Wide-field in vivo background free imaging by selective magnetic modulation of nanodiamond fluorescence

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Abstract: The sensitivity and resolution of fluorescence-based imaging in vivo is often limited by autofluorescence and other background noise. To overcome these limitations, we have developed a wide-field background-free imaging technique based on magnetic modulation of fluorescent nanodiamond emission. Fluorescent nanodiamonds are bright, photo-stable, biocompatible nanoparticles that are promising probes for a wide range of in vitro and in vivo imaging applications. Our readily applied background-free imaging technique improves the signal-to-background ratio for in vivo imaging up to 100-fold. This technique has the potential to significantly improve and extend fluorescent nanodiamond imaging capabilities on diverse fluorescence imaging platforms.

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1. Introduction

Fluorescent nanodiamonds (FNDs) containing negatively charged nitrogen-vacancy (NV−) centers are promising optical imaging probes. The NV− center is a defect in the diamond lattice consisting of a substitutional nitrogen and a lattice vacancy that form a nearest-neighbor pair [1, 2]. NV centers are fluorescent sources with remarkable optical properties including indefinite photo-stability, broad excitation and emission spectra in the visible and near infrared range [3, 4], and magnetic field-dependent fluorescence emission [5, 6]. The extraordinary optical properties of FNDs have been harnessed for extended dynamic intracellular tracking in HeLa cells [3, 7], for long-term tracking within the nematode
Caenorhabditis elegans including following the transfer of FNDs from one generation of organism to the next [8], and for long-term imaging in mice [9]. These and other studies also demonstrated that FNDs are biocompatible imaging probes that do not produce significant short- or medium-term adverse effects in cultured cells, nematodes, or mice [7–10]. More recently, nanodiamonds grafted with chemotherapeutic agents have been shown to be effective, biocompatible drug delivery particles that increase therapeutic effectiveness by overcoming drug efflux [11]. These studies suggest that FNDs could be developed into dual use therapeutic and diagnostic, or theranostic, particles. Whereas these examples illustrate the potential of FNDs for in vivo optical imaging, a significant limitation of this potential is the difficulty of exciting and detecting FND emission through skin and tissue due to autofluorescence.

Autofluorescence from naturally occurring fluorescent biomolecules limits in vivo fluorescence imaging below ~700 nm because of overlap between the fluorescent probe and background emission spectra [12, 13]. Background fluorescence generally decreases at longer wavelengths in the near infrared region (~700-1000 nm), facilitating imaging in this portion of the spectrum [12]. Because the majority of the FND emission spectrum falls below 700 nm, there is significant background for in vivo imaging that is difficult to eliminate with existing methods. Spectral unmixing [14] techniques that decompose fluorescence emission into a linear superposition of reference spectra cannot always reliably and accurately separate signal from background fluorescence. Fluorescence lifetime imaging techniques [15] that distinguish signal from background based on fluorescence lifetime differences are limited if the lifetimes are similar. They also require sophisticated excitation and detection instrumentation. Phase-sensitive or lock-in detection is a well-known signal recovery technique that dramatically improves the signal-to-background ratio [16, 17]. However, this technique requires selective modulation of the imaging probe emission, which has limited progress in using this technique for imaging applications. Magnetically modulated optical nanoprobes (MagMOONs) have been developed with optical emission that can be modulated by a rotating magnetic field [18]. Optically modulated emission of nitrospirobenzopyran-based chemical probes, Dronpa [19], and Cy5-Ag nanodot systems [20] has also been used in combination with lock-in detection to improve the signal-to-background ratio. However, these probes suffer from intrinsic intensity fluctuations, i.e. blinking, and/or photobleaching, both of which limit their use in imaging applications.

Here, we report the implementation of wide-field background-free imaging through magnetic modulation of FND emission combined with computational phase-sensitive signal recovery techniques that improve the signal to background ratio up to 100-fold. Background-free imaging has been recently demonstrated in vivo employing microwave modulation of FND emission [21], and for isolated diamonds in a point-scanning configuration employing magnetic modulation of FND emission [22]. We developed a wide-field magnetic modulation background-free imaging scheme and demonstrate in vivo background-free detection of FNDs by imaging sentinel lymph nodes in mice. We also implemented a phase-sensitive detection scheme based on pixel-by-pixel analysis of wide-field images as an alternative to image subtraction schemes employed previously [21, 22].

Imaging the lymphatic system is of medical interest because it is a route for vaccine delivery [23] and is altered in diseases related to infection, blockage, and cancer [24]. Evaluating the presence of metastasis in the primary draining or sentinel lymph nodes (SLNs) from a tumor basin informs the prognosis and therapy of carcinomas [23].

