INVITED PAPER

Elucidating Structure and Function *In Vivo* With Hybrid Fluorescence and Magnetic Resonance Imaging

Combining these two imaging techniques can offer new dimensions in the visualization of functional and molecular tissue characteristics and improve the treatment outcome of interventional approaches.

By Mark Niedre and Vasilis Ntziachristos

ABSTRACT | While the mathematics, physics, and technology behind magnetic resonance (MR) and fluorescence image formation are distinctively different, the two modalities have significant complementary features to impart strong preclinical and clinical application synergies. Traditionally, hybrid MR and fluorescence imaging implied the use of a system where optical and MR signals can be concurrently acquired. In this case, the common geometry allows for the superposition of fluorescence images of cellular and subcellular processes onto anatomical and functional MR images. More recently, a different hybrid imaging paradigm is strongly evolving by utilizing hybrid MR-fluorescence nanoparticles. This approach offers a second paradigm of hybrid visualization where the common underlying contrast enables the coregistration of MR and fluorescence images acquired under different geometries. We review herein progress with the evolving field of multimodality MR and fluorescence imaging and discuss how these strategies offer a highly promising outlook in established and in novel preclinical and clinical applications.

KEYWORDS | Dual modality; fluorescence; imaging; magnetic resonance imaging (MRI); optical

I. INTRODUCTION

Fluorescence imaging and magnetic resonance imaging (MRI) are each highly successful modalities in their own merit. Fluorescence imaging has become a workhorse of biomedical research, routinely employed in the biomedical laboratory *in vitro*, and more recently increasingly applied *in vivo* to gain information at the cellular and subcellular level in unperturbed environments. The enabling technologies for *in vivo* use have been the development of fluorescent probes with molecular specificity as well as transgenic technologies that utilize fluorescent proteins, combined with the parallel emergence of advanced microscopic and macroscopic optical imaging techniques, such as confocal and multiphoton microscopy or whole-body fluorescence tomography [1]–[6]. This strategy has led to a renaissance in elucidating structural, functional, and molecular information associated with critical biomedical problems such as disease formation and progression and *in vivo* evaluation of novel therapeutics.

Fluorescence imaging comes with several attractive characteristics, such as:

i) the ability to concurrently resolve several distinct targets by utilizing fluorophores emitting at different spectral bands;
ii) high flexibility in developing and utilizing fluorescent reporters for many different and diverse cellular processes and molecular pathways;
iii) high detection sensitivity reaching limits in the subpicomole range for common organic fluorochromes in the case of whole mouse imaging, and even higher sensitivity when imaging superficial

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activity, for example, in endoscopic and intraoperative applications;

iv) high resolution imaging when using microscopy for imaging events at superficial depths.

On the other hand, adoption of fluorescence imaging at large animal and human scales has been impeded by the high degree of light scatter and absorption in tissue, which ultimately places physical limits on the achievable image resolution and depth of light penetration. Progress with advanced illumination schemes and appropriate inversion algorithms has recently allowed *in vivo* quantitative macroscopic imaging at the whole-animal level by means of fluorescence molecular tomography (FMT). The method has been shown capable of resolving biomarkers (proteases, receptors, etc.) in a number of cancer models, three-dimensional resolving of fluorescently labeled deep-seated lung tumors, imaging of apoptosis (cell surface receptors) in response to anticancer chemotherapy, protease activity in lung tumors, imaging of macrophage infiltration in cardiac infarctions, and imaging of murine breast cancer using a number of cell-surface molecular targets [7]–[12].

MRI has similarly seen remarkable progress over the last two decades with significant technical developments improving the imaging resolution, sensitivity, and acquisition speed. In addition, a number of advanced contrast imparting agents and techniques have emerged that render MRI a highly versatile tool for visualizing both anatomy and function [13], [14]. Novel contrast agents that modulate T_1 and T_2 relaxivities, combined with molecular targeting strategies provided by advances in nanotechnology, have yielded significant progress in developing MRI as a tool for visualizing cellular and subcellular events [15], [16]. Included in this category of novel MRI contrast agents are superparamagnetic iron-oxide-based nanoparticles—such as monodisperse iron oxide (MION) and cross-linked iron oxide (CLIO) [17], [18]—as well as gadolinium-chelates encapsulated in liposomes, micelles, polyacrylamide, or incorporated into high-density lipoprotein (HDL)-like nanoparticles [20]–[22]. Use of these novel MRI agents has been demonstrated *in vivo* in wide-ranging applications, including imaging of gene expression in mice [17] and *xenopus laevis* embryos [23], annexin-V binding in cells in response to anticancer therapy [18], and her2/neu cell surface receptors in cell culture [19].

In light of these recent advancements, imaging strategies that synergistically utilize both MRI and fluorescence are emerging as particularly attractive. Two general approaches have emerged in recent years in combining the two technologies, specifically: i) the use of concurrent dual-modality systems and ii) the use of dual-modality probes. The former strategy has generally been applied when the penetration depth of fluorescence imaging is appropriate for the application, for example, in imaging small animals, as in the work of Pogue *et al.* [24] and Nalcioglu *et al.* [25]; the human breast [26]–[28]; or functional brain imaging studies [29], [30]. In this case, hybrid systems allow the combination of the molecular targeting capacity imparted by fluorescent reporter probes with the high resolution and highly versatile tissue-function contrast achieved with MRI. Hybrid systems further enable the utilization of MR information to improve the accuracy of the fluorescence tomographic inversion problem, resulting in a potentially superior imaging modality to either individual modality alone [31], [32]. The second probe-based hybrid MR-fluorescence imaging approach utilizes agents that impart both MR and fluorescence contrast (i.e., hybrid probes). This approach can be used to merge noninvasive MR imaging findings with corresponding highly sensitive intraoperative or endoscopic detection of optical signals during follow-up procedures.

In practice, these two different hybrid imaging paradigms offer fundamentally different visualization strategies: it is the common geometry of hybrid systems that allows the coregistration of different contrast mechanisms obtained from MR and fluorescence in order to yield higher information content from the combined imaging session versus either modality used as stand alone. When using hybrid probes, however, these roles are reversed; in this case it is the common contrast that allows the coregistration of two different imaging modalities and geometries. For example, preoperative MR can be used to identify tumor spread and possible lymph node involvement, whereas intraoperative fluorescence imaging can be used to guide the surgeon towards activity identified with MR and in parallel further inspect for locoregional metastatic foci. In the latter case, the fluorescence imaging offers superior sensitivity and resolution compared to MRI.

In this paper, we review these two different hybrid MR-fluorescence imaging strategies and describe recent advances and key applications associated with their development, highlighting pertinent examples from the large and constantly expanding body of work in the field. We then discuss the outlook of these approaches for preclinical and clinical use.

II. HYBRID FLUORESCENCE—MAGNETIC RESONANCE IMAGING SYSTEMS

The concept of a "multimodality" hybrid imaging system has emerged in recent years from the observation that the combination of the strengths of different modalities in a single imaging system can yield images that contain information significantly superior to those produced by any of the systems alone as a result of the fundamentally different contrast that can be imparted. MRI, for example, offers excellent anatomical and functional information but is relatively insensitive to cellular and subcellular molecular information. In contrast, optical and fluorescence tomographic techniques can yield excellent cellular and subcellular information with high sensitivity but relatively

limited anatomical resolution. As such, a great deal of potential synergy exists in designing hybrid instruments to perform simultaneous fluorescence and MR data acquisition and through combining information yielded from the two modalities. Due to its excellent resolution and soft-tissue contrast, MRI has been the imaging technique most commonly used in conjunction with optical tomography, although similar multimodality approaches to those described below have also been used with ultrasound and X-ray computed tomography (CT) [33]–[35].

