Assessing Antibody Pharmacokinetics in Mice with In Vivo Imaging

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Received July 29, 2010; accepted October 14, 2010

ABSTRACT

Recent advances in small-animal molecular imaging instrumentation combined with well characterized antibody-labeling chemistry have enabled detailed in vivo measurements of antibody distribution in mouse models. This article reviews the strengths and limitations of in vivo antibody imaging methods with a focus on positron emission tomography and single-photon emission computed tomography and a brief discussion of the role of optical imaging in this application. A description of the basic principles behind the imaging techniques is provided along with a discussion of radiolabeling methods relevant to antibodies. Practical considerations of study design and execution are presented through a discussion of sensitivity and resolution tradeoffs for these techniques as defined by modality, signaling probe (isotope or fluorophore) selection, labeling method, and radiation dosimetry. Images and analysis results from a case study are presented with a discussion of output data content and relevant informatics gained with this approach to studying antibody pharmacokinetics.

Introduction

Researchers use numerous techniques for assessing the pharmacokinetics of antibodies in mice. The majority of these techniques are ex vivo, including organ biodistribution of radiolabeled antibodies after necropsy, quantitative whole-body autoradiography, histology and serum concentration analysis (enzyme-linked immunosorbent assays). Gamma scintigraphy has afforded dynamic two-dimensional imaging of radiolabeled antibody localization in vivo; however, its use has been primarily qualitative in nature (Levine et al., 1980; Khaw et al., 1984; De Santes et al., 1992; Camera et al., 1993). The advance of commercially available small-animal molecular imaging instrumentation combined with well characterized labeling chemistry for antibodies has enabled detailed in vivo, dynamic, quantitative measurements of radio- or fluorophore-labeled antibodies in mouse models (Smith-Jones et al., 2004; Robinson et al., 2005; Cai et al., 2007a,b; Sosabowski et al., 2009). The primary modalities used for in vivo imaging of antibody kinetics in mice currently are single-photon emission computed tomography (SPECT), positron emission tomography (PET), and fluorescence imaging. This article examines the strengths and limitations of these imaging modalities through the assessment of sensitivity and resolution tradeoffs as defined by modality, isotope (or fluorophore) selection, labeling method, and dosimetry. A description of the basic principles behind the imaging techniques is provided along with a discussion of labeling methods relevant to antibodies with a focus on the role of modality and isotope selection on study design and execution. Image and select analysis results from a case study are presented with a discussion of output data content and relevant informatics gained in such studies.

Imaging Physics

SPECT and PET are nuclear medicine imaging techniques used to measure the three-dimensional distribution of a ra-
diopharmaceutical administered to a patient or subject. In both SPECT and PET, the decay of a radioactive probe conjugated to the antibody of interest results in detectable signals outside the patient or subject. Specifically, projection data are generated through the estimation of the location and energy of high-energy photons interacting with a detector material, typically via the photoelectric effect in high-density crystals or electron-hole pair production in semiconductors (Wagner et al., 1968; Barrett and Myers, 2004; Vernick and Aarsvold, 2004; Barrett, 2005). The three-dimensional spatial distribution of the injected radionuclide is estimated through reconstruction of the projection data into an image, either by a filtered backprojection or a statistical-based iterative algorithm (Gullberg, 1979; Shepp and Vardi, 1982; Feldkamp et al., 1984; Lange and Carson, 1984; Kaufman, 1987; Fessler et al., 1991; Hudson and Larkin, 1994). In other words, the three-dimensional image created from a SPECT or PET scan maps the radionuclide-antibody conjugate within the patient or subject.