2. Materials and methods

2.1. Magnetic modulation of FND emission

Synthetic type Ib FNDs (ND-15NV-40nm and ND-NV-100nm, Adamas Nanotechnologies) at concentrations of 1 mg/ml were mixed with 1 mg/ml poly-L-Lysine. The mixture was
pipetted onto a clean quartz slide (SPI 25 mm x 76 mm x 1 mm) and incubated overnight. Excess solution was rinsed with DI water. A flow cell was made with double-sided tape (SA-S-1L 0.12 mm Secure-Seal adhesive double sided tape, Grace BioLabs) sandwiched between a clean coverslip (Gold Seal 22 mm x 40 mm No.1) and the FND-coated quartz slide. The flow cell was filled with DI water. Scanning confocal images were acquired with a Carl Zeiss LSM5 LIVE microscope with an acquisition time of 250 ms per image. A 10X Zeiss objective with 0.3 NA (EC Plan-Neoflura) was used to excite the FNDs and collect FND emission. Samples were excited at 532 nm, emission was filtered using a long pass filter LP650 and detected with a photomultiplier tube (PMT). An electromagnet (APW Company, catalog# EM400-12-212, 4.0” Diameter Round Electromagnet) was energized with a 12 V, 1.6 A square wave. The flow cell was placed ~13 mm away from the magnet face where the peak magnetic field strength was ~100 Gauss.

2.2. Silica coating of nanodiamonds

Previously published procedures were followed to stabilize nanodiamonds with a silica coating [25]. Briefly, 10 mg (0.4 mL) of phospholipid 16:0-18:1 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) (Catalog#: 850457C, 25 mg/mL in chloroform, Avanti) was dried under a nitrogen stream to form a thin layer on the walls of a glass vial and further dried under vacuum desiccation for 45 min. 100 nm type Ib fluorescent nanodiamonds (Catalog#: ND-NV-100nm, 1mg/mL suspension, Adamas Nanotechnologies) were sonicated in a water bath for 30 min. 2.5 mL of 1% tetraethylorthosilicate (TEOS) (Catalog#: 333859, Sigma-Aldrich) in ethanol was added to 2.5 mL of the FND solution and immediately transferred to the glass vial with POPC. The phospholipid was re-suspended using a water bath sonicator for 10 min. 7.5 µL of triethylamine (TEA) (Catalog#: T0886, Sigma-Aldrich) was added to the reaction to catalyze silanization. The solution was then ultrasonicated (Model: Ultrasonic Cleaner, Laboratory Supplies Company Inc.) for 40 min and free TEOS and TEA were removed by dialysis in DI water with a molecular weight cut-off of 100 kDa (Catalog#: G235059, Spectrum Laboratories) and multiple changes of water over a period of 48 h. To dissolve the liposomes, 10% SDS (500 µL) was added and the solution was sonicated in a water bath for 2 h. Dialysis was repeated to remove dissolved POPC and SDS.

2.3. Sentinel lymph node imaging

Female athymic (nu/nu) mice were purchased from Charles River Laboratories at 4–6 weeks of age and housed in a specific pathogen-free American Association for Laboratory Animal Care approved facility. All experiments were approved by the National Cancer Institute’s Animal Care and Use Committee. Mice were anesthetized using gas mixtures of 1.5-2.5% isoflurane in O2 to maintain a respiration rate of ~30 bpm during the injection procedure. A volume of 10 µL of an ~80 mg/mL silica-coated nanodiamond (~100 nm diameter) solution in PBS (pH7.4) was intradermally injected into the front foot pad of each mouse. Previous studies have shown that the primary draining lymph nodes (LNs) from this injection site are the axillary and lateral thoracic LNs [23, 26]. At 24 h post-injection, the mice were sacrificed and optical imaging was performed using a Maestro CRi spectroscopic optical camera (excitation filter 523 nm, emission filter 675 nm long pass). Spectral unmixing of the FND and background signals was performed following the Maestro protocol. Briefly, spectral libraries were captured for the FNDs and the tissue autofluorescence by recording individual spectral curves of isolated FNDs and mouse skin independently. A series of images of the sample containing FNDs were then captured at specific wavelengths covering the emission range of the FNDs, generating an image cube. Proprietary spectral unmixing algorithms employing the previously recorded spectral libraries were then run on the image cubes to generate spectrally unmixed images.

Images were also taken with magnetic modulation in a UVP BioSpectrum Imaging System equipped with a BioLite MultiSpectral Light Source (excitation filter 525 nm,
emission filter 650 nm long pass) with additional illumination provided by a 532 nm laser (Z-Bolt, #DPSS-100) achieving ~1 mW/cm² intensity. A 3” diameter × 1/2” thick permanent magnet (Catalog# DZ08-N52, K&J Magnetics) was placed directly under the non-metallic stage containing the mouse. The field strength in the center of the sample was ~1000 G with a ~47 G/mm field gradient. Magnetic field modulation was accomplished by mechanically moving the magnet, resulting in 100% modulation depth, i.e., 0 to ~1000 G. Each mouse was imaged in the instrument on the non-magnetic stage under which the permanent magnet could be slid in and out. Series of images were captured with and without an applied magnetic field. Individual images were assembled into synthetic movies in ImageJ for subsequent analysis. This process assures absolute synchronization of the magnetic field modulation with the movie frames.