A number of multimodality imaging strategies have been described in the literature, including hybrid instruments for simultaneous data acquisition and coregistration, as well as hybrid image reconstruction algorithms. Pertinent examples of each strategy, as well as discussion of design considerations in multimodality systems, are given in this section. In order to properly review these strategies, a brief review of the major concepts of optical tomographic imaging is first given here.

Fluorescence Tomographic Imaging: In contrast to simple planar fluorescence reflectance imaging (FRI)—wherein a tissue sample is irradiated with a light source such as a laser and the emitted fluorescent light is detected using, for example, a charge-coupled device (CCD) camera—FMT and diffuse optical tomography (DOT) allow three-dimensional rendering of fluorochromes, absorbers, and light scatterers in macroscopic tissue samples [6], [36]–[40]. This is shown conceptually in Fig. 1 and can be viewed as the optical analog of, for example, X-ray computed tomography. Briefly, optical tomography requires the use of i) multiple optical measurements between light-source and detector pairs through bulk tissue, ii) modeling of light propagation through the optically diffusive media, and iii) solving the subsequent inverse problem to yield the distribution of the quantity of interest. Tomographic optical techniques have therefore benefited greatly from major developments in models of photon propagation in biological tissue, handling of inverse problems, and new classes of targeted fluorescent probes. Fluorescence tomography is generally performed using either steady-state (continuous wave) or frequency modulated light sources, although pulsed light sources and time-domain measurements have also been reported [41]–[43]. Detectors that have been utilized for fluorescence tomography include appropriately filtered CCD cameras, optical fiber-coupled photomultiplier tubes, and streak camera detectors.

Light propagation in tissue is generally well modeled with the diffusion approximation to the Boltzmann transport equation [44], i.e.,

$$\frac{1}{c}\frac{\partial}{\partial t}\phi(\mathbf{r},t) - D(\mathbf{r})\nabla\phi(\mathbf{r},t) + \mu_a(\mathbf{r})\phi(\mathbf{r},t) = S(\mathbf{r},t) \quad (1)$$

where $\Phi(\mathbf{r},t)$ is the diffuse intensity at position $\mathbf{r}$ inside the media at time t, $S(\mathbf{r},t)$ is the photon source, $\mu_a(\mathbf{r})$ is the absorption coefficient, c is the speed of light in tissue, and $D(\mathbf{r})$ is the diffusion coefficient. The diffusion approximation can therefore be applied using a number of analytical or finite element solutions for continuous wave, frequency-modulated or time-domain experimental cases for the specific geometry of the FMT instrument. More exact solutions to the Boltzmann transport equation may be required to model light propagation under particular circumstances, for example, in the case of very early times following an infinitesimally short laser pulse [45], [46].

The approach is then to set up and solve a system of linear equations that couples the set of source-detector

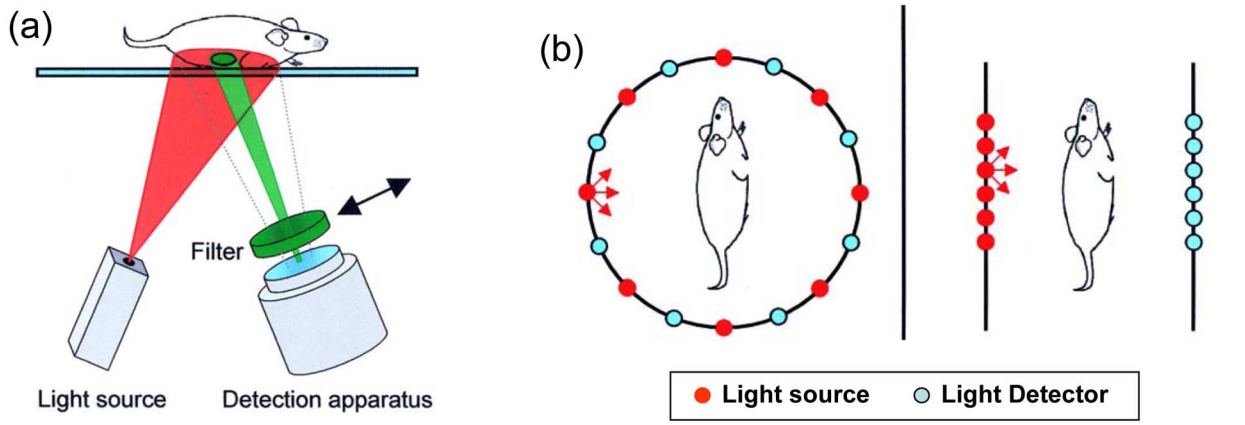

Fig. 1. *(a) Schematic of a simple FRI system commonly used for macroscopic fluorescence imaging applications but suffering from poor resolution and nonlinear signal attenuation in deep tissues. (b) Schematics of FMT systems using ring or planar geometry. Multiple source (red dots) and detector (blue dots) projections are made through the animal, and the fluorescence distribution is calculated by back-projecting physical models of light propagation through tissue. Adapted from [36].*

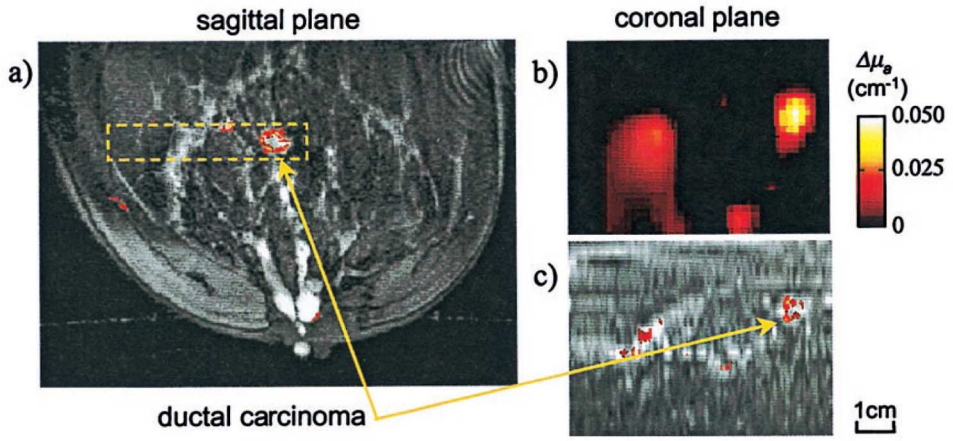

Fig. 2. *A hybrid dual-modality MRI and optical-tomographic instrument. (a) Sagittal MRI image of a human female breast with an invasive ductal carcinoma. (b) Coronal slice of an optical tomographic image showing the change in absorption due to injection of an ICG contrast agent. Comparison with the coronal MRI slice (c) shows excellent coregistration of the ductal carcinomas with the optical reconstruction. Adapted from [26].*

measurements to the parameter of interest (i.e., the fluorochrome concentration). In particular, the system of equations can be expressed as a matrix problem $\boldsymbol{y} = \boldsymbol{W} \cdot \boldsymbol{x}$, where $\boldsymbol{y}$ are the source-detector measurements, $\boldsymbol{x}$ is the fluorochrome concentration at each position in the volume, and $\boldsymbol{W}$ is the "weight matrix" or sensitivity function describing light propagation in the media. This matrix problem is generally ill-posed and can be inverted using a number of numerical techniques including singular value decomposition (SVD), the algebraic reconstruction technique (ART), and conjugant-gradient type methods. Similar methodology can be applied in the case of diffuse optical tomography, wherein fluorochromes are not used and images are reconstructed from intrinsic tissue contrast. This has been shown to be useful in a number of applications, including imaging of oxygenated and deoxygenated hemoglobin, water fraction, lipid content, and functional imaging of tissue hemodynamics [40], [47], [48].

