A fluorescent imaging method for analyzing the biodistribution of therapeutic monoclonal antibodies that can distinguish intact antibodies from their breakdown products

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Keywords: antibody, biodistribution, breakdown, fluorescence imaging, fluorescent resonance energy transfer (FRET)

Many monoclonal antibodies have been developed for therapy over the last 2 decades. In the development of therapeutic antibodies, the preclinical assessment of an antibody’s biodistribution is important for the prediction of the antibody’s efficacy and safety. For imaging analyses of such biodistributions, radioisotope (RI) labeling and fluorescence labeling methods are typically used, but the resulting data are limited because these methods cannot distinguish breakdown products from intact antibodies. To resolve this problem, we developed a novel method using fluorescent resonance energy transfer (FRET)-type labeling and a spectral unmixing tool. With FRET-type labeling (labeling with 2 species of fluorophore), different fluorescence properties of labeled intact antibodies and their breakdown products (the hydrolyzed/digested type of breakdown products) are made visible. With the spectral unmixing tool, the fluorescence of a solution containing the intact antibody and its breakdown products could be unmixed in proportion to their contents. Moreover, when labeled antibodies that targeted either human epidermal growth factor receptor-2 or epidermal growth factor receptor were injected into nude mice implanted subcutaneously with tumor cells, the accumulation of the injected labeled antibodies and their breakdown products in the tumor could be separately analyzed by both whole-mouse imaging and a tumor homogenate analysis. These results suggest that our method using FRET-type labeling and a spectral unmixing tool could be useful in distinguishing breakdown products from intact antibodies.

Introduction

In the development of therapeutic monoclonal antibodies, biodistribution is a critical factor because the accumulation of an antibody at the targeted region and the lack of accumulation in all other regions are highly related to the efficacy and safety of monoclonal antibody therapy. Antibodies engineered for increasing the efficacy and decreasing the adverse effects of monoclonal antibody therapies are now being actively developed, e.g., antibodies engineered for increased affinity to antigens, Fc gamma receptor (FcγR), or neonatal Fc receptor (FcRn); bispecific antibodies; antibody-drug conjugates (ADCs).1-2 Because these antibodies might have unique pharmacokinetic (PK) or pharmacodynamic (PD) properties that are different from those of conventional antibodies, the preclinical assessment of such novel antibodies’ biodistribution is especially important for prediction of their efficacy and safety.

For analyses of the biodistribution of antibodies, radioisotope (RI) labeling and fluorescent labeling methods are typically used.3-8 However, since breakdown products of antibodies are co-detected with the intact antibodies by these methods, important information regarding the state of the accumulated antibodies is not available. Although researchers have developed fluorescent probe systems that are activated by the breakdown of antibodies in order to detect tumors with high tumor-to-background ratios,9,10 there is no method to analyze the distribution of intact antibodies and their breakdown products simultaneously, and distinguish them.

Fluorescent resonance energy transfer (FRET) is the phenomenon by which an excited donor transfers energy to an acceptor. FRET can be used as a powerful tool to detect signaling processes, especially in vitro.11-13 The excitation of the donor fluorophores of proteins labeled with 2 species of fluorophores is expected to generate FRET, and the acceptor fluorophores are expected to yield fluorescence. Since the labeled proteins that have degraded might not generate FRET, it is possible that labeled intact proteins and their breakdown products can be resolved depending on the difference in their emission spectra.

For in vivo fluorescence imaging, near-infrared fluorescence is used because of its better tissue penetration.14 In vivo imaging...
apparatuses have been developed, and a spectral unmixing tool has come to be used for imaging.\textsuperscript{8,15,16} It is thus possible to resolve different near-infrared fluorophores with overlapping spectra.

We have developed a fluorescence analysis method that would be useful for biodistribution studies of monoclonal antibodies because it can distinguish between intact antibodies and their breakdown products using a spectral unmixing tool. As models, we used 2 therapeutic antibodies, trastuzumab and cetuximab. Trastuzumab targets human epidermal growth factor receptor-2 (HER2) and it is used for breast cancer therapy. Cetuximab is an anti-epidermal growth factor receptor (EGFR) antibody used in therapies for colon and head and neck cancer. Trastuzumab and cetuximab targeted to antigen-expressing tumors are thought to be internalized into tumor cells, and subsequently either recycled to the cell surface or subjected to lysosomal breakdown.\textsuperscript{17,18} We used these antibodies as models of tumor-targeting or non-targeting antibodies to show the usefulness of our new imaging method.