**SPECT.** As the acronym implies, SPECT cameras image individual high-energy photons (γ-rays or characteristic x-rays) resulting from radionuclide decays. These single-photon emissions follow a 4π-geometry, i.e., radiate outward in all directions with equal probability. Therefore, an aperture comprised of highly attenuating material is required to identify the path of origin of each emission before creation of an image with a gamma camera (Copeland and Benjamin, 1949; Anger, 1952, 1967). The two most common aperture or “γ-ray collimator” types are parallel-hole and pinhole collimators. The former is used most often in the clinic, whereas the latter is used when the field of view (FOV) of the patient or subject is small with respect to the size of the detector FOV, e.g., a human thyroid or a mouse. The volumetric resolution of pinhole SPECT imaging systems is a function of the magnification and diameter of the pinhole aperture as well as the intrinsic resolution of the gamma camera (Jaszczak et al., 1994; Fessler, 1998; Acton and Kung, 2003; Bequê et al., 2003; Schramm et al., 2003; Accorsi and Metzler, 2004; Beekman and Vastenhouw, 2004; Furenlid et al., 2004; Meikle et al., 2004; Aoi et al., 2006; Funk et al., 2006).

**PET.** In contrast to SPECT, wherein the radionuclide emits a detectable photon, PET imaging uses a radionuclide that emits a positron, also known as the antimatter of an electron. The positron travels some distance (typically on the order of a millimeter) before encountering an electron, transiently combining and annihilating. The annihilation produces two photons of identical energy (511 keV) traveling in nearly opposite directions (i.e., ~180° apart), a process referred to as coincidence. The identification of this coincident photon pair is executed by a ring of crystal detectors (typically lutetium yttrium orthosilicate or bismuth germinate) coupled to photomultiplier or photodiode detectors. The localization of this photon pair enables the estimation of the origin of the annihilation and thus radionuclide distribution within the subject without the need for a high-density, attenuating aperture. The ability in PET to image without the use of gamma collimators or apertures results in a higher sensitivity of PET compared with SPECT instrumentation. The volumetric resolution of PET imaging systems is a function of positron range before annihilation (proportional to the energy of the positron emission), detector geometry and intrinsic resolution, and the temporal resolution of the gamma detection process (Cherry et al., 1997, 2006; Jeavons et al., 1999; Chatziioannou et al., 1999; Phelps, 2009).

**Physical Characteristics of SPECT and PET Isotopes.** A summary of factors pertinent to the isotopes most commonly used for SPECT and PET imaging of radiolabeled antibodies is shown in Tables 1 and 2. Six SPECT isotopes [gallium-67 (67Ga), iodine-123 (123I), iodine-125 (125I), indium-111 (111In), lutetium-177 (177Lu), and technetium-99m (99mTc)] and four PET isotopes [copper-64 (64Cu), fluorine-18 (18F), gallium-68 (68Ga), and iodine-124 (124I)] were selected based on their physical properties, availability, and current use in the field.

A primary physical property for consideration of any isotope used in an imaging study is its radioactive half-life. In practice, the half-life of the isotope selected should be at a minimum 2-fold longer than the biological half-life of the event kinetics to be imaged (Gambhir, 2002). Because of relatively long-lived circulation, imaging antibody pharmacokinetics can be a challenge. The maximum possible duration of an imaging study depends on the effective half-life of the labeled antibody: 

\[ T_E = T_B \times t/(T_B + T_I) \]

where \( T_E \) is the effective half-life of the radiolabeled antibody, \( T_B \) is the biological half-life of the antibody, and \( T_I \) is the physical half-life of the isotope. In practice, given typical injected doses and acquisition times, small-animal SPECT and PET systems are able to image out to approximately four effective half-lives. For example, an antibody with a 72-h biological half-life labeled with \( 111\text{In} \) (67.2-h physical half-life) has an effective half-life of 34.7 h, permitting imaging out to approximately 6 days. The differences between the half-lives of SPECT and PET isotopes are significant with the oft-preferred longer-lived half-lives belonging to SPECT. An exception to this is \( 124\text{I} \) for PET, with a 4.2-day half-life (Table 2). Unfortunately, the benefits offered by the long half-life of \( 124\text{I} \) are overshadowed when imaging in mice by the significant negative contribution of its large positron range to reconstructed resolution.