A. Hybrid Optical-MRI Imaging Systems

In the most direct hybrid approach, optical and MRI imaging can be performed simultaneously using a dual-modality instrument. Herein, optical and MRI instrumentation and systems are integrated to allow simultaneous data acquisition without physical displacement of the subject or temporal lag between scans. An example hybrid imaging prototype was presented by Ntziachristos *et al.* [26], wherein concurrent MRI and DOT imaging of human female breasts was performed. In this case, an exogenous imaging agent, indocyanine green (ICG), was administered to the imaging subjects in order to create optical (absorption) contrast. For DOT imaging, subjects were scanned before and after administration of ICG, and the resulting difference in absorption coefficient (μ_a) was calculated using the "perturbation" reconstruction method [38], [49], [50]. Structural and functional MRI scans were simultaneously performed so that correlation between the two modalities could be achieved. Accurate coregistration of the MRI and optical images was achieved using H_2O-$CuSO_4$-filled fiducials mounted at known locations on the DOT instrument. These appeared as bright spots on the MRI, so that, combined with *a priori* knowledge of the instrument geometry, precise physical coregistration was possible.

An example image from this work is shown in Fig. 2, wherein the sagittal MRI, differential DOT (i.e., $\Delta\mu_a$), and functional MR images of a patient with an invasive ductal carcinoma are shown. The area with the largest increase in μ_a after ICG enhancement corresponded to a cancerous lesion also apparent on the MR Gd-enhanced images. The multimodality information in this case allowed validation of the DOT images from the prototype instrument.

Optical spectroscopic techniques have also been utilized in hybrid MRI systems. For example, Tromberg *et al.* [51], [52] described the use of a handheld broadband diffuse optical spectroscopy scanner with a contrast-enhanced magnetic resonance imager to detect changes in physiology in human breast tumors. In this case, external fiducials were used to enable image coregistration between the two modalities. Similarly, Paley *et al.* [53] utilized optical spectroscopy combined with an MRI system to perform functional investigations of activation of rat brains in response to whisker stimulation.

It should be noted that in all of the above examples, the optical and MRI data sets were treated independently, such that *a priori* information from the MRI images were not used in the analysis of the optical data or vice versa. As discussed in detail below, however, the importance of

hybrid systems lies in the utilization of MR contrast into the optical inversion method to improve the imaging performance and yield a hybrid imaging method as well.

B. Dual-Modality Imaging Systems Versus Sequential Imaging Sessions

Dual-modality hybrid imaging systems have a number of advantages over sequential imaging sessions—i.e., imaging an object with an instrument dedicated to one modality, followed by a session with a second, independent system. Of particular importance is the ability to simultaneously perform imaging with each modality under identical physiological and geometric conditions. This avoids displacement of soft tissues due to movement of the subject between separate instruments, which can complicate image coregistration and interpretation of results, for example, in the case of small-animal or human breast imaging. The use of MRI and optical fiducials, combined with knowledge of fixed instrument geometry, allows coregistration of images from each modality with excellent accuracy, since no physical movement of the object imaged occurs. Simultaneous imaging also has the advantage that it avoids complications in possible temporal changes in tissue characteristics between scans, for example, due to pharmacokinetics of exogenously applied contrast agents. This is particularly important when considering dual-modality molecular imaging probes discussed in Section III. Furthermore, independent measurements with two imaging modalities enable validation of measurements obtained with experimental prototype imaging systems.

On the other hand, MRI instrumentation is generally larger, more expensive, and poses more physical and material constraints than optical tomographic imaging systems. Therefore, in most hybrid systems, optical imaging components have normally been adapted to work in the MRI environment versus the other way around. Practically, this implies that optical tomographic imaging may be performed under suboptimal conditions due to necessary design constraints. This concept is illustrated in Fig. 3, which depicts the number of usable singular values (i.e., the number of values above a given noise threshold) in an inverse problem, determined using the SVD inversion technique [54]–[56]. In this figure, adapted from Lasser *et al.* [54], as well as in similar work by Graves *et al.* [55] and Culver *et al.* [56], the importance of key optical tomographic instrument parameters such as the number of light sources, number of optical projections (i.e., angular rotations of the imaging subject), and detector separation distance on the generation of usable data sets is shown. The important implication of these figures is that key