**Results**

**The properties of labeled antibodies**

The model of an antibody labeled with 2 species of fluorophores is shown in Figure 1A. Excitation of the donor fluorophores of the labeled antibody is expected to generate FRET, and the acceptor fluorophores to yield fluorescence. In contrast, the breakdown labeled-antibodies are expected not to generate FRET. Our aim was to distinguish between intact proteins and their breakdown products using these different fluorescence properties.

Trastuzumab (anti-HER2) and cetuximab (anti-EGFR) were labeled with XenoLight680 (absorption/emission max., 681/698 nm) and XenoLight750 (absorption/emission max., 755/775 nm), and unbound fluorophores were removed by size exclusion chromatography as detailed in the Materials and Methods section. The spectra of trastuzumab labeled with XenoLight680 and XenoLight750 are shown in Figure 1B. The spectra of the labeled antibodies were changed by digestion using proteinase K. In the case in which the antibody was labeled with a single species of fluorophore, the spectra of the labeled antibodies were less changed by digestion. We thus considered the spectral change between intact antibody and the breakdown products to be due mainly to the change of FRET efficiency.

Despite the change of FRET efficiency, the emission intensity at the emission maximum of the acceptor fluorophore (~780 nm) was not so different between the intact and broken-down antibody. Possible reasons for this are as follows. The donor fluorophore partly emits fluorescence at around 780 nm. Since the emission intensity of the donor fluorophore of the breakdown products was roughly 3 times higher than that of the intact antibody (Fig. 1B), the fluorescence intensity of the donor fluorophore at ~780 nm was increased by the breakdown. Moreover, since the acceptor fluorophore, XenoLight750, is directly excited by the wavelength at 640 nm, there is a possibility that the static quenching of XenoLight750 partly contributed to the low emission intensity at ~780 nm of the labeled intact antibody. These factors are thought to have produced the phenomenon in which the fluorescence intensity of broken-down antibody at ~780 nm was not so different from that of the intact antibody.

The binding of the labeled antibodies to cells was confirmed by confocal laser scanning microscopy. The labeled trastuzumab bound to HER2-expressing SKOV3-luc-D3 cells. The labeled cetuximab did not bind to HER2-expressing SKOV3-luc-D3 cells, but bound to EGFR-expressing A549-luc-C8 cells (Fig. S1). Moreover, we also confirmed that the labeled antibodies could bind to antigens by surface plasmon resonance (Fig. S1). We used these labeled antibodies in the following experiments as models of the tumor-targeting or non-targeting antibodies.

**Spectral unmixing analysis of a mixture of intact antibodies and breakdown products**

The labeled intact antibodies and their breakdown products digested by proteinase
K were mixed in predefined ratios of breakdown products of 0%, 20%, 40%, 60%, 80%, and 100%, and 22 shots of fluorescent images were acquired with a series of excitation and emission wavelengths as described in the Materials and Methods section. To spectrally unmix the fluorescence, we used the unmixing tool and the separately obtained solution spectra of intact antibodies and breakdown products. Figure 2A shows the unmixed fluorescence of the intact antibodies and breakdown products and a merged image of the intact antibody (green) and the breakdown products (red). The fluorescence could be unmixed in proportion to the content of the intact antibodies and breakdown products.

The radiant efficiency, i.e., the relative fluorescence units of the analytic software (Living Image, Perkin Elmer, Boston, MA), is plotted in Figure 2B. The radiant efficiency of breakdown products was linearly increased and that of the intact antibodies was linearly decreased with the elevation of the content of breakdown products. These results indicated that the fluorescent signals of the mixture of intact antibodies and breakdown products could be effectively unmixed with the spectral unmixing tool.

As described later, we also observed the accumulation of the fluorescence-labeled antibodies in mice. Since the mice that had not been injected with the fluorescence-labeled antibodies emitted auto-fluorescence, it was preferable that the spectral unmixing analyses were performed using images in which the auto-fluorescence/signal ratio was low. As shown in Figure 2C, the fluorescence of intact antibodies and breakdown products could also be separated using 7 images (excitation 640 nm/emission filter 700, 720, 740 and 760 nm; excitation 675 nm/emission filter 740 and 760 nm; and excitation 710 nm/emission filter 760 nm) whose auto-fluorescence/signal ratio was low; thus, we used the 7 images to unmix the fluorescence of the in vivo images.

