared to the excitation photons (e.g., for two photon fluorescence twice higher energy). Using excitation light in the near infrared region improves the penetration depth and reduces the autofluorescence of the background tissues. The spatial resolution of the method is higher than that of other *in-vivo* fluorescence imaging techniques that work in diffuse mode. However, even in the best case scenario, the imaging depth is limited to 1mm (37). Thus it can be applied potentially to characterize only the most superficial tumors.

On the other hand, the fluorescence tomography is being developed to characterize deeper tissue layers up to several centimeters. However, since the number of measurements is usually less than the number of reconstructed voxels, the inversion matrices that are involved in tomography reconstruction algorithms, are ill-posed. In order to improve the quantitative values in the reconstructed image, the fluorescence imaging needs to be combined with second imaging modalities such as, MRI, CT or ultrasound (13). More detailed reviews of these fluorescence imaging techniques can be found in references 13 and 37-38. More detailed discussion of these approaches to *in vivo* fluorescence imaging is beyond the scope of this review, which is focused mainly on *in-vivo* targeted fluorescence imaging for cancer diagnostics and treatment.

Fluorescence lifetime imaging (frequency domain, time domain)

Fluorescence lifetime imaging is based on the average time that excited fluorophore stays in the excitation state before its transition to the ground state accompanied by emission of a photon. Fluorescence lifetime can be measured either by time-domain or frequency domain techniques. In the frequency domain technique, the excitation light intensity is sinusoidally modulated at 100's of MHz. Passing the modulated light through the tissue changes the amplitude and phase of the fluorescence signal. Fluorophores with larger lifetimes have larger phase shifts and amplitude attenuation.

In the frequency domain method, based on the amplitude and phase measurements, two fluorescence lifetime can be defined, phase lifetime (τ_p) and modulation lifetime (τ_M). The definitions of these two lifetimes are as follows,

$$\tau_p = \tan(\varphi) / \omega; \tau_M = \left(\frac{1}{\omega}\right) \sqrt{\left(\frac{1}{M^2} - 1\right)} \quad [1]$$

where φ is the phase shift, ω is the angular modulation frequency of the excitation light and M is the attenuation in modulation depth of the received signal (39).

If the media contains only one kind of fluorophore, the phase and modulation lifetime will be equal. However, if the medium contains different fluorophores, then the value of these two parameters can be different and can be used to distinguish and separate the images of each fluorophore.

In the time domain system, a very short laser pulse (sub-nsec) illuminates the target. The detector should have a very fast time response and can be implemented by a time gated ICCD or a fast PMT with a time correlated single photon counter (TCSPC).

In time domain method (40), the measured signal can be written as

$$I(t) = \text{IRF} \otimes I_0 e^{-t/\tau} \quad [2]$$

where IRF is the impulse response function of the system, $\otimes$ is the convolution, I_0 is the intensity of the excitation light and τ is the fluorescence lifetime. The fluorescence lifetime can be estimated by curve fitting algorithms, such as a least-squares algorithm. If the medium contains more than one kind of fluorophore, a multi-exponential curve fitting algorithm needs to be applied.

Equations [1] and [2] are valid if the effects of photon migration on the apparent fluorescence lifetime τ' (determined as an observed exponential decay time of emission intensity $I(t)$) are negligible. If the fluorophore is embedded deeper than several scattering lengths $l_s \sim 1/\mu_s'$ in the turbid medium (μ_s' is the transport-corrected scattering coefficient), measured values of τ' are larger than intrinsic fluorescence lifetime τ . Time-resolved fluorescence intensity distribution presents the convolution of the exponential decay curve, similar to Eq. (2), and Green functions, describing photon migration from the source to the fluorophore inside the turbid medium and from the fluorophore to detector [see., e.g., refs. (41-43)]. Reconstruction algorithms presented in these papers, use analytical formulas, derived from a diffusion approximation and random walk model to evaluate τ' for the fluorophore inside the turbid medium. Several simpler approaches to find the intrinsic fluorescence lifetime have been suggested in the literature, for example, analyzing the asymptotic behavior of emitted intensity that, in the case of the lifetime longer than the photon migration time scale, reveals τ (44), or using random walk analytical formulas in the opposite case of relatively short lifetimes (45).