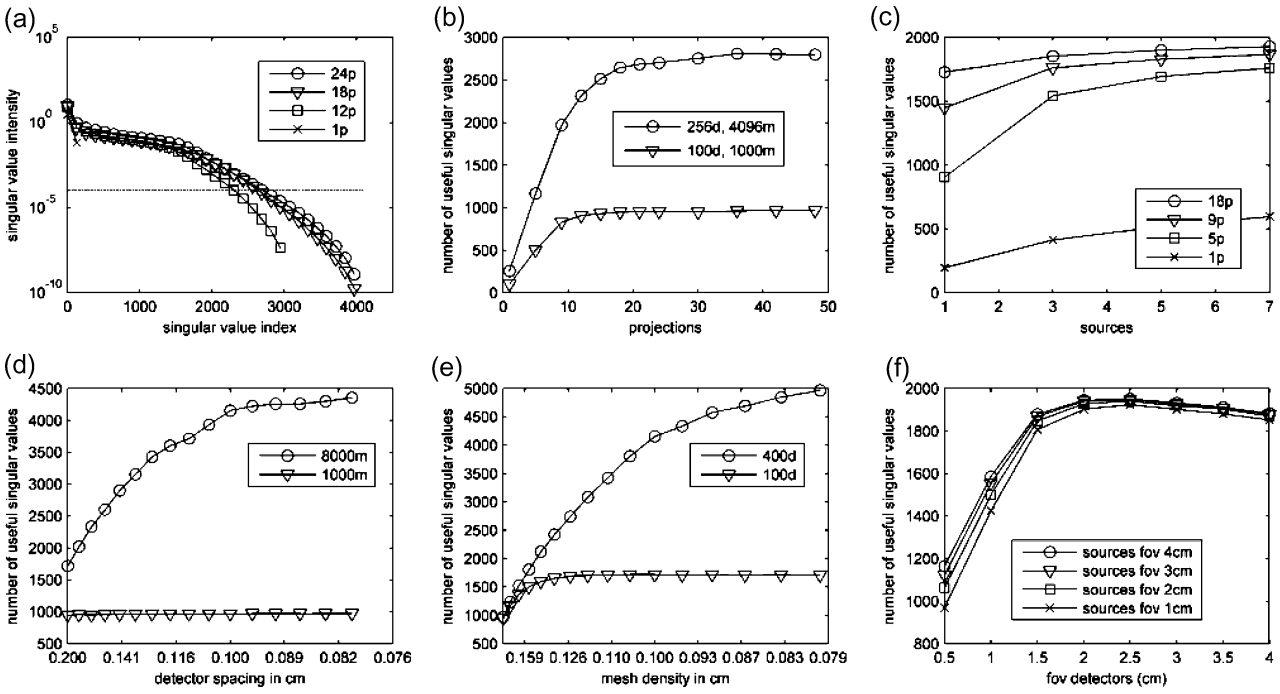

Fig. 3. *The effect of fluorescence tomography instrument design parameters on the number of useful singular values obtained with SVD matrix inversion. (a) Singular values for a given number of evenly distributed projections p around a cylindrical object. The number of singular values exceeding the noise threshold (10^{-4}) (b) as a function of number of projections, (c) sources, (d) detector spacing, (e) mesh density, and (f) field-of-view of the detectors. Parameters varied include the number of detectors d and number of mesh points m. Spatial and material constraints inherent in the design of an integrated optical-MR imaging system may result in suboptimal data acquisition in the optical instrument. Adapted from [54].*

parameters in instrument design have a large impact on the quality of the data set obtained and the eventual accuracy of the image reconstruction.

For example, in hybrid imaging systems, spatial and geometric constraints posed by an MRI magnet coil may limit the number of detectors that can be implemented in the instrument and therefore result in a suboptimal detector spacing arrangement [Fig. 3(d)]. Ultimately, this will limit the number of singular values available in the inversion problem and compromise the overall fidelity of optical tomographic reconstructions generated with the system. Conversely, the design of dedicated instruments can be optimized for the particular imaging modality so that better overall image reconstructions may actually be obtained versus a hybrid instrument. Therefore, unless transient signals need to be simultaneously recorded by a hybrid system, the use of sequential imaging systems—each optimized for delivering the best possible imaging performance—may be a preferred dual-modality imaging approach. This latter case is common in molecular imaging applications where, after original biodistribution, the fluorescence contrast persists for several minutes or hours before being biodegraded and eliminated by the organism.

C. Optical/Fluorescence Imaging With *a Priori* Spatial Information

In order to better utilize information generated from hybrid-imaging systems and improve the image fidelity possible with optical tomography, "prior knowledge" can be incorporated from one imaging modality into the reconstruction algorithm of the other. In this approach, high-resolution anatomical or functional MR images are spatially segmented according to tissue type, structure, or function in order to more accurately guide the optical reconstruction. Different methods of using spatial priors have been reported in DOT imaging by Barbour *et al.* [31] as well as Arridge and Schweiger [32] and followed the general concept and methodology reported previously to improve imaging performance in positron emission tomography and single photon emission CT [57], [58]. The overall approach has been increasingly adopted and refined in subsequent years, with differing approaches to utilizing the "priors" proposed. This is an evolving field of optical imaging reconstruction that has not yet been fully evaluated; however, the more common approaches that have emerged in the literature include the following.

1) Preassignment of "Known" Optical Properties by Region: In this strategy, optical properties are assigned *a priori* according to each region (tissue type) as defined in the segmented image. These are incorporated in the forward model to calculate a more accurate model of photon propagation through the volume of interest. The implicit assumption is therefore that the optical properties (i.e., μ_s and μ_a) of each tissue type are accurately known *a priori*. This strategy has often been used in conjunction with the "perturbation method" to more precisely model the light propagation through the reference media.

For example, Barbour *et al.* [31], [59] utilized a segmented MRI image of a human breast and assigned optical coefficients to three regions corresponding to fat, parenchyma, and tumor tissue. To illustrate the principle, simulated source-detector measurements were calculated for the reference tissue, as well as a second set for the breast with a simulated tumor included. The authors demonstrated the feasibility of the concept by showing that the use of the "known" optical parameters in the image inversion resulted in good localization of tumor locations with these simulated data sets. Other similar results and variations on the approach have been shown, including Pogue *et al.*, who utilized preassumed optical properties according to region as an initial guess in the optical reconstruction of a rat cranium with a simulated inclusion [24] as well as in a simulated breast model [60].

While this strategy represents a relatively conservative approach to utilizing spatial priors, most demonstrations of the technique have been proof-of-principle using simulated data. In practice, obtaining accurate *a priori* knowledge of the optical properties of each tissue type is a challenging inversion problem on its own.

2) Reduction of Unknown Parameters by Region: The parameter reduction strategy aims to reduce the total number of unknowns in the system of coupled linear equations posed by the inverse problem. Herein, the optical contrast is assumed to be highly correlated to the MRI contrast, so that the optical properties are assumed constant inside a segmented tissue type. Hence, instead of assuming unknown and independent scattering and absorption coefficients for each position inside the reconstruction mesh, a single set of unknown optical properties for each assigned "tissue type" is determined. In this way, the number of unknown parameters can be reduced by several orders of magnitude and the inversion problem is therefore significantly less ill-posed [61]. For example, Ntziachristos *et al.* [27] used a hybrid MRI and dual-wavelength DOT system to investigate malignant and benign breast lesions in humans. MRI images were segmented into tumor and background regions in order to quantify the tumor optical attenuation at different wavelengths over average background optical properties that could be accurately determined via time-resolved methods. Similar approaches have also been used by Brooksby *et al.* with simulated breast models [57] and Zhu *et al.* [34] using a combined ultrasound-DOT imager with phantom studies. Schweiger and Arridge [62] proposed a variation on this concept with simulated data from a human brain model, wherein a two-stage reconstruction scheme was used to first solve for global optical property averages per tissue type, which was then used as an initial guess for a second stage that recovered localized perturbations within the regions.

3) Use of Spatially Varying Regularization Parameters by Region: While the previous strategy has been shown to be successful in increasing reconstruction accuracy, the use of stringent priors may bias the reconstruction toward the *a priori* assumed distribution [60]. This has driven the exploration of less stringent application of spatial priors by assigning inversion regularization parameters according to tissue type [28], [33], [63]–[66]. Regularization parameters are commonly employed during numerical inversions of large systems of equations to improve numerical stability [55]. Normally, these are single, scalar values, but in the case of spatially varying regularization, individual parameters are assigned according to each position inside the reconstruction mesh, often according to tissue type determined from a segmented structural image. Physically, this can have multiple implementations. In the work of Li *et al.* [33], distinct regularization parameters were assigned to one of two tissue types in simulated data sets, as well as in the breast of the human volunteer. Herein, the use of separate regularization parameters implied an assigned probability of optical contrast occurring in one tissue type versus another.