Although the results using XenoLight680&750-trastuzumab are shown as examples, fluorescent signals of intact protein and breakdown products of XenoLight680&750-cetuximab were also unmixed in a similar fashion (Fig. S2).

Spectral unmixing analysis of the accumulation of labeled antibodies in tumor xenografts in mice

We analyzed the biodistribution of labeled antibodies and their breakdown products in vivo. Since the antibodies are internalized into cells and broken down by acidic lysosomes, we checked the pH stability of the fluorescence of the labeled antibodies. We found that the fluorescent spectra of XenoLight680&750-trastuzumab and XenoLight680&750-cetuximab were not altered between pH 7.2 and pH 5.6, with or without breakdown (Fig. S3). We thus confirmed that the labeled antibodies could be applied for in vivo analyses.

The labeled anti-HER2 antibody XenoLight680&750-trastuzumab or the labeled anti-EGFR antibody XenoLight680&750-cetuximab were injected intravenously into nude mice implanted subcutaneously with HER2-expressing cells (SKOV3-luc-D3). As examples, the images of mice at 3 days after an injection of labeled antibodies are shown in Figure 3A. The solutions poured into the wells of the plates to the left of the imaged mice are labeled intact antibodies (upper well) and their breakdown products digested by proteinase K (lower well). The acquired images were unmixed by the unmixing tool using the spectra of labeled antibodies and breakdown products (Fig. 3B). Since the solutions of the intact antibodies and their breakdown products were well distinguished, the spectral unmixing analysis was confirmed to have worked. As shown in the images, XenoLight680&750-trastuzumab and their breakdown products clearly accumulated in the SKOV3 tumors.

We analyzed the radiant efficiency of fluorescence in the tumors (whose regions were detected by luminescence) over a time course as described in Materials and Methods (Fig. 4).

![Figure 2. Unmixing analyses of the mixture of labeled trastuzumab and its products broken down by proteinase K treatment. (A) The unmixed images of the mixture of labeled intact trastuzumab and the breakdown products. Six mixtures with predefined ratios of breakdown products of 0%, 20%, 40%, 60%, 80% and 100% were unmixed using 22 shots of fluorescent images as described in Materials and Methods. The green and red of the merged images represent intact antibodies and breakdown products, respectively. (B, C) The radiant efficiency, i.e., the relative fluorescent units in the analyzed software. The plots unmixed using 22 images are shown in (B) and that using 7 images are shown in (C). In proportion to the mixture ratio, the fluorescence could be unmixed.](image-url)
The labeled intact trastuzumab accumulated in the tumor with a peak at 2–4 days after the injection. In contrast, the breakdown products of the labeled trastuzumab accumulated in the tumor gradually. For cetuximab, a model of the non-targeting antibody to SKOV3 tumor, the accumulations of intact antibodies and breakdown products were lower than those of trastuzumab. Since radiant efficiency is influenced by the number and sites of the fluorophores, the radiant

Figure 3. Images of nude mice implanted with HER2-expressing cells at 3 days after an injection of labeled anti-HER2 (XenoLight680&750-trastuzumab) or anti-EGFR antibody (XenoLight680&750-cetuximab). (A) The images acquired by different excitation or emission wavelengths. (B) The unmixed images using the images shown in (A). XenoLight680&750-trastuzumab and their breakdown products were highly accumulated in the implanted cells.
efficiencies of labeled trastuzumab and labeled cetuximab are not necessarily the same.

It should be noted that, although the radiant efficiency of broken-down trastuzumab was ~10% lower than that of broken-down cetuximab, that of intact cetuximab was ~25% lower than that of intact trastuzumab. Even considering this difference in radiant efficiency, the accumulations of intact antibodies and breakdown products of cetuximab were lower than those of trastuzumab.