Reconstructed resolution is paramount to both qualitative and quantitative image analysis. Multiple factors contribute to reconstructed resolution, but for most SPECT isotopes, the resolution is approximately half a cubic millimeter (0.5 mm³) in mouse imaging (Table 1). The notable exception to this pattern is \( 125\text{I} \) with a reconstructed resolution of one cubic millimeter (1 mm³). The primary reason for this larger value is the low photon energy (multiple emissions approximately 28 keV) that translates to a large intrinsic detector resolution in conventional scintillation detectors (Weber et al., 1994; Beekman et al., 2002; Accorsi and Metzler, 2004; Lackas et al., 2005; Beekman and Have, 2007). For PET isotopes, reconstructed resolution depends primarily on two factors: the positron range (i.e., the distance traveled by a positron before annihilation with an electron) and photon noncollinearity (the deviation from 180° in the emitted photon paths caused by residual momentum of the electron-hole pair at annihilation) (Chatziioannou et al., 1999). Because this article is concerned with small-animal scanners, it is primarily the disparity in positron range that is responsible for the large variation in reconstructed resolution for the isotopes shown in Table 2.

In the clinical setting, PET resolution tends to exceed SPECT resolution for numerous reasons associated with the need for SPECT imaging apertures. For example, in clinical SPECT imaging, because the patient size and associated FOV are on the order of the size of the available detectors,
parallel-hole collimators are used for most applications. The degradation in spatial resolution and sensitivity attributable to these collimators exceeds the similar detrimental effects of the physical limitations associated with PET imaging, resulting in higher spatial resolutions in PET. However, the opposite is true in preclinical imaging in small-animal models. The effects of the aforementioned positron range and the difficulty in accurately estimating the interaction location of the relatively higher energy photons associated with PET isotopes are more pronounced because of the smaller subject volumes (Levin and Hoffman, 1999). Conversely, these small imaging volumes enable the use of high magnification apertures in SPECT imaging, increasing sensitivity and resolution relative to their clinical counterparts (Schramm et al., 2003; Beekman et al., 2005).

Nuclear medicine imaging physics follows Poisson statistics, as the nature of radioactive decay is that of an independent counting event. That the mean equals the variance for a Poisson random variable translates into reduced noise and improved estimation of the radioisotope distribution as the number of detected photons increases. Achieving the physical limits of reconstructed resolution also depends strongly on the number of acquired counts. In both PET and SPECT, the number of acquired counts is dictated by the geometric efficiency and resolution settings of the system (how many photons emitting from the field of view are collected by the system) and the physical properties of the isotopes used in the study (Wilson and Tsui, 1993; Barrett et al., 1994; Wilson et al., 1994; Kupinski et al., 2003; Barrett and Myers, 2004; Barrett, 2005). Differences in sensitivity between isotopes are governed primarily by the number of decays resulting in relevant photons or photon-creating positrons (intensity) correlated with positron intensity. Furthermore, the absence of a significant biological clearance (i.e., no biological clearance, dose is limited only by isotope half-life). The radioisotope-to-antibody ratio is calculated from reported specific activity measurements of radiolabeled antibodies and antibody fragments and the theoretical specific activity of the isotope.