In our preclinical studies, since the tumor is located in mouse forelimb subcutaneously, we deal with the case of superficial fluorophores, when the depth of the tumor does not have a significant effect on the lifetime. It should be noted that in clinical applications, related to the shallow tumors such as skin or head and neck tumors, the exponential approximation of Eq. (2) can still be applied. However, for applications dealing with the deeply embedded tumors, the effect of the photon diffusion on the observed time-resolved fluorescence intensities should be taken into account, using some reconstruction algorithms, for example, mentioned above (45). Sung-Ho Han et al. (43) presented recently diffusion-based simulations of the apparent fluorescence lifetime, τ' , determined as an observed exponential

decay time of emission intensity, as a function of the fluorophore depth z . The found dependence proved to be close to linear.

As with CW fluorescence imaging, *in-vivo* lifetime imaging can be implemented with multi-photon microscopy, epi-illumination and fluorescence tomography (46-50). Limitations of both the time domain and frequency domain techniques are similar to those discussed in the previous section on CW intensity imaging technique.

In comparing the time and frequency domain techniques, it should be noted that since in practice implementation of the frequency-domain is usually limited to one or few modulation frequencies, the time domain data can provide more information about the probed media, since a short laser pulse contains much broader range of the modulation frequencies, including the zero-frequency component. The effect of measurement noise is less in the time domain method, since its lifetime calculation is based on the slope of the detected signal and the slope of a signal is less susceptible to noise than its amplitude (51). On the other hand, instrumentation for the time-resolved imaging is considerably more expensive, and data collection time is longer relative to a frequency domain system. Therefore, to calculate the lifetime in applications that need very fast imaging, the frequency domain method is more suitable.

As we mentioned before, fluorescence lifetime does not depend on fluorophore concentration (before saturation), however it may be sensitive to other factors, such as temperature, quenching, viscosity, solvent polarity and pH (30, 32). The sensitivity lifetimes to these parameters is based on the structure of the dye. Therefore, to probe specific properties of the probe environment in tissue, it is important to choose a proper dye with lifetime sensitive mainly to one specific factor. Lifetime imaging has been used extensively in cell biology and in *in-vitro* studies. It has also been applied to endogenous and exogenous fluorophores in both *ex-vivo* tissue and *in-vivo* animal studies (30, 52-53). Fluorescence lifetime imaging has not been conducted with exogenous contrast agents in clinical studies due to the lack of FDA-approved exogenous contrast agents, but some companies e.g., Li-Cor Corp. have started pre-clinical/clinical studies to get FDA approval for their near infrared fluorescent dyes. It should be noted that the lifetime imaging, based on endogenous fluorophores has been already used in clinical studies of the human skin (54).

Applications: Image and treatment paradigm in cancer

In this section we review the setup and some of the applications of fluorescence intensity and lifetime imaging, for characterization and monitoring of tumors, developed in our group.

Instrumentation

Our fluorescence small animal imager consisted of a CW and a time domain fluorescence systems. A cooled, charge-coupled device (CCD) camera was used in CW mode with a bandpass filter ($800\text{nm} \pm 20\text{nm}$) to find the location of the tumor (ROI) and position the scanner of the time domain system to the ROI. The same camera was used without the filter to capture the white light image of the small animal. The field of view of the CCD camera was $12 \times 12 \text{ cm}^2$. The time domain system consisted of a tunable Ti-sapphire pulse laser with a pulse width of 100 fs and repetition rate of 80 MHz, (Tsunami, Spectra Physics, Mountain View, CA). The laser peak was set at excitation wavelength of 750 nm. The femto-second laser pulse scanned the target (tumor or contralateral site) of the animal in a raster pattern through a scanning head with the source and detector fibers at 2 mm distance. The scanner was programmed to scan any area in the field of view of the CCD camera ($12 \times 12 \text{ cm}^2$). In most experiments, the integration time for each pixel was set to 2 seconds based on the maximum intensity of the detected fluorescence signal and the saturation point of the