The accumulation of labeled cetuximab in an A549 tumor expressing EGFR, the target of cetuximab,19 was also analyzed (Fig. 5). Images of mice at 3 days after an injection of labeled cetuximab are shown in Figure 5A. As with the case of the SKOV3 tumor, intact antibodies and breakdown products could be distinguished. The time course of the accumulation is shown in Figure 5B, C. The labeled intact cetuximab accumulated in the tumor with a peak ~1 day after injection. In contrast, breakdown products of the labeled cetuximab accumulated to the tumor gradually and seemed to reach a plateau at 7–10 days after injection. Whereas the maximum radiant efficiency of labeled intact cetuximab in the A549 tumor was similar to that in the SKOV3 tumor, the breakdown products accumulated to a greater extent in the A549 tumor. These results indicated that cetuximab highly accumulated in the A549 tumor. Since the maximum radiant efficiency of labeled intact cetuximab in the A549 tumor was not higher than that in the SKOV3 tumor, the intact cetuximab accumulated in the A549 tumor might be promptly broken down.

As described above, we were able to assess the relative quantity of intact antibodies and breakdown products by whole-mouse imaging. However, it should be noted that the accumulation of intact antibodies could not be compared simply with that of breakdown products even in the same animal by whole-mouse imaging. The reason is that the penetration of light into tissue might differ between labeled intact antibodies and their breakdown products because of the difference in their distributions in the tumor. To compare the accumulation of intact antibodies with that of breakdown products, a quantification method using the homogenate of tumor, as described below, is useful.

The spectral unmixing analyses of the accumulation of labeled antibodies using tumor homogenate

The absolute accumulations of intact antibodies and their breakdown products also provide important information for PK assessments, but there is no method that easily distinguishes their accumulation in tissues. We next tried to quantify the intact antibodies and their breakdown products in tumors using the unmixing method developed here.

Using the homogenate of the tumor, we quantified the accumulations of XenoLight680&750-trastuzumab and XenoLight680&750-cetuximab, whose accumulation levels in SKOV3 tumors differed in whole-mouse imaging. The SKOV3 tumors of the mice were extirpated at 3 days after an injection of XenoLight680&750-trastuzumab or XenoLight680&750-cetuximab as described in the Materials and Methods section, and then homogenized with 10 volumes of phosphate-buffered saline (PBS). We calculated the amounts of intact antibodies and breakdown products accumulated in the tumors from the fluorescence of the homogenate using standard curves. To prepare the samples used for the standard curves, the tumors of mice that were not injected with labeled antibodies were homogenized with 10 volumes of PBS, and known concentrations of the labeled antibodies were mixed with the homogenate.

We had to consider the possibility that the intact antibodies were denatured by physical damage or broken down by proteases released from cells during homogenization. We thus examined the recovery of the fluorescence of the intact antibodies as follows. We mixed the known concentrations of antibodies with the homogenate of the tumor extracted from mice that had not been administered antibodies, and the mixture was re-homogenized under the same conditions as those used for the homogenization of the tumors.

Since the fluorescence of the intact antibodies was not decreased by the homogenization, the intact antibodies were not denatured or broken down during homogenization. Moreover, since the fluorescence of the intact antibodies was not changed after 2 h incubation at room temperature, the intact antibodies were rarely broken down in the homogenate. These results indicated that it is possible to measure the amount of intact antibodies and breakdown products using the homogenate of tumors.

The standard curves of labeled intact antibodies and their breakdown products are shown in Figures 6A–F. When the fluorescence of the tumor homogenate containing labeled intact antibodies was spectrally unmixed, it was unmixed as the
fluorescence signal of intact antibodies, and their intensity was linearly increased according to the concentrations (Fig. 6A, D). As described below, the concentrations of intact antibodies in the tumor were lower. Since the plots of fluorescence intensity against the concentration curved at lower concentrations, we used quadratic regression curves as the standard curves (Fig. 6B, E). Similarly, the fluorescence of tumor homogenate containing the breakdown products was also unmixed as the fluorescence signal of breakdown products, and their intensity increased linearly according to the concentrations (Fig. 6C, F).

The homogenates of tumors extirpated from the mice at 3 days after the injection of labeled antibodies were measured for fluorescence, and the concentrations of intact antibodies and breakdown products were calculated from the standard curves. Moreover, the total accumulations in the tumors were calculated from the homogenate volume, and the percentage of injected dose per gram (%ID/g) was calculated from the total amount of intact antibodies plus their breakdown products (Fig. 6G).