In the work of Brooksby *et al.* [28], shown in Fig. 4, MRI images of the breasts of human volunteers were segmented according to either adipose of glandular tissue types. Regularization parameters were calculated according to tissue region and included in the optical reconstructions of a multispectral near-infrared (NIR) DOT scanner. In this case, the regularization parameters were defined so as to allow sharp transitions at tissue boundaries while at the same time smoothing reconstructed values inside a given region. Spatial priors were incorporated into the reconstruction using the matrix equation

$$(J^T J + \beta L^T L)\partial\mu = \bar{J}^T \partial\phi \tag{2}$$

where J is the Jacobian matrix for the diffusion equation solution, B is the regularizing factor, and L is a matrix generated by segmentation of the MRI spatial data. The reconstructed absorption values (μ_a) at multiple wavelengths were then used to generate images of hemoglobin concentration and saturation as well as tissue water fraction. More recently, Carpenter *et al.* [66] demonstrated the use of this technique in tomographic reconstruction of a breast tumor in humans.

The spatially varying regularization scheme has been demonstrated to be a flexible method for inclusion of spatial priors into optical reconstructions. However, since choice of regularization parameters can have a very large effect on the eventual optical reconstruction obtained, care must therefore be taken to not overbias the solution toward the assumed distribution, and biophysical assumptions should be carefully considered.

Fluorescence Tomography With Spatial A Priori Information: As a general comment, it is noted that, to date, most

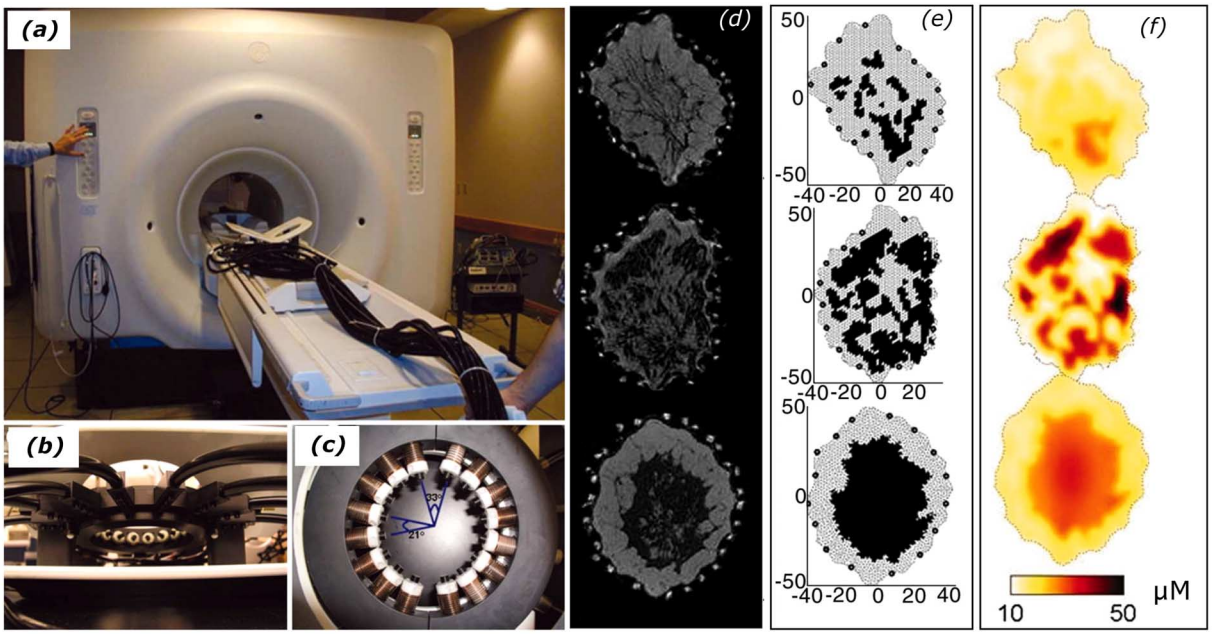

Fig. 4. ***A hybrid MRI-optical tomographic instrument that utilizes spatial MRI data a priori in the optical reconstruction. (a)–(c) Photographs of a hybrid MRI-optical tomographic scanner prototype using a ring-geometry optical illumination-detection scheme. (d) Axial MRI slices were acquired and (e) segmented according to tissue type. The spatial priors were incorporated into the optical tomographic reconstruction algorithms using a spatially varying regularization scheme, and (f) the total hemoglobin concentration was calculated. Adapted from [28].***

optical tomographic studies utilizing spatial priors have been performed using changes in absorption and scattering as contrast, as opposed to the use of fluorescent constructs as in FMT. Limited work has been reported in the literature, including that of Davis *et al.*, wherein fluorescence reconstructions of phantoms and simulated data were performed [67], as well as that by Hyde *et al.* in the crania of mice [68]. The use of fluorescence versus endogenous tissue contrast presents some complications in using *a priori* data, and the algorithms that have been developed for the endogenous contrast case will likely require reexamination and modification for use in the fluorescence domain. For example, the assumption that the fluorophore concentration inside a given tissue type will be homogenous or "smooth" (as is commonly done when reconstructing optical properties in DOT) may be physically incorrect. Likewise, while certain tissues can be associated with attenuation, based on their overall vascularization and hemoglobin content, there is no apparent correlation between fluorescence content and tissue type unless the biodistribution of a fluorochrome is known *a priori* with certainty, which, if the case, makes the need for *in vivo* imaging less relevant. In the general fluorescence imaging case, however, where no knowledge exists on fluorochome biodistribution, it is important to make use of anatomical and functional priors without stringent assumptions on fluorescence—tissue correlations. In fluorescence optical tomography, this approach is unquestionably of tremendous potential value and is therefore is an important area of future research.

In summary, while development of instrumentation and image reconstruction algorithms for dual-modality optical-MRI imaging systems is largely still in its infancy, the ability of hybrid systems to yield higher information content than standalone instruments in small-animal research and clinical applications has been clearly demonstrated by a number of researchers, and continued refinement of hybrid technologies is expected in the future.

III. HYBRID FLUORESCENCE–MAGNETIC RESONANCE PROBES

The utility of hybrid imaging probes follows a different principle than that of hybrid imaging systems. In the context of fluorescence-MR imaging, a hybrid probe—i.e., a probe that imparts contrast in both imaging modalities—may be resolved by MR and optical instrumentation, both operating under optimal detection arrangements, for example, using MRI for noninvasive disease detection and optical imaging during subsequent surgical intervention. While important recent developments in MR radiofrequency/coil technology, field strength, and novel T_1 and T_2-relaxivity modulating MRI contrast agents have consistently improved the detection sensitivity possible with MRI probes, the MRI detection sensitivity lies in the micro- to nanomolar range [15]–[23]. Conversely, with fluorescence imaging applied in microscopic, endoscopic, or whole-body small-animal imaging applications, sensitivity in the pico- to femtomolar range or better is possible, and therefore offers significant improvement in detection of cellular and subcellular processes [1]–[6].

A growing number of dual-modality probes have been described in the literature in recent years. In general, it is the common imaging contrast between modalities combined with the probe localization characteristics that allows image coregistration and validation that can be exploited for different uses. The three major categories of applications that have evolved for this emerging technology as well as pertinent examples of probe design are described in the following sections. As the field continues to mature, it is anticipated that novel targeting strategies, probe designs, and imaging applications will emerge in the future.