PMT. The reflected fluorescence signal was filtered by a high-pass emission filter at 780 nm and was detected by a photomultiplier tube, (R7422, Hamamatsu Corporation, Hamamatsu City, Japan). Detected photons were counted by a time-correlated single-photon counter, (SPC-730, Becker & Hickl, Berlin, Germany). Initialization, scanning, and acquisition were controlled by the Labview software. The animal was placed within a dark chamber on a temperature-controlled scanning stage and was anesthetized through a nose cone (47).

Fluorescence intensity imaging

Diagnostics—To track the HER2 cancer biomarker, each HER2 specific affibody molecule was attached to a NIR fluorescence dye. Affibody molecules were kindly provided by affibody AB, Bromma, Sweden. Labeling of HER2 specific affibody molecules with Alexa Fluor 750 fluorophores were described in detail in ref. (23).

In the first phase, we studied the toxicity and selective binding of the affibody probe to the HER2 receptors *in-vitro*. In these experiments, the HER2 specific affibody fluorescent probes were studied on SKBR-3 and U251 cell lines. SKBR-3 is a human breast adenocarcinoma cell line and has high expression of HER2 receptors. U251 is a human glioblastoma MG cell line and was selected as a non-expressing HER2 tumor model. After incubation of the cultured cells mixed with 1nmol/L HER2 specific affibody labeled with alexa fluor 750 at 37°C for one hour, cells were washed twice with media to remove the non-attached fluorophores and imaged by confocal fluorescence microscopy. Figure 3 shows that HER2 specific affibody fluorescent probes were bound on the surface of SKBR-3 cells which had high HER2 expression (fig.2(a)), however, they did not bind to U251 cells which did not have any HER2 expression (fig. 2(b)).

In the next step, HER2 specific affibody fluorescent probes were tested *in-vivo* by our time-domain fluorescence small-animal imager. In this experiment, BT474 and MDA-MB468 cell lines were used as high HER2 and no HER2 expressing human tumor models, respectively. The cells were implanted into the right forelimb of xenograft female nude mice. The study was approved by the Animal Safety and Use Committee of NIH. Five million cells were injected in 0.1 mL of 50% Matrigel into their right forelimb. The mice were imaged after the tumors grew to 0.5–1 cm in size and were anesthetized by Isoflurane before imaging. 10µg of HER2 specific affibody conjugated with Dylight 750 was injected in mouse tail vein. Figure 3 shows the fluorescence intensity of the tumor, 3 hours after injection. As expected the HER2 specific affibody fluorescent probes bound to the HER2 receptors in the mouse tumor with BT474 tumor model and showed very high fluorescence intensity in the tumor region. However, the fluorescence intensity detected from MDA-MB468 tumor which has no HER2 expression is almost negligible. The small fluorescence intensity seen in MDA-MB468 tumor region is due to the accumulation of the fluorophores in the tumor region because of the leakiness of tumor vascularization.

Monitoring tumor vascularity—Another application of fluorescence imaging is monitoring the tumor vascularization (angiogenesis). Tumor vascularization was imaged using AngioSense 750 (VisEn Medical, Inc., Bedford, MA). Anesthetized mice with N87 human xenograft, which is a tumor model with high HER2 expression, were injected intravenously with 150µL of 2 nmol of AngioSense 750. Figure 4 shows higher accumulation of Angiosense 750 fluorophores in the tumor area compared to contralateral site.

Estimates of HER2 receptor expression in-vivo—Even though the fluorescence intensity in tumors with high HER2 expressing tumor models is much higher than no HER2

expressing tumor models, using the fluorescence intensity information alone to quantify the HER2 receptors has some limitations.