The average %ID/g value calculated from the mice injected with labeled trastuzumab was ~35 %ID/g. Although the reported %ID/g of RI-labeled antibodies in tumors have varied depending on the labeling methods, the %ID/g of RI-labeled trastuzumab in an SKOV3 tumor of the mice at 2 or 3 days after injection has generally been reported to be ~25–40.20-22 The value calculated in this study was similar to that calculated using radioisotopes.

The quantified accumulation of both labeled intact trastuzumab and its breakdown products was significantly higher than that of cetuximab in an SKOV3 tumor (Fig. 6G, H), as was the case with the relative accumulation estimated by whole-mouse imaging. Moreover, by comparing the accumulation of intact antibodies and their breakdown products, it was clarified that the labeled antibodies accumulated in the tumor were mostly broken down at 3 days after injection.

As shown here, the unmixing method could be used to quantify the accumulation of intact antibodies and their breakdown products using homogenized samples.
Figure 6. For figure legend, see next page.
Discussion

We developed a method that can distinguish between intact antibodies and breakdown products using FRET-type labeling and a spectral unmixing tool. To the best of our knowledge, this is the first report demonstrating that the amounts of labeled intact antibodies and their breakdown products could be analyzed simultaneously by an imaging method. Here, we showed that the accumulation of the intact antibodies and their breakdown products in a tumor can be separately analyzed by both whole-mouse imaging and a tumor homogenate analysis.

Whole-mouse imaging can be used to compare the relative accumulations of different antibodies or to analyze the time course of the accumulation of a single type of antibody. In contrast, a homogenate analysis is suitable to analyze the absolute accumulation of the antibodies or to compare the accumulations of intact antibodies and their breakdown products. A homogenate analysis might also be suitable when the fluorescence intensity is low because of the low accumulation of an antibody or the high tissue absorption.

In this study, we were able to separately analyze the intact antibodies and their breakdown products by whole-mouse imaging. However, in the case of high tissue absorption (e.g., the analysis of the accumulation in a deeper tumor), whole-mouse imaging might be insufficient. Depending on the tissue, the absorption might be different between the wavelengths. For example, when the tissue penetration of the fluorescence of XenoLight750 is higher than that of XenoLight680, part of the breakdown products might be misinterpreted as intact antibody. In this study, the amount of the breakdown products did not influence the results of the intact antibodies, as is shown by the results at 15 days from injection (Fig. 4) and at 7–18 days from injection (Fig. 5). Conversely, the amount of the intact antibodies did not influence the results of the breakdown products, as shown by the results at 5 h from injection (Figs. 4, 5). However, when the tissue absorption is high, the results of the whole-mouse imaging might need to be confirmed by the homogenate analysis.

Although trastuzumab and cetuximab were used as models in this study, we have confirmed that the method can be used for other antibodies labeled with the fluorophores used here, i.e., the fluorescent signals of mixtures of intact antibodies and breakdown products could also be unmixed. Examples using anti-tumor necrosis factor (TNF) antibodies, infliximab and adalimumab, are shown in Fig. S4A, B. Our present findings also confirmed that the accumulation of the labeled anti-TNF antibodies and their breakdown products in tissue (e.g., liver) could be analyzed using homogenate samples. Our new unmixing method may thus be useful for analyzing biodistributions other than tumor accumulation. The method might also be applicable to proteins other than antibodies.

For in vivo imaging, the use of fluorophores emitting in the near-infrared region of the spectrum is recommended, considering the penetration of light in living tissue. Although we used XenoLight680 and XenoLight750 as labeling fluorophores, the fluorescence could also be unmixed in proportion to the content of the intact antibody (infliximab) and their breakdown products using other pairs of fluorophores. The use of fluorophores with too far or too distant emission wavelengths, however, tends to make the unmixing more difficult (Fig. S4C–H). It is important that the spectrum of labeled intact antibody and that of its breakdown products can be distinguished.