A. Validation of MRI Findings With Correlative Fluorescence Microscopy

In preclinical research and development applications, the ability to perform imaging with multiple modalities is useful in studying and validating probe activity and localization along with underlying biological processes. The pharmacokinetics and localization of molecularly targeted MRI-based probes can be imaged with sub-100-μm resolution in laboratory animals using small-animal MRI imaging systems. The resolution achieved sheds unparalleled insights into disease and tissue function but does not allow for a precise understanding of probe biodistribution and interaction with tissue at the cellular and subcellular level. Hybrid MR-fluorescent probes address this shortcoming by allowing noninvasive *in vivo* imaging of probe distribution and accumulation based on the MR contrast and subsequent invasive microscopic imaging, for example, using fluorescence endoscopy or confocal microscopy of surgically excised tissue samples.

For example, Frias *et al.* developed a dual-modality recombinant HDL-like nanoparticle for *in vivo* imaging of atherosclerotic plaques [20], [21]. Sample images from these works are shown in Fig. 5. The nanoparticle design consisted of phospholipid-based MRI contrast agents (Gd-DTPA-DMPE) as well as fluorescently labeled phospholipids (NBD-DPPE). During probe synthesis, both types of molecules became reconstituted into the nanoparticle, which measured approximately 9 nm in diameter. As an *in vivo* model for probe validation, apoE-knockout mice were used. This model has been widely used previously as a standard model for development of atherosclerotic plaques. As shown in Fig. 5(b), the probe was successfully imaged as a positive contrast agent using MRI, and the maximum contrast occurred 24 h after probe injection. Subsequently, *in vitro* fluorescence imaging was performed using confocal microscopy on sections of resected arterial tissue, although in principle the nanoparticle could be imaged *in vivo* in clinical applications using a fiber-optic-based intravascular probe. Using multichannel microscopy [Fig. 5(c)], it was

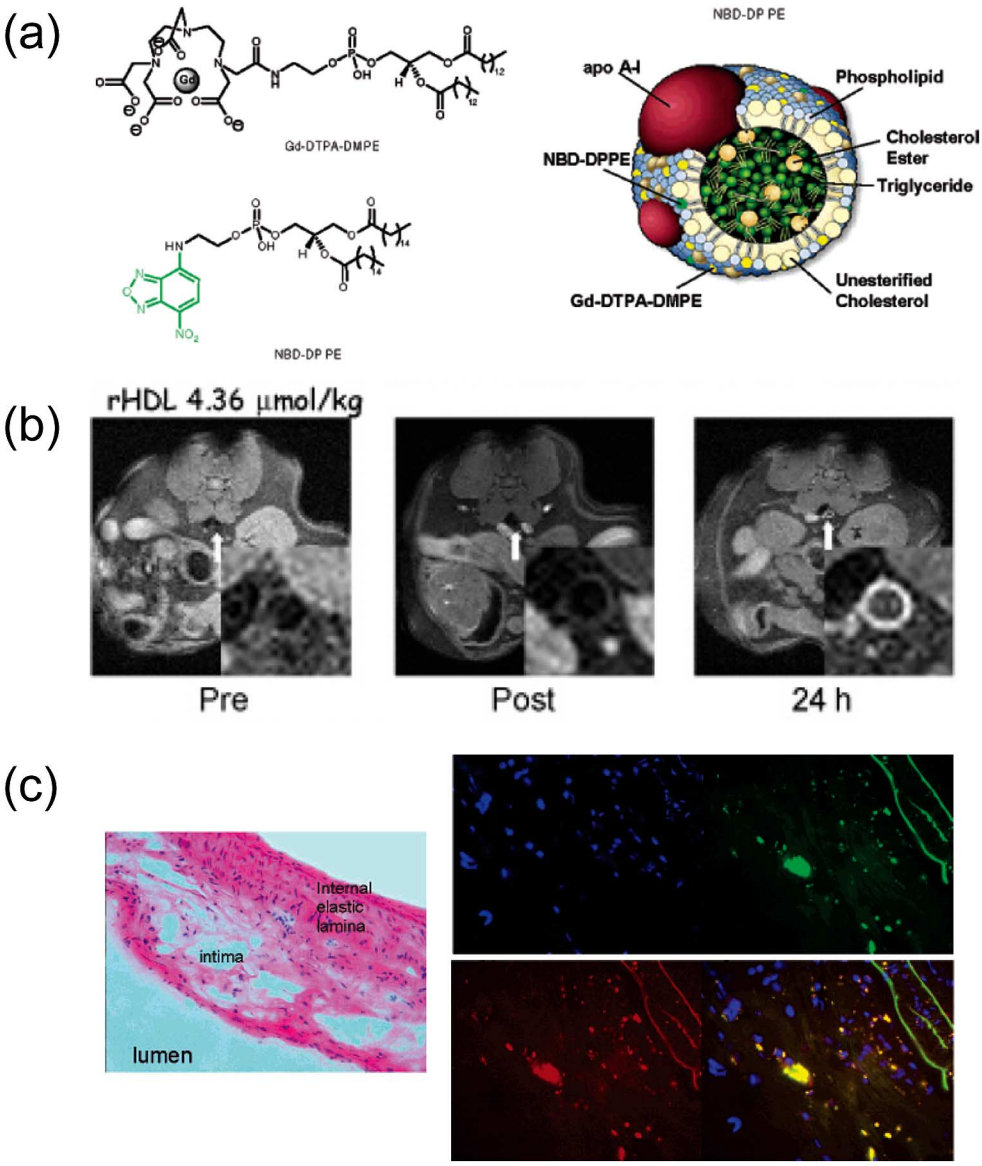

Fig. 5. ***(a) Recombinant HDL-like nanoparticle composed of phospholipid-based MRI (Gd-DTPA-DMPE) and fluorescent (NBD-DPPE) contrast agents. The nanoparticle was used for in vivo MRI imaging of artherosclerotic plaques in apoE-knockout mice (b). Aortas were removed 24 h after injection and imaged using confocal fluorescence microscopy (c). Hematoxylin and Eosin (H&E) staining is shown, as well as 4'-6-Diamidino-2-phenylidole (DAPI) staining (blue), nanoparticle fluorescence (green), and macrophage-specific antibody (red). Colocalization (yellow on merged image) of macrophage-antibody and nanoparticle fluorescence indicated macrophage uptake of the probe. Adapted from [20] and [21].***

verified that the nanoparticles colocalized strongly with a fluorescently labeled macrophage-specific antibody, in this case consistent with imaging of arterial plaques with high macrophage content. This work demonstrated the feasibility of noninvasively studying atherosclerosis based on the MR contrast of a molecular dual-modality nanoparticle and understanding the underlying microdistribution and probe uptake mechanism using fluorescence imaging.

Many other investigators have used dual-modality probes to merge noninvasive MR imaging with subsequent *in vitro* fluorescence microscopy of the same nanoparticle, with each imaging modality applied under different geometric and operating modes. For example, Shan *et al.* constructed a dual-modality liposomal-encapsulated nanoparticle that was surface-linked with transferrin ligands, since up-regulated levels of the transferring receptor has been associated with a number of human cancers [69]. The activity of the probe was validated by MR imaging of nude mice with human breast cancer (MDA-MB-231) xenografts. Probe uptake and cellular localization was also

verified with confocal fluorescence microscopy as well as whole-animal epifluorescence imaging. Similarly, Li *et al.* [70] labeled human low-density lipoproteins with PTIR267, a dual-modality MR-fluorescent contrast agent, and verified its operation in MR imaging of mice with a xenografted B16 melanoma tumor line and *in vitro* confocal imaging of labeled tumor cells. Evgenov *et al.* [71] used a dual-modality superparamagnetic ironoxide (SPIO)-Cy5.5 nanoparticle to label human pancreatic islets and performed longitudinal imaging studies with a small animal MRI scanner, as well as epifluorescence and confocal microscopic imaging of resected tissues. Numerous other studies have reported on novel probes, applications, and molecular targets, including dual-modality imaging of cells undergoing apoptosis (Annexin-V) in response to anticancer therapy [72], as well as labeling of the bombesin (pancreatic) cell surface receptor [73].