Fluorescence intensity is very sensitive to the intensity variation of excitation source, total blood volume in circulation, changes in system parameters and concentration of the injected dye. To overcome this problem we introduced an algorithm based on the compartmental ligand-receptor model (28). Considering that the dissociation rate of bound fluorophores is very low, the fluorescence intensity can be written as

$$I_f = I_{\text{free ligands in blood}} + I_{\text{free ligands in the tumor}} + I_{\text{bound ligand - receptor}} \\ = \alpha F_{b1} \exp\left(-\frac{t}{\tau}\right) + \beta F_T + \gamma B_{\max} [1 - \exp(-k_{\text{on}} t F_T)] \quad [4]$$

where α , β and γ are constants, F_{b1} , F_T and $B_{\max}$ are the number of free ligands in the blood, and free and bound ligands to the receptors in the tumor region, respectively, τ is the time constant of the clearance of the fluorophores from the normal tissue, and k_{on} is the kinetics rate of ligand-receptor binding. In this approximation, we assumed that after the initial time t_1 , the concentration of local free ligands in the tumor stays constant. The intensity of the free ligands in blood can be considered as the same as the intensity measured at the contralateral site. Therefore, if we subtract the measurements at the contra-lateral site from the tumor site, the two remaining components will be the free and bound ligands to the receptors in the tumor region, which can be simplified as

$$y(t) = y_0 + a[1 - \exp(-bt)] \quad [5]$$

To eliminate the system variations between different experiments, we normalized the measurement data at different time points to the first measurement data, when binding was almost negligible and the accumulation of the free ligands in the tumor was stabilized. The

derivative $\left.\frac{dy}{dt}\right|_{t=0} = ab$ presents the normalized rate of accumulation (NRA). By using a fitting algorithm, NRA can be estimated from a time series of fluorescence intensity measurements. To confirm our results, we compared the estimated NRA of several BT474 tumors with different HER2 expression by the values obtained *ex vivo* for the same tumor by ELISA assay. The results show a good linear correlation between the NRA and HER2 concentration obtain by ELISA. The ELISA assay was performed according to the protocol provided by the manufacturer (Calbiochem, Gibbstown, NJ) and HER2 concentration is expressed in nanograms of HER2 per milligram of total protein.

Treatment—To monitor the cancer biomarkers during therapy, we have pursued two methods. In the first method we labeled the drug molecules (in this case trastuzumab (54-56)) directly and measured the intensity and lifetime of the reflected fluorescence signal from the tissue (figure 6). The advantage of this method is its potential to monitor the effect of drug molecules directly and *in-vivo*.

The main goal of the second experiment was to make a fluorescent probe that has minimal effect on the therapy and does not interfere with the binding of therapeutic agent (23). HER2 receptor has two epitopes and the HER2 specific affibody and trastuzumab (MAB drug) molecules bind to different epitopes. The first step was to confirm these two separate bindings in an *in-vitro* study. The results were shown in figure 7. In the first experiment, HER2 specific affibody and trastuzumab molecules were labeled with different fluorophores. HER2 specific affibody molecules were labeled with Alexafluor 488 (emission peak at 488nm) and trastuzumab molecules were labeled with Alexafluor 630

(maximum emission at 630nm). Tumor cells with high HER2 expression (SKBR-3) were mixed and incubated with both labeled HER2 specific affibody and labeled trastuzumab. Since the emission wavelengths of Alexafluor 488 and Alexafluor 630 are different, different emission filters were used to separate their images. Figure 7(a) shows binding of both HER2 specific affibody and trastuzumab molecules to the cells. In the next experiment, 100-fold excess unlabeled trastuzumab was added to the media to block the HER2 receptors. After one hour incubation, the labeled affibody and labeled trastuzumab were added to the cell media and were incubated for one hour before imaging. Figure 7(b) shows that the unlabeled-trastuzumab molecules completely blocked all the HER2 epitopes that could attach to labeled trastuzumab molecules. Therefore, from labeled trastuzumab and labeled affibody experiments, only labeled affibody molecules were bound to the cells confirming that the HER2 epitope that binds to HER2 specific affibody molecule is different from the one that binds to trastuzumab.