### Table 1

<table>
<thead>
<tr>
<th>Radioisotope</th>
<th>67Ga</th>
<th>123I</th>
<th>125I</th>
<th>111In</th>
<th>177Lu</th>
<th>99mTc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Half-life (days)</td>
<td>3.26</td>
<td>3.02</td>
<td>5.94</td>
<td>2.80</td>
<td>56.7</td>
<td>0.25</td>
</tr>
<tr>
<td>Energy in keV (mean no. of gamma emissions per 100 decays)</td>
<td>93.2 (42.4), 184.6 (21.2)</td>
<td>28.0 (86.7), 159.0 (83.0)</td>
<td>27–32 (144.6)</td>
<td>171.3 (90), 245.4 (94.0)</td>
<td>56.7 (5.4), 112.9 (6.4), 208.4 (11.0)</td>
<td>140.5 (89)</td>
</tr>
<tr>
<td>Reconstructed resolution (mm²) 1-mm pinhole w/Jasz</td>
<td>0.73</td>
<td>0.51</td>
<td>1.00</td>
<td>0.42</td>
<td>0.51</td>
<td>0.41</td>
</tr>
<tr>
<td>Average sensitivity in 20-g mouse (cps/μCi/cc, 1.0-mm pinhole array)</td>
<td>0.182</td>
<td>0.242</td>
<td>0.243</td>
<td>0.285</td>
<td>0.052</td>
<td>0.226</td>
</tr>
<tr>
<td>Average sensitivity in 20-g mouse (cps/μCi/cc, 2.0-mm pinhole array, -μPET resolution)</td>
<td>0.73</td>
<td>1.77</td>
<td>1.77</td>
<td>1.14</td>
<td>0.21</td>
<td>0.90</td>
</tr>
<tr>
<td>Whole-body absorbed dose (Gy) per 100 μCi and a 72-h biological half-life (maximum dose, no biological clearance)</td>
<td>0.258 (0.542)</td>
<td>0.065 (0.077)</td>
<td>0.276 (5.726)</td>
<td>0.284 (0.556)</td>
<td>1.117 (3.846)</td>
<td>0.017 (0.019)</td>
</tr>
<tr>
<td>Specific activity (theoretical) Ci/μmol Radioisotope/antibody</td>
<td>40</td>
<td>237</td>
<td>2.2</td>
<td>47</td>
<td>19</td>
<td>522</td>
</tr>
<tr>
<td>Primary labeling method</td>
<td>DOTA</td>
<td>Direct labeling (iodination)</td>
<td>Direct labeling (iodination)</td>
<td>DTPA</td>
<td>Imageable and therapeutic</td>
<td>HYNIC, MAG3, hydrazinonicotinamide</td>
</tr>
<tr>
<td>Primary strength/use</td>
<td>Translatable to Ga-68 for PET</td>
<td>Neuroimaging</td>
<td>Long half-life</td>
<td>Availability and translation</td>
<td>Low price</td>
<td></td>
</tr>
<tr>
<td>Primary shortcoming</td>
<td>Contamination with Zn</td>
<td>Cost, half-life</td>
<td>Long half-life</td>
<td>High cost</td>
<td>Potential therapeutic effect</td>
<td>Short half-life</td>
</tr>
<tr>
<td>Cost/mCi ($)</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>++++</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>

DOTA, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; DTPA, diethylenetriaminepentaacetic acid; HYNAC, hydrazinonicotinamide. The number of + signs indicates relative expense, where + is on the order of less than $10 per mCi and +++++ is on the order of hundreds of dollars per mCi (note that costs will vary according to location and institution).
TABLE 2
Physical and radiochemical properties of four PET isotopes commonly used in antibody imaging

| Radioisotope/antibody (does not include cold or contaminant label) | Half-life (days) | Mean annihilation distance (mm) | Reconstructed resolution (mm³) | Positron abundance (mean no. of positrons created per 100 decays) | Average sensitivity in 20-g mouse (cps/μCi/cc) | Whole-body absorbed dose (Gy) per 100 μCi with 72-h biological half-life (maximum dose, no biological clearance) | Theoretical specific activity (Ci/μmol isotope) | Radioisotope/antibody (does not include cold or contaminant label) | Primary labeling method | Primary strength/usage | Primary shortcoming | Cost/mCi ($) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 64Cu | 0.542 | 0.67 | 2.85 | 17 | 1.573 | 0.217 (0.256) | 241 | 0.02085 | Chelation (TETA) | Half-life | In vivo transcomplexation | ++ | ++++ |
| 18F | 0.076 | 0.65 | 2.80 | 97 | 8.97 | 0.076 (0.078) | 1718 | 0.00003–0.00002 | Via prosthetic group | Low positron emission energy (short annihilation distance ~0.2 mm) | Short half-life | ++ |
| 68Ga | 0.047 | 27.3 | 90 | 8.33 | 2779 | 0.123 (0.125) | 31 | 0.00003–0.001 | Chelation (DOTA, NOTA) | Potential availability (from 68Ge/68Ga generator) | Contaminating γ, short half-life | +++ |
| 124I | 4.200 | 2.35 | 42.2 | 25.6 | 4.46 (3.486) | 1.17 (0.542) | 3.25 | 0.00242 | Direct labeling (iodination) | Long half-life | Poor imaging resolution | +++ |

*Reconstructed resolution computed by convolving the mean annihilation distance (1.25 mm) with optimal isotropic resolution of 1.25 mm, which is typical of modern scanners.