Moreover, for the selection of fluorophores, low permeability of the cellular membrane is an important criterion. Since the permeability of XenoLight680 and XenoLight750 was low, these fluorophores were mostly retained in the tumor after breakdown, and the fluorescence (e.g., ex. 675 nm/em. 720 nm and ex.710 nm/em. 760 nm) of the tumor was not decreased over a long term after the injection of the labeled antibody. In contrast, rapid diffusion of high-permeability fluorophores released from the antibody by breakdown would result in a significant miscalculation. Additionally, along with the conventional fluorescent labeling, it should also be noted that the excess labeling of a highly hydrophobic fluorophore may decrease stability or cause aggregation of the protein.

In our preparation of the labeled tumor-targeting or non-targeting antibodies, fluorophores were randomly labeled to primary amines of the antibodies, but other methods (e.g., labeling sulphydryl groups) might be available. To enable labeling of sulphydryl groups, most antibodies must be reduced. Since two labeling sites are generated by the reduction of a disulfide bond, 2 fluorophores may bind to the antibody with high FRET efficiency. In cases in which the labeled number of fluorophores should be decreased, the labeling with sulphydryl groups might be effective. Because any type of labeling, including labeling with fluorophores, might affect the function of proteins, labeling methods should be selected depending on the protein structure and purpose of the study. For example, to elucidate the impact of the target receptor binding of an antibody on its distribution and

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**Figure 6 (See previous page).** Quantitative analyses using homogenate of tumors. (A–F) The standard curves of labeled intact antibodies and their breakdown products. The fluorescence of the tumor homogenates containing labeled intact antibodies or breakdown products was spectrally unmixed. Each value represents the means and standard deviation from 3 wells of the plate. (A) intact trastuzumab, (B) low concentrations of intact trastuzumab, (C) breakdown products of trastuzumab, (D) intact cetuximab, (E) low concentrations of intact cetuximab, (F) breakdown products of cetuximab. The radiant efficiency unmixed as intact antibodies (black circles) and breakdown products (gray circles). Since the fluorescence intensity of intact antibodies was low, the quadratic regression curves were used for the standard curves. (G) The amounts of intact antibodies and breakdown products in homogenates of tumor extracted from the mice at 3 days after an injection of labeled antibodies. *Calculated on the assumption that the specific gravity of the tumor is 1. (H) Comparison of the relative accumulations of labeled trastuzumab and labeled cetuximab between the homogenate analyses and whole-mouse imaging. The accumulation of trastuzumab was set to 100%. Black bars: relative accumulation of trastuzumab (n = 3). Gray bars: relative accumulation of cetuximab (n = 3). Concerning both intact antibodies and their breakdown products, similar results were obtained from the homogenate analyses and whole-mouse imaging.
breakdown, it is preferable to select the method with low influence on the binding property of the antibody.

As discussed below, there are numerous applications for the unmixing analysis of antibody accumulations in tumors. Antibody-dependent cell-mediated cytotoxicity (ADCC) is an important mechanism underlying monoclonal antibody-based cancer therapies. ADCC, which involves the killing of target cells by cytotoxic effector cells (e.g., natural killer cells), is induced through an interaction of target-bound antibodies with the FcγR of effector cells. In cases in which ADCC is critically important for therapy, antibodies that highly accumulate in tumors and remain on the cell surface without breakdown may be effective because effector cells bind antibodies located on the cell surface.

On the other hand, the method may also be applied to ADCs, which are currently being actively developed for cancer therapy. Since ADCs show their therapeutic efficacy by internalizing into tumor cells and releasing the conjugated drugs, antibodies that highly accumulate in tumors and are prone to internalize and degrade in cells may be effective as ADCs. Since the method reported here can analyze both intact antibodies and their breakdown products, it may useful for the selection of candidates of these antibodies.

Moreover, antigen-mediated clearance is significant in the PK of antibodies whose targets are membrane-associated antigens. Since the analytic method described in this study was able to detect the differences in breakdown between tumor-targeting and non-targeting antibodies in a SKOV3 tumor, the method could also be used for analyzing the magnitude of the antigen-mediated clearance of antibodies.

Our novel unmixing method can be applied to the analysis of biodistributions other than tumor accumulation, and so the method may be useful for clarifying the effect of antibodies’ properties (e.g., binding to antigen or receptors) on distribution and breakdown. Antibodies have recently been engineered to have increased efficacy and decreased adverse effects (e.g., antibodies engineered for increased affinity to antigens, FcγR, or FcRn, bispecific antibodies, and ADCs). Since these antibodies might have PK or PD properties that are different from those of conventional antibodies, preclinical assessments of their accumulation to the target region and other regions using our new method might be useful for predicting the efficacy and safety of these antibodies.