Other examples of interest include the work of Mulder *et al.* [74], [75], who demonstrated the use of dual-modality nanoparticles targeted to the $\alpha_v\beta_3$-integrin cell surface receptor, the up-regulation of which is implicated in angiogenic processes. In this work, the dual modality imaging capability was achieved using T_1-modulating gadolinium-based contrast agents and either fluorescein or CdSe/ZnS quantum dots. Wang *et al.* [76] similarly developed dual-modality $CdSe/Zn_{1-x}Mn_xS$ quantum-dot based nanoparticles and demonstrated T_1 contrast MRI and confocal fluorescence imaging of labeled mouse macrophage cells. Huh *et al.* [77] conjugated SPIO nanocrystals with fluorescently labeled Herceptin antibodies and demonstrated *in vivo* MR detection of breast cancer in mice, as well as subsequent fluorescence microscopy of excised tissue. Choi *et al.* [78] similarly utilized SPIO nanoparticles tethered with fluorescent isothiocyanate targeted to folate receptors and demonstrated their use in murine tumor models. Many other examples of the dual-modality nanoparticles have been described in the literature in recent years, but in all cases the ability to image with both fluorescence and MRI instruments has been demonstrated to be instrumental in investigating probe accumulation in target tissue, pharmacokinetics, diffusion from blood vessels, colocalization with immunohistochemical targets, etc. The resulting expanding library promises to continue to increase the applications and versatility of multimodality imaging in the future.

B. Interventional Imaging With MRI and Fluorescence

Dual-modality molecular probes are well suited to a number of important potential clinical applications. A particular emerging strategy is to utilize fluorescence *in vivo* imaging for intervention as a followup to MR examination. MRI-based contrast agents can be utilized for diagnostic and for surgical planning purposes due to the superior imaging resolution achievable in deep tissue. In cancer applications, for example, the ability of appropriate MR agents to outline tumor borders [79], [80] and aid in disease staging by identifying lymph-node involvement is particularly attractive [81]. In this case, the significantly improved sensitivity of fluorescence imaging relative to preoperative MR is well suited to real-time interventional or intraoperative imaging. One overall approach is therefore the use of preoperative MR imaging for tumor identification and staging followed by MR-guided fluorescence imaging, for example, for accurate removal of tumor borders, inspection for remnant invasive diseased tissue and locoregional metastatic foci (that are frequently not visible under white-light examination [82]), and interrogation and excision of lymph nodes including sentinel lymph nodes (SLNs).

A pertinent example is the identification of brain tumor margins by fluorescence imaging of a dual-modality nanoparticle as in the work of Kircher *et al.* [83], wherein the feasibility of the concept of preoperative MR and intraoperative fluorescence imaging of a CLIO-Cy5.5 probe was successfully demonstrated in animal models *in vivo*. Imaging of SLNs for staging of breast cancers is another important application for dual-modality probes [84]. For example, in the work of Koyama *et al.* [85], [86], the use of a magnetofluorescent nanoparticle—a dendrimer-based dual gadolinium-chelate and cyanine-5.5 probe—was investigated for MRI and fluorescence imaging of SLNs in preclinical animal models. It was demonstrated that the contrast agent accumulated efficiently in the SLN and could be imaged with MRI prior to surgery as well as with a planar fluorescence imaging system during surgical resection of the node. As expected, it was observed that while MRI could be used to detect the nanoparticle with higher three-dimensional accuracy in deep tissue, NIR fluorescence imaging could be used to detect the nanoparticle at significantly lower concentrations during surgery, illustrating the value of using information from the complementary modalities in a "treatment planning—guided surgery" concept.

Using a technique that could allow interrogation of a larger number of lymph nodes adjacent to the SLN, de Kleine *et al.* [87] similarly utilized the fluorescence-guided surgery approach to identify affected lymph nodes in mice with implanted breast cancer and fibrosarcoma cells. As shown in Fig. 6, mice were injected with a dual-contrast CLIO-Cy5.5 nanoparticle and imaged with an epi-illumination interventional system. Using this methodology, it was shown that lymph nodes could be easily identified in fluorescence mode and that cancer-positive lymph nodes exhibited lower probe uptake and less fluorescence than normal, unaffected lymph nodes. It is anticipated that the concept will help in more accurate cancer staging and treatment intervention in the future. Overall, the use of MR-guided *in vivo* fluorescence imaging intervention may find significant utility in future invasive procedures based on the dual-modality nanoparticle approach.

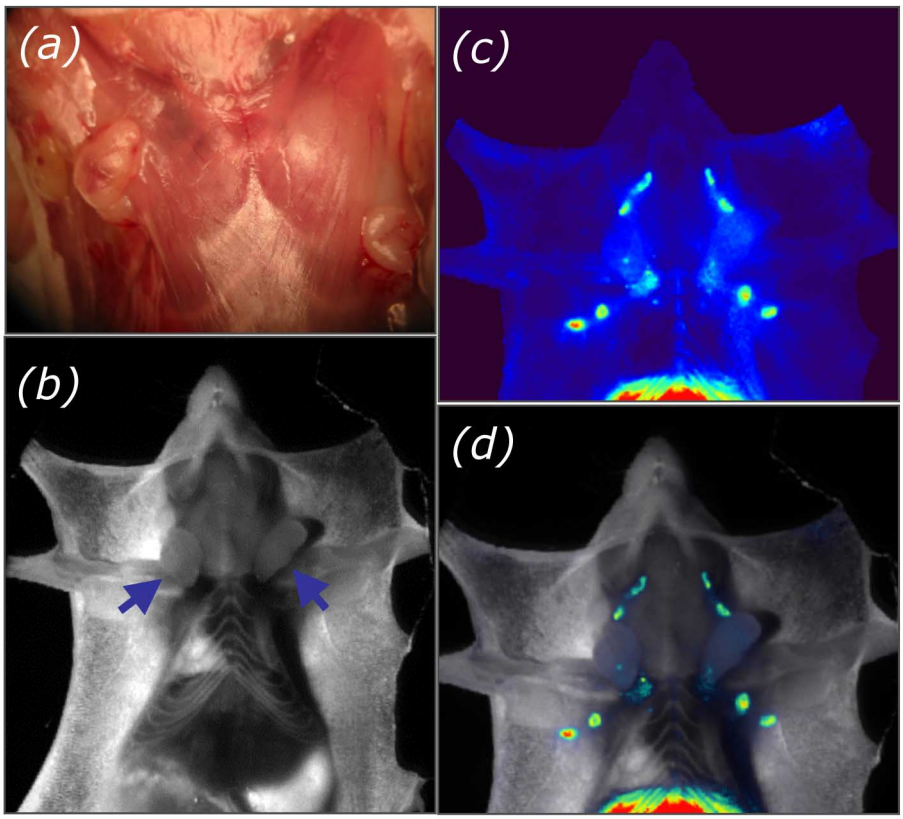

Fig. 6. ***Fluorescence-guided lymph-node resection in a mouse with two implanted HT-1080 fibrosarcoma tumors and injected with a CLIO-Cy5.5 dual-modality contrast agent. (a) White-light image of the exposed tumor bed, showing the surgeon's field of view. (b) Grayscale image of the mouse, showing location of the tumors (arrows). The location of the lymph nodes is not readily apparent. (c) Fluorescence image of the mouse, clearly showing the location of the lymph nodes. (d) Fluorescence white-light overlay image, which can be used by the surgeon to guide the intervention [87].***