The sensitivity is presented in terms of average photons detected per microCurie per cubic centimeter where the object volume is represented by a 20-g mouse (i.e., 80-mm axial extent, 30-mm transaxial extent). Dose values are provided in terms of the whole-body absorbed dose assuming a 100-μCi injected dose and either 72-h biological half-life or infinite biological half-life (i.e., no biological clearance; dose is limited only by isotope half-life).

30 to 70%, between the center of the field of view and the edge of the field of view (Beekman et al., 2002; Schramm et al., 2003; Chatziioannou, 2005; Cherry, 2006).

The relationship between sensitivity and imaging volume as a function of the field of view is presented in Tables 1 and 2. Values are provided in terms of average photons counted per second in SPECT or coincidence events measured per second (cps) in PET per micro-Curie (μCi) per cubic centimeter (cc) (cps/μCi/cc) assuming a 20-g mouse as the subject. The values for the PET table were calculated assuming a 0.5% average sensitivity (namely, 0.5% of the 511-keV photon pairs created are collected as coincidence events by the detector) for a cylindrical FOV of length 8 cm and diameter 3 cm. As all positron-electron annihilations produce 511-keV photons for PET imaging, variation in sensitivity among isotopes is driven by the percentage of decays resulting in positron emission. For example, 18F nuclear decay produces 97% positrons, whereas 64Cu produces only 17%. Calculating the values for SPECT is more complicated because isotopes vary not only in their emission intensities (percentage of decays resulting in gamma emissions), but also as a function of the associated energy. The values are estimated for the same FOV defined above using the configuration of a commercial microSPECT (NanoSPECT/CT, Bioscan, Washington, DC). The average sensitivities were calculated using a ray-tracing technique (Schramm et al., 2003) and validated with 99mTc and 111In.

*Poor imaging resolution, however, is accompanied by a 4-fold loss in sensitivity.

Given that nuclear medicine imaging involves the administration of radioactive probes, it is also necessary to consider the radiation dose to the subject. As defined above, the unit of cps/μCi/cc provides a measure of expected counting statistics as a function of the amount of radioactivity injected and the animal mass. The amount and type of isotope as well as the effective half-life of the radiolabeled antibody determines the absorbed dose [expressed in units of gray (Gy)] received by the mouse from the administered radiolabeled agent (Funk et al., 2004; Hindorf et al., 2004). The absorbed dose to the animal is a function of the cumulated activity (μCi·s) and the mean dose per unit cumulated activity (Gy/μCi·s) or S-value (Weber, 1991; Stabin and Siegel, 2003). Dose values are provided for each isotope in Tables 1 and 2 using whole-body S-values for a 20-g (spherical) mouse as provided in Stabin and Siegel (2003).
The first value in the tables represents an estimate of absorbed dose per 100 μCi of radiolabeled antibody with an effective half-life ($T_{1/2}$) of 72 h. The second value provides an estimate of the maximum absorbed dose as measured in a theoretical case in which there exists zero clearance of the radiolabeled antibody ($T_B = 0$) and the effective half-life is the radiative half-life of the isotope ($T_E = T_I$).

Estimating the effects of low radiation doses over an extended period of time in mice is difficult to fully characterize. There has been significant investigation into the effect of radiation dose from multiple microCT studies or $[^{18}F]$-fluorodeoxyglucose PET with absorbed dose values equivalent to the studies described here (Daibes et al., 2004; Laforest et al., 2004; Taschereau and Chatziioannou, 2007). For reference, the whole-body lethal dose to 50% of mice in a cohort within 30 days [LD(50/30)] is on the order of 6 Gy when generated with a single (<10 min) exposure to radiation (Prosser, 1947; Samarth and Kumar, 2003), and tumor eradication doses in xenograft tumor models are on the order of 50 Gy (Govindan et al., 2000). In practice, the absorbed doses for a typical $^{111}$In- or $^{125}$I-labeled antibody study are equivalent to longitudinal multidose $[^{18}F]$-fluorodeoxyglucose studies with four to six time points with microCT image collection (approximately 0.5–2.5 Gy over the course of a couple of weeks). There does not exist a consensus in the literature as to the impact of absorbed dose in such studies (Carlson et al., 2007).