The unmixing method reported here might be applicable to imaging using confocal laser scanning microscopy by employing an unmixing algorithm similar to that used with the in vivo imaging apparatus. At present, some of the analytic software for microscopy is equipped with a spectral unmixing analysis program that can be used for the unmixing of fluorescence from multiple fluorophores (i.e., fluorescent proteins and chemical fluorophores) and autofluorescence. However, since the unmixing by microscopy is performed using spectra obtained by excitation with a single-wavelength laser, the unmixing algorithms for microscopy and in vivo imaging are different. Therefore, in our experiment, the intact proteins and the breakdown products of the mixture could not be distinguished by a confocal laser scanning microscope (LSM780, Carl Zeiss). However, by employing the unmixing algorithm using multiple excitation wavelengths, microscopy-based unmixing analyses of intact proteins and breakdown products may be possible. When microscopes become available for the unmixing analyses described here, the analysis of the detailed distribution of intact antibodies (or the other proteins) and their breakdown products in tissue sections will be possible, and other applications (e.g., in cultured cells) may be possible.

In this study, we developed a method for analyzing the biodistribution of antibodies using FRET-type labeling and a spectral unmixing tool. Since it is difficult to distinguish between the intact antibody and their breakdown products by other methods, this approach is a significant advancement.

Materials and Methods

Materials and animals for fluorescence imaging
Trastuzumab (Chugai Pharmaceutical, Tokyo) and cetuximab (Bristol-Myers Squibb, New York, NY) were purchased via reagent distributors. The fluorophores (XenoLight CF680 and XenoLight CF750), the SKOV3-luc-D3 human ovarian carcinoma cell line, and the A549-luc-C8 human lung carcinoma cell line were purchased from Caliper Life Sciences (Hopkinton, MA). BALB/c athymic nude mice were purchased from CLEA Japan (Tokyo).

Fluorescence labeling of therapeutic antibodies
Both the reactive fluorophores XenoLight680 (XenoLight CF680) and XenoLight750 (XenoLight CF750) have a succimidyl ester group which reacts with an amine group of the protein to form a stable amide linkage. Trastuzumab and cetuximab were labeled with XenoLight680 and XenoLight750 according to the manufacturer’s instructions with a slight modification. Two mg of IgG was incubated typically with 50 nmol of XenoLight680 and XenoLight750 in PBS (pH7.2) with 0.1 M NaHCO3 (Sigma-Aldrich, St. Louis, MO) with continual stirring in the dark at room temperature for 1 h. The labeled antibody was separated from the unbound fluorophores by size exclusion chromatography using Bio-Gel P-30 gel (Bio-Rad Laboratories, Hercules, CA).

The protein concentration and the labeling efficiency were calculated based on the absorbances at 280, 681 and 755 nm. The average numbers of XenoLight680 and XenoLight750 were 2.3 and 2.5 per labeled trastuzumab, and 2.3 and 2.3 per labeled cetuximab, respectively.

Tumor cell implantation
The SKOV3-luc-D3 human ovarian carcinoma cell line, which expresses HER2 and luciferase, was maintained in MacCoy’s 5A medium (In Vitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 IU/mL of penicillin, and 100 µg/mL of streptomycin. We subcutaneously implanted 1×107 cells mixed with an equal volume of Matrigel Phenol Red Free (BD Biosciences, Bedford, MA) into the right flank of 5- to 6-wk-old female nude mice that were maintained on
a low-fluorescence diet, iVid#1 (Oriental Yeast, Tokyo). In addition, the A549-luc-C8 human lung carcinoma cell line, which expresses EGFR and luciferase, was maintained in RPMI medium 1640 (Invitrogen) supplemented with 10% fetal bovine serum, 100 IU/mL of penicillin, and 100 μg/mL of streptomycin, and 1 x 10^7 cells without Matrigel were implanted as described above.

Tumors were allowed to grow from 4 to 6 mm in dia., and then each mouse received a single intravenous dose of the labeled trastuzumab or the labeled cetuximab at 10 mg/kg via a lateral tail vain. Fluorescence images and luminescence images were obtained prior to dosing (day 0) and at 0.21, 1, 2, 3, 4, 7, 9, 11 and 15 days after dosing.