The third experiment was the same of the second experiment, however, instead of unlabeled trastuzumab, we added 100-fold excess unlabeled HER2 specific affibody to the cells. The results shown in figure 7(c) also confirm that the HER2 epitope that binds to HER2 specific affibody molecule is different from the epitope that binds to trastuzumab. In the last experiment, 100-fold excess unlabeled HER2 specific affibody and unlabeled trastuzumab were added to the cell media. After one hour incubation, labeled HER2 specific affibody and labeled trastuzumab were added and incubated for one hour. The results in figure 7(d) shows that all HER2 epitopes were blocked with unlabeled affibody and unlabeled trastuzumab, therefore, none of the labeled molecules could bind to HER2 receptors.

We repeated the same experiment *in-vivo* in a live animal. In this experiment, we used only one fluorescent dye (Alexa Fluor 750) to label the HER2 specific affibody and/or trastuzumab molecules. Our results showed that the tumor uptake of HER2 specific affibody (ABD-(Z_{HER2:342})₂) labeled with fluorescent probe is almost the same as when it was injected with 100-fold excess unlabeled trastuzumab. However, its uptake decreases when the mouse was injected with unlabeled HER2 specific affibody in advance. This *in-vivo* study also confirmed that the epitope that binds to HER2 specific affibody molecules is different from the one that binds to trastuzumab. It shows that labeled HER2 specific affibody can be used to monitor the changes in the HER2 receptors without interfering with a HER2 specific drug like trastuzumab (figure 8).

Fluorescence lifetime imaging

Cancer cells have a high level of anaerobic metabolism which generates hyperacidity inside the cell. Since cells need to maintain their intracellular pH to survive, these cells release the acidic products to the extracellular space. Therefore, their pH of extracellular fluid is different compared to the normal tissues (34). The pH of extracellular fluid in normal tissues is around 7.4, however, it can be as low as 5.4 for tumors, depending on their metabolic level. Thus, pH sensors can be useful to study the metabolic status of tumors. However, for an *in-vivo* study this sensor should be non-invasive and does not affect the measurement environment. Using a pH sensitive dye combined with optical imaging methods can provide this opportunity to measure the pH *in-vivo* and monitor the metabolic status of the tumor.

Another important challenge in cancer treatment is to quantify the rate of internalization of the cancer drugs into the malignant cells. Monitoring of this process *in-vivo* is crucial for development of new therapeutic drugs. The pH inside the lysosome has been reported as low as 4.8, which is much lower than the extracellular environment of the normal cells which is 7.4. Therefore, if a pH sensitive fluorescence dye can be internalized within the lysosome, its lifetime will change and, by measuring its value, we can determine the localization of the drug or imaging agents outside or inside the cells.

pH sensitive fluorescent probes can indicate a change in environmental pH based on different mechanisms. They can be designed in a way that by changing the environmental pH, their intensity changes or their maximum emission or excitation spectrum shifts. The problem of the first group is that the tissue heterogeneity can also change the fluorescence intensity and it is difficult to separate these two effects. On the other hand, designing a probe with high sensitivity and broad spectrum shift is difficult. A third approach is to design a fluorescent probe that its lifetimes changes with pH.

Recently, Achilefu, et al. (32) have synthesized a pH sensitive probe for lifetime imaging, by modifying a hexamethotindotricarbocyanine with a tertiary amino functionality that was coupled to the fluorophore. By substitution of indole ring at the meso position, they were able to change fluorescence lifetime from 1.16 ns in acidic DMSO to 1.4 ns in basic DMSO. The next step is conjugating this pH sensitive dye directly with HER2 specific affibody and cancer drugs like Affitoxin (58) or trastuzumab to monitor its performance *in-vivo*.