### Chemistry and Biology

**Trivalent Metal Cations ($^{111}$In, $^{67}$Ga, $^{68}$Ga, $^{64}$Cu, $^{177}$Lu).** Antibodies, antibody fragments, and other protein scaffolds can be radiolabeled with metal cations using bifunctional metal chelates, the most common of which are various functional derivatives of 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid and diethylenetriaminepentaacetic acid. The functional groups for conjugation to a protein include primary amine reactive groups such as cyclic anhydride, isothiocyanate, and N-hydroxy succinimidy l ester (NHS). Many of these compounds are available commercially (Macrocyclics, Dallas, TX and Sigma-Aldrich, St. Louis, MO), and reaction with primary amines on lysine side chains and the N terminus is relatively straightforward (Sosabowski and Mather, 2006). After chelate conjugation, the average number of chelate molecules per protein can be determined experimentally (Smith-Jones et al., 2000). Conjugation ratios of one to three chelates per antibody molecule are preferred such as not to interfere with biological function. Conjugation with bifunctional metal chelates results in a heterogeneous mixture of molecules with different number of chelators, attached at different positions. Although not used commonly, site-specific labeling methods have been developed (Li et al., 2002; Ahlgren et al., 2008).

Radioiodination is performed by incubating the chelate-conjugated protein with the radioisotope usually at room temperature or mildly elevated temperatures in mildly acidic conditions (Sosabowski and Mather, 2006). The time required for radioiodination will depend on the chelate/isotope pair and concentrations, but is typically performed within 1 h.

**Iodination ($^{125}$I, $^{123}$I, $^{120}$I).** Iodination is typically performed using one of two methods, direct labeling in the presence of an oxidizing agent or indirect labeling with an intermediate iodinated compound (Hermanson, 1996). Direct labeling methods are the more common of the two, and oxidizing agents used in solution or immobilized to solid supports are available commercially (Thermo Fisher Scientific, Waltham, MA). Immobilized oxidizing agent and enzymatic iodination via lactoperoxidase result in a slower and more controllable iodination process. Direct iodination results in the modification of tyrosine phenolic groups and histidine imidazole groups. Indirect iodination methods allow conjugation to other functional groups such as primary amines and have been described previously (Hermanson, 1996). As with bifunctional metal chelates, iodination results in a heterogeneous mixture of radiolabeled protein with radioiodide incorporated at various locations. Low levels of iodination via the direct labeling method does not typically affect protein activity, because of the relatively small size of iodine, unless the active site contains a large number of surface-accessible tyrosine and/or histidine residues (Kung et al., 2000). Site-specific iodination has been described previously (Arano et al., 1994).

$^{99m}$Tc, $^{111}$In, $^{125}$I can be incorporated into proteins using direct and indirect labeling methods (Banerjee et al., 2001). In direct labeling, disulfide bonds are reduced under mild conditions and the $^{99m}$Tc is coordinated to the sulphydryls. Indirect labeling is typically preferred because of improved stability (Hnatowich et al., 1993) and is performed using bifunctional metal chelates such as mercaptoacetyltriglycine (MAG3) (Winnard et al., 1997), S-acetylm mercaptoacetyltriglycine (Chang et al., 1999), or hydrazinonicotinamide (Babich et al., 1993), or it can be accomplished through the incorporation into the protein a hexahistidine peptide (Waibel et al., 1999). The latter approach is used only with proteins produced recombinantly and will result in site-specific modifications. NHS derivatives of hydrazinonicotinamide are available commercially (Solulink, San Diego, CA). NHS derivatives of MAG3 and S-acetylm mercaptoacetyltriglycine can be prepared in-house (Winnard et al., 1997; Chang et al., 1999; Misra et al., 2007) and conjugated to proteins in a straightforward manner under mildly basic conditions. Conjugation results in a heterogeneous mixture of chelate modified proteins as in the other methods discussed above.