**Fluorescent and luminescent imaging**

**Analyses of a mixture of intact antibodies and breakdown products**

The mixture of intact and breakdown products digested by proteinase K (Sigma-Aldrich) was poured into the wells of black plates (Sumitomo Bakelite, Tokyo), and fluorescent imaging was performed using IVIS Lumina II imaging system equipped with Mid-High Emission Filter set (Caliper Life Sciences). Twenty-two shots of fluorescence images were acquired using 5 excitation filters and 7 emission filters. The concrete conditions were excitation 570 nm/emission filter 640, 660, 680, 700, 720, 740, and 760 nm; excitation 605 nm/emission filter 660, 680, 700, 720, 740, and 760 nm; excitation 640 nm/emission filter 680, 700, 720, 740, and 760 nm; excitation 675 nm/emission filter 720, 740, and 760 nm, and excitation 710 nm/emission filter 760 nm.

**Animal imaging**

For animal imaging, mice were anesthetized by their inhalation of 3% isoflurane (Dainippon Sumitomo Pharma, Osaka, Japan) and placed on heated imaging platform of the IVIS Lumina II imaging system with inhalational isoflurane anesthesia during image acquisition. For the animal imaging, 7 shots of fluorescence images were acquired (excitation 640 nm/emission filter 700, 720, 740 and 760 nm; excitation 675 nm/emission filter 740 and 760 nm, and excitation 710 nm/emission filter 760 nm). To generate a spectrum library for unmixing, we acquired fluorescence images of labeled antibodies and their breakdown products digested by proteinase K (Sigma-Aldrich) using the same excitation and emission filter sets.

To visualize the regions of the implanted cells that express luciferase, luminescence imaging was performed. The mixture of intact and breakdown products digested by proteinase K (Sigma-Aldrich) was poured into the wells of black plates and 22 shots of fluorescence images were acquired with the same conditions as those used in the analyses of the mixtures of intact antibodies and breakdown products as described above.

Animal experiments were humanely conducted under the regulation and permission of the Animal Care and Use Committee of the National Institute of Health Sciences (Japan).

**Homogenate analyses**

For the homogenate analyses, mice were anesthetized by the inhalation of 3% isoflurane and blood was exsanguinated from the abdominal aorta at 3 days after injection of Xeno-Light680&750-trastuzumab or XenoLight680&750-cetuximab. The SKOV3 tumors were extirpated, cut with a surgical knife, and homogenized for ~30 sec with a polytron homogenizer (Kinematica, Luzern, Switzerland) in 10 volumes of PBS (pH7.2). Next, 100 μL of the homogenate was poured into the wells of the black plates and 22 shots of fluorescence images were acquired with the same conditions as those used in the analyses of the mixtures of intact antibodies and breakdown products as described above.

Animal experiments were humanely conducted under the regulation and permission of the Animal Care and Use Committee of the National Institute of Health Sciences (Japan).

**Spectral unmixing of fluorescence**

The acquired images were analyzed using Living Image Software (Caliper Life Sciences). To create libraries of reference spectra for spectral unmixing, we measured the fluorescence of labeled antibodies and their breakdown products as described above, and saved the data as a set of reference spectra. Then, fluorescent images of the mixture solution, animals, and tumor homogenates were unmixed using the reference spectra.

In the case of animal imaging, the tumor regions were selected as regions of interest (ROIs) by luminescence, and the radiant efficiency (the relative fluorescent units in the analyzed software) of the ROIs were analyzed. Background regions whose size and shape were the same as the ROIs of the tumor were selected from the contralateral areas of the tumor in unmixed images. We calculated the fluorescence signals of intact antibodies and breakdown products by subtracting the radiant efficiency of the background from that of the tumor at each time point.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Funding**

This work was supported in part by JSPS KAKENHI Grants #21790172 and #24590229, the Program for the Promotion of Fundamental Studies in Health Sciences from the National Institute of Biomedical Innovation, and by Health and Labor Sciences Research Grants from the Ministry of Health, Labor and Welfare of Japan.

**Supplemental Material**

Supplemental data for this article can be accessed on the publisher’s website.

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