C. Validation of Fluorescence Tomography Technology With MRI

Capitalizing on the ability of dual probes to report the same underlying activity with two different detection methods, fluorescently labeled CLIO nanoparticles have also been used to validate the performance of novel fluorescence tomography systems and methods against MR, the latter serving as the imaging gold-standard. This approach can serve as the most accurate way to validate developments and improvements in fluorescence imaging technology, specifically, the ability to spatially resolve fluorescence biodistribution *in vivo*.

For example, Montet *et al.* [88] used a dual-modality magnetofluorescent probe to validate a newly developed FMT system and methodology for imaging angiogenesis and treatment response *in vivo*. Imaging sessions were performed sequentially using MR and FMT on mice with either 9L gliosarcoma or MDA-MB-468 breast cancer cells implanted in the mammary fat pad. Better than 95% correlation was observed in imaging the tumor vascular volume fraction (VVF) between the two modalities, demonstrating FMT as an accurate method for visualization of VVF in tumors *in vivo*. In a similar study, Sosnovik *et al.* [9] used CLIO-Cy5.5 to investigate the appropriateness of using FMT to image cardiovascular disease versus MRI. It was demonstrated that surgically induced myocardial infarctions could be visualized by FMT, as was confirmed by MR imaging and correlative histology. As shown in Fig. 7, FMT images of the heart region in infarcted and control (sham surgery) animals demonstrated increased probe uptake and fluorescence in the infarcted mice, a finding confirmed *in vivo* by sequential MR imaging of the same nanoparticle as shown in Fig. 7(d) and (e); in this case the CLIO nanoparticle provided *negative* contrast. Validation of the probe microdistribution in this case was performed *in vitro* using microscopic analysis on excised tissue samples, as described in Section III-A, further revealing that probe uptake was primarily due to macrophage infiltration in the ischemic parts of the cardiac muscle.

IV. CONCLUSIONS

Fluorescence imaging is a powerful biomedical tool for noninvasive interrogation of biomedical systems at the

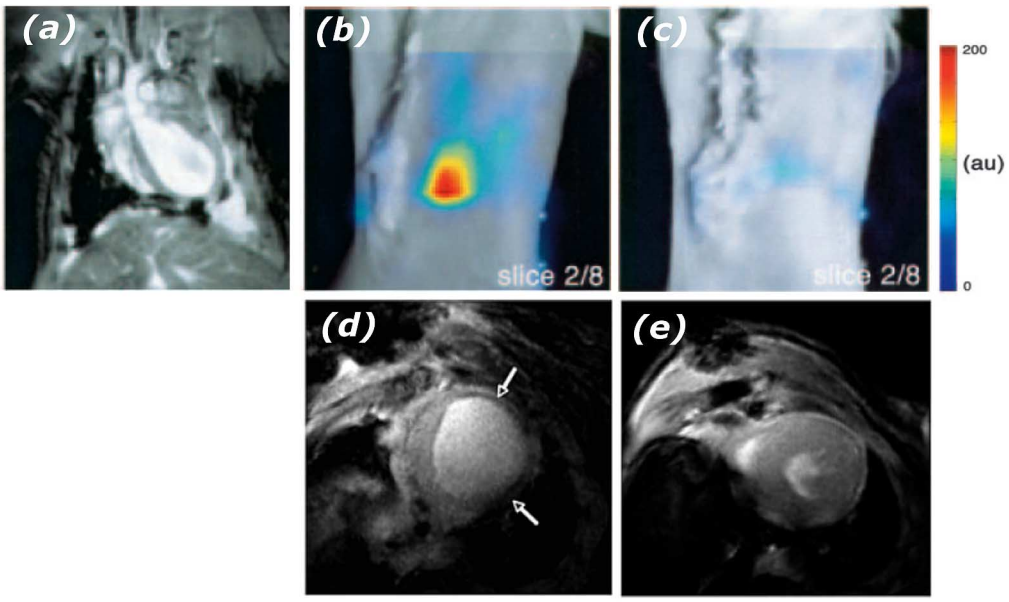

Fig. 7. ***Dual-modality imaging of mice with surgically infarcted myocardia and sham-surgery controls, injected with a CLIO-Cy5.5 based nanoparticle. (a) Coronal MRI slice of a mouse is shown, (b) corresponding to the FMT images of a mouse with an infarction as well as (c) a control showing accumulation of the probe in myocardial macrophages. MRI images with a 3.5 ms echo time are shown for (d) infarcted and (e) control mice. In the MRI case, probe accumulation is indicated by negative contrast around the anterolateral myocardium (arrows). Adapted from [9].***

cellular and subcellular levels. The advent of targeted molecular probes and tomographic fluorescence imaging techniques such as FMT have enabled three-dimensional whole-animal imaging of, for example, cell surface receptors, protease activity and gene expression *in vivo*. Magnetic resonance imaging allows exceptional high-resolution anatomical and functional imaging and, to a lesser extent, imaging at the molecular level. The multimodality imaging approach promises to extend the versatility of both MRI and fluorescence imaging by combining the information provided by each technique to yield significantly higher versatility than either modality versus standalone approaches. Simultaneous data acquisition using hybrid imaging systems allows accurate image coregistration and more meaningful interpretation of data, while inclusion of spatial priors into tomographic optical reconstructions can markedly improve the resolution achievable with fluorescence imaging in deep tissues. Nonetheless, the spatial and material constraints inherent in hybrid instruments can lead to implementations where data acquisition with each modality is performed suboptimally compared to standalone instruments, and therefore special care must be taken in the integrated system design versus a sequential operation strategy with optimized systems.

Multimodality targeted molecular probes give a new perspective in hybrid MR-fluorescence imaging by enabling accurate imaging using distinct modes and geometries, by means of the common contrast imparted by the probe. In preclinical research applications, validation of MR findings at the cellular and subcellular levels can be accomplished by combining MR imaging with fluorescence microscopy applied invasively or on excised specimens. Moreover, MR-guided fluorescence-enhanced intervention offers significant advantages during surgical procedures facilitated by the ability to perform parallel multimodality imaging at different physical scales, including MRI, macroscopic fluorescence imaging, and confocal and two-photon microscopy, with the optical methods more accurately relating to the surgeon's vision. Finally, validation of new fluorescence macroscopic imaging methods against MRI can be accurately achieved using dual- or multimodality probes. The many different implementations and applications of hybrid fluorescence imaging demonstrate an emerging and highly promising area of imaging. ■

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