$^{18}$F. A variety of methods to label proteins with $^{18}$F have been described previously (Guhlke et al., 1994; Cai et al., 2008; McBride et al., 2010); however, labeling with this particular isotope generally remains a challenge because of its short half-life, low radiochemical yields, and low specific activities. A commonly used approach uses the $^{18}$F-labeled amine-reactive intermediate N-succinimidyl 4-[$^{18}$F]fluorobenzoate (Vaidyanathan and Zalutsky, 2006). This approach involves the $^{18}$F-labeling of the intermediate followed by conjugation to the protein. An approach using a bifunctional chelate has also been described previously (McBride et al., 2009, 2010).

Radioisotope incorporation for all approaches described above can be determined by thin-layer chromatography or size-exclusion chromatography. It is generally good practice to evaluate the modified protein for its biological activity by in vitro assays specific to the particular application. In addition, protein and label stability can be assessed by incubation in serum and analysis by appropriate methods such as size-exclusion chromatography to determine extent of degradation.

**Isotope Selection.** The selection of an appropriate isotope for imaging antibody pharmacokinetics depends on a variety of factors including 1) antibody blood clearance half-life,
2) imaging time points desired and study design, and 3) antibody dose (low doses will require high specific activity and/or high gamma abundance to achieve high-quality images). As outlined in Tables 1 and 2, the physical properties of different isotopes are important in study design given their significant influence on sensitivity and reconstructed resolution as well as the temporal window of measurement allotted by their radioactive half-life. Compromises are inevitable.

In vivo activity is associated with the biodistribution of the isotope, a signal that is not exclusively correlated with the biodistribution of the full-length protein of interest. Thus it is important to take into consideration the stability and in vivo behavior of the isotope conjugation. For example, enzymes in the blood will slowly dehalogenate iodinated compounds and the dissociated iodine will result in increased uptake in the thyroid and lower uptake in the blood and other organs. Antibody internalized by cells will undergo degradation by proteases in endosomes and lysosomes. Directly iodinated compounds generally result in relatively low cellular iodine retention (Press et al., 1996). Indirect iodination using residualizing labels has been reported to result in greater retention (van Schaik et al., 2005; Vaidyanathan et al., 2009).

99mTc-MAG3 labels also exit the cell more slowly (Engfeldt et al., 2007), whereas chelated metal cations exhibit even shorter cellular release with a half-life of ~120 h (Press et al., 1996). These residualization kinetics must be taken into account when analyzing data because they can have a large impact on the apparent biodistribution especially at longer time points. Likewise, for tumor targeting antibodies, tumor activity levels will not accurately represent the concentration of intact antibody in the tumor because the total activity will be a sum of extracellular-localized (radiolabel associated with free and surface-bound antibody) and intracellular-localized isotope (Shih et al., 1994; Ferl et al., 2006).

Optical Imaging

Optical imaging relies on the detection of photons in the visible to near-infrared portion of the electromagnetic spectrum produced by bioluminescence or fluorescence (Weissleder, 2002). Bioluminescence is a chemical process wherein light is emitted during the interaction of a protein produced in engineered cells and an administered substrate, for example firefly luciferase and luciferin (de Wet et al., 1987). Fluorescence imaging uses probes that emit light (fluoresce) after the excitation at a fluorophore-specific wavelength. The subsequent emission of light is shifted to a longer wavelength by an amount known as the Stokes shift. For example, widely used green fluorescent protein, mutated to improve fluorescence and photostability by Helm et al. (1995), has a primary excitation peak at 489 nm and primary emission peak at 509 nm.

The pharmacokinetics of fluorophore-labeled antibodies can be assessed with fluorescence imaging; however, determination of antibody localization is limited by the absorption properties of tissues. Developments in fluorophore chemistries have resulted in a series of fluorochromes with emission extending from the ultraviolet through the visible spectrum and into the near-infrared (Weissleder et al., 1999; Weissleder and Mahmood, 2001). The latter is advantageous because tissue absorption of signal and auto fluorescence is minimized at these wavelengths. But even in the near-infrared range, organs at depth remain difficult to detect. Three-dimensional image reconstruction of fluorophores has been demonstrated in both academic and commercial environments; however, results with this modality are almost exclusively two-dimensional. Generally, only organs/areas of interest on or near the surface will be detected reliably (Arridge, 1999; Ntziachristos and Weissleder, 2001; Ntziachristos et al., 2002, 2005; Grimm et al., 2005; Morimoto, 2007; Weissleder and Pittet, 2008).