Prospects and challenges

In 2004, the Food and Drug Administration (FDA) started the Critical Path Initiative program to establish nationwide projects and gather different scientific and technological areas to create better drug development systems (59-60). Developing better molecular imaging probes and improving the *in-vivo* imaging systems are key elements in these projects.

There are many criteria that should be considered in an imaging system for cancer. It should be able to detect and quantify the specific cancer biomarkers and monitor the interaction of the drug with the cancer cells. To pursue this, more efficient and specific molecular imaging probes need to be designed to target the biochemical and pathophysiological features of the tumor. The molecular imaging probe should have minimum level of toxicity and side effects on the normal tissue and organs. They should give enough contrast and signal to background ratio at low concentration levels (in nanomolar to micromolar range). They should have a stable specific binding, high accumulation in the target region and fast washout time from the blood and normal tissues. On the other side the clearance of the probes from the body has to be slow enough to allow accumulation in the target sites and obtain a good affinity and binding to the receptors. It is also important that the imaging probe does not interfere with the drug functionality (61-63).

Several targeted ionized probes for positron emission tomography (PET) have been adapted to clinical trials (64), however, the main studies on fluorescent probes and, in particular, targeted fluorescent probes has been restricted to preclinical studies. The next and important step is to move these studies to the clinic, since the preclinical studies cannot always predict the clinical potential of an imaging probe. The critical step is to investigate the toxicity of these probes in humans. So far, the only FDA approved fluorescent dye in the NIR spectrum is Indocyanine Green (ICG) as a nontargeted fluorescent probe for ophthalmology and cardiology applications.

Each targeted molecular probe usually consists of three parts, a marker (e.g. radionuclide or fluorescent dye), a targeted binder (e.g. HER2 affibody or antibody) and a linker. The parameters that have to be considered in designing a fluorescent probe (marker) are the emission and excitation wavelengths, quantum yield, stability, toxicity and molecular size. The excitation wavelength determines the detection depth. Since both tissue chromophores and water absorption are lower in NIR region, fluorescent dyes in this spectral region have higher penetration depth in tissue than other wavelengths. The emission spectrum is desired to have minimal overlap with the excitation spectrum. Fluorescent probes need to have high quantum yield to generate brighter fluorescence signal. The molecular size of the fluorescent

probe is also important since it determines the pharmacokinetics and clearance time of the probe from the body. Using fluorescent probes that can turn on in specific conditions and turn off in other conditions can improve the image quality and signal to background ratio (61). Fluorescence imaging has the potential to monitor multiple biomarkers simultaneously by using different fluorescent probes with different emission wavelengths (11,66-67). In these applications, the excitation spectrum of each dye has to be narrow and as separate as possible. Multicolor imaging can be used to characterize several cancer biomarkers and/or to evaluate the effect of multiple drugs *in-vivo*. It can also be used to study the pharmacokinetics of several drugs or probes with different clearance rates at the same time, which is unique among other imaging modalities.

As an alternative approach to fluorescence imaging, Wilson, et.al (67,68) have introduced phosphorescence probes for non-invasive monitoring the hypoxic condition of the tumors. Phosphorescence probes have longer temporal response (ms range) compared to fluorescent probes (ns). Due to its longer temporal response, the separation of the phosphorescence signal from the excitation and autofluorescence signals (background noise) can be obtained by simple time gating. Also, implementation of a time resolved phosphorescence imaging system is simpler and less expensive than a time resolved fluorescence system.

As was mentioned before, one of the challenges in deep tissue imaging is the high scattering properties of the tissue. The tissue scattering reduces the resolution of the optical imaging significantly. Co-registration of optical imaging with high resolution imaging modalities, such as CT, MRI and US can provide functional information within the framework of anatomical structures (66-75). In deep tissue imaging, the absorption and scattering of the tissue has a big impact on the detected fluorescence intensity and lifetime and has to be considered in the imaging reconstruction algorithms. The absorption and scattering properties of the tissue can be obtained by diffuse optical tomography (DOT) methods (76). In fluorescence tomography, anatomical structures can help to decrease the unknown variables in the fluorescence reconstruction algorithms and improve the 2D and 3D images of the fluorescent probes (77-79).