The advantages of optical imaging for assessing antibody pharmacokinetics in vivo are the conjugation chemistry, cost, and throughput. The fluorophore conjugation chemistry is often carried out with a commercially available kit and can be performed in most laboratories without special license, equipment, hazardous precautions, or specific training. The detection systems are generally 30 to 50% as expensive as those for small-animal SPECT or PET. Planar fluorescence images are generated within seconds or minutes, typically with a photograph overlay. Optical imaging experiments also can accommodate four to five animals per scan, allowing an efficient researcher to perform 100+ animal images per hour, whereas PET and SPECT imaging is often limited to one to two animals per 10- to 30-min scan. Such throughput and cost combined with expected improvements in quantification from advances in instrumentation provide an exciting outlook for this approach. The reader is referred to a review by Leblond et al. (2010) for a thorough overview of the current state of preclinical fluorescence imaging.

Example Data

Results from an experiment comparing the kinetics of two different 111In-labeled antibodies (specific and nonspecific) in a xenograft tumor model are presented in Fig. 1. SPECT and CT images (NanoSPECT/CT; Bioscan) were collected at 3, 24, 48, 72, and 96 h after injection. Whole-body helical SPECT studies using 36 pinholes (1.4 mm diameter) were collected with 16-min acquisition times at hour 3, scaling up to 24 min acquisition times at hour 96 (Fig. 1A). Whole-body helical CT studies were collected with acquisition times of 3 min at a tube setting of 45 kVp (average X-ray energy of just under 45 keV). Antibodies were conjugated with diethylentriaminepentaacetic acid followed by radiolabeling with 111In. Each animal received an intravenous injection of 111In-labeled antibody (600 μCi, 15 μg) in 100 μl of sterile saline.

At each time point for each animal in the study (in this case 16 animals were imaged daily) region-of-interest analysis was performed on each of the following organs: tumor, brain, spleen, heart, liver, kidneys, bladder, muscle, and whole body through a combination of manual, semiautomated, and fully automated segmentation routines. An example of a suborgan analysis technique possible with small-animal nuclear imaging is presented in Fig. 1B. Longitudinal radial profiles of tumors provide a means of assessing changes in distribution and concentration as well as a means to visualize the heterogeneity of antibody distribution within the tumor.

Both microSPECT and microPET systems provide images in units of radioactivity per volume. A broad range of output data are available with these nuclear techniques including activity (μCi or kBq), activity per volume (μCi/mm³ or kBq/mm³), standardized uptake value, a measure of region-of-interest activity per volume normalized by the injected dose per subject mass (i.e., “whole-body” concentration), percent-
age of injected dose, percentage of injected dose per gram, and concentration (nM) when the specific activity of the radiolabeled antibody is available. Typically, ex vivo biodistribution analyses are performed after collection of the final image. Extensive work has been performed assessing the precision of the quantification in these techniques as well as correlating in vivo and ex vivo results (Forrer et al., 2006; Rolleman et al., 2008).

**Discussion**

Advances in preclinical imaging instrumentation and analysis methods have created the means for assessing antibody pharmacokinetics in vivo. This review has focused on a basic description of the imaging modalities and labeling methods used in these studies with a focus on the role of modality and isotope selection in study design. Although some of the information provided from such studies is attainable with in vitro and ex vivo techniques, the opportunity to measure organ and suborgan antibody kinetics longitudinally in a mouse resides exclusively with the molecular imaging approaches outlined in this review. SPECT and PET offer radiolabeled antibody imaging resolution and sensitivity that translates from mouse to human. The cost of laboratory instrumentation and infrastructure for these modalities is significant, although labeling and imaging facilities are widely available. Designing and executing such studies is a multidisciplinary effort encompassing physics, mathematics, biology, pharmacology, engineering, and chemistry. Ultimately, the selection of a labeling method and imaging modality must be made expertly and with sound understanding of the complex factors governing signal origin, detection, and contamination.

**Acknowledgments**

We thank Ky Harlin and Mary Germino for assistance with figure preparation.

**Authorship Contributions**

Conducted experiments: Cheng and Rusckowski.
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