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Abbreviations

MAB	Monoclonal AntiBodies
HER2	Human Epidermal Growth Factor 2
IHC	ImmunoHistoChemistry
NIR	Near-InfraRed
FISH	Fluorescent In situ Hybridization
ELISA	Enzyme-Linked Immunosorbent Assay
CW	Continuous Wave
ROI	Region of Interest
NRA	Normalized Rate of Accumulation
DOT	Diffuse Optical Tomography

Figure 1.
Schematic of our small animal imaging system.

(a)

(b)

Figure 2. Confocal microscopy studies show (a) binding of HER2 specific affibody fluorescent probe with HER2 receptors in SKBR-3. (b) no binding was observed between HER2 specific affibody fluorescent probe and U251 cells (23).

Figure 3.

In-vivo fluorescence intensity map of xenograft mouse with (a) high HER2 expressing human tumor model (BT474) and (b) no HER2 expressing human tumor model (MDA-MB468), 2 hours after injection of the HER2 specific affibody® (His6-Z_{Taq}-GS3-Cys) conjugated to DyLight 750.

Figure 4. Monitoring the uptake of AngioSense 750 at the tumor compared to the contralateral site (control). The higher uptake at tumor site indicates its higher vascularization. The fluorescence intensity at the tumor and contralateral sites was normalized **to the background intensity of tumor area before injection.**

Figure 5.

a) *In-vivo* measurements of optical intensity at the tumor and contralateral sites over time (BT474 tumor) after injection of ABD-(Z_{HER2:342})₂ affibody labeled with alexafluor 750, (b) Estimated NRA of several BT474 tumors with different HER2 expression vs. *ex vivo* measurement of HER2 concentration obtained by ELISA assay (28).

Figure 6.

Fluorescence intensity (a) and lifetime (b) at the tumor site after injection of trastuzumab labeled with Alexafluor 750. The fluorescence lifetime, defined, as the decay time of time-resolved fluorescence signal, and intensity of contralateral site were estimated, using average values over 15 pixels and compared to the tumor region. The maximum accumulation of the dye at the tumor area occurs at about 20 hours after injection. At this time, the mean fluorescence lifetimes, corresponding to the tumor region was measured as 1.25 ps and 10% higher at contralateral site, while the mean fluorescence intensity at the tumor region was about two times higher than that of the contralateral site.

Figure 7.

In-vitro study of binding affibody (probe) and trastuzumab (MAB drug) to different epitopes of HER2 receptors. (a) Image of SKBR-3 cells after adding the labeled HER2 specific affibody and labeled trastuzumab to the cell media. (b) Blocking HER2 epitopes with unlabeled 100-fold excess trastuzumab and image the high HER2 expressing cancer cells after adding the labeled HER2 specific affibody and labeled trastuzumab. (c) Blocking HER2 epitopes with unlabeled 100-fold excess HER2 specific affibody molecules and image the high HER2 expressing cancer cells after adding the labeled HER2 specific affibody and labeled trastuzumab. (d) Blocking HER2 epitopes with unlabeled 100-fold excess HER2 specific affibody molecules and unlabeled 100-fold excess trastuzumab and imaging the cells after adding the labeled HER2 specific affibody and labeled trastuzumab (23).

Figure 8.

In-vivo study of binding HER2 specific affibody (probe) and trastuzumab (drug) to different epitopes of HER2 receptors. Y-axis shows the maximum fluorescent uptake at tumor site for labeled trastuzumab and labeled affibody alone, labeled affibody after blocking with unlabeled trastuzumab and labeled affibody after blocking with unlabeled affibody.