2.4. Motion correction of movies

Any movement or drift of the stage, mouse, or detector leads to changes in pixel values between image frames and introduces noise which can be as high as 100% if the movements are more than few pixels between frames. To correct for these errors, motion correction was applied to the time series of images to correct for in-plane motion at the sub-pixel level. A non-rigid deformation map was calculated pairwise between each image and a common reference image using an optical flow method, which iteratively maximized the local cross-correlation image subsets at different resolutions [27, 28]. The initial image (frame 0) was chosen as a common reference for both image series acquired with external field ON and OFF ensuring that the ON and OFF images were co-registered. A sub-pixel spline based interpolation was used for application of the non-rigid deformation to minimize loss of spatial resolution. Following motion correction, the time series of images were averaged to reduce random fluctuations due to noise thereby creating average ON and OFF images. A difference image between ON and OFF motion corrected averages was used to analyze the contrast of the probe signal by subtraction of the background tissue. This signal from the nanodiamond was then overlaid on the initial image (Fig. 4). The temporal intensity fluctuations were dominated by laser intensity fluctuations, which could be as high as 10%. Drift and motion artifact was a combination of slow stage drift, on the order of pixels, and small pixel-scale deformations of the mouse during imaging.

2.5. Computational lock-in detection

Synthetic lock-in post-processing of modulated diamond emission images was implemented in Matlab. Time series corresponding to the pixel values of the synthetic movies were multiplied by a sine wave with the same frequency as the modulation of the applied magnetic field. The magnitude of the temporal Fast Fourier Transform (FFT) of the resultant values was calculated (Fig. 4). Multiplication of sine waves of the same frequency (f) results in signals at the sum (2f), and difference (0) frequencies. Either signal can be used for lock-in detection. A clear peak was observed at 2f, and the amplitude of this peak averaged over three points of the FFT centered at 2f was calculated. To eliminate the effects of any potential phase shift between the signal and reference signals, the process was repeated with a cosine reference wave. The square root of the sum of the squares of the FFT magnitudes obtained from the sine wave and cosine wave multiplications was calculated for each pixel to obtain the final lock-in image. Averaging the amplitude of the FFT over a small frequency range is equivalent to temporal averaging over a period of time approximately equal to the reciprocal of the frequency range.

2.6. Signal-to-background ratio calculations

For in vitro measurements, the background consisted of non-specific fluorescent molecules (Fig. 2(a) and 3) or from Alexa 647 dye molecules (Fig. 2(b)). For in vivo measurements (Fig. 4) the background consisted of intrinsic fluorescence from the tissue and skin. The signal-to-
background ratio was calculated as the ratio of the total counts in 2 × 2 pixel regions centered on a nanodiamond and at a location without nanodiamonds. For both Figs. 2 and 3, the positions of the boxes are same before and after the image processing to determine the improvement in the signal-to-background ratio. In the animal images, the average intensity of ten 4 × 4 pixel boxes drawn over the lymph node regions containing signal were compared to the average intensity of ten 4 × 4 pixel boxes drawn over skin background signal regions.

3. Results and discussion

The fluorescence emission of FNDs was modulated by exploiting the energy level structure of fluorescent NV− centers (Fig. 1(a)). Excitation and emission pathways for an NV− center can be described by a triplet ground state ($m_s = 0$, $m_t = \pm 1$), a triplet excited state ($m_s = 0$, $m_t = \pm 1$), and a cascading pair of metastable singlet states [1, 2, 6, 29]; where the spin quantum number $m_s$ is quantized along the N–V symmetry axis. The degeneracy of the spin sublevels is lifted due to spin-spin and spin-orbit interactions in addition to crystal strain. Optical transitions between the ground and excited states preserve the spin state, i.e., $\Delta m_s = 0$ (Fig. 1(a)). However, an NV− center in the excited state spin sublevels $m_s = \pm 1$ can undergo a non-radiative transition to the ground state spin sublevel $m_s = 0$ via the singlet metastable state pathway (Fig. 1(a)). When the FND is excited, this non-radiative decay pathway effectively pumps NV− centers into the $m_s = 0$ spin state, which corresponds to the maximum emission intensity. Application of an external magnetic field lifts the degeneracy of the $m_s = \pm 1$ levels and, more importantly, mixes the $m_s = \pm 1$ and $m_s = 0$ states if the external magnetic field is not perfectly aligned with the N-V symmetry axis [30]. As a result of the mixing of the spin states, some of the excited state centers will be in the $m_s = \pm 1$ spin sublevel and a fraction will decay via the non-radiative pathway, decreasing the fluorescence from the NV− centers. Therefore, the fluorescence emission of the NV− centers decreases in the presence of a magnetic field and returns to its maximum intensity when the field is eliminated. This process can occur on a time-scale of 0.5 µs or less [31].

To measure the magnetic-field induced change in NV− center fluorescence, FNDs were immobilized on a slide and imaged with 532 excitation and a 650 nm long pass emission filter in a scanning confocal microscope (Zeiss LSM 5 LIVE) (Fig. 1(b)). In the presence of a ~100 Gauss magnetic field modulated with a 0.1 Hz square wave, the fluorescence of a typical FND was modulated by ~6% (Fig. 1(c)).
Fig. 1. Magnetic modulation of FND emission. (a) Energy level diagram of NV\(^{-}\) centers in diamond showing spin-triplet \((m_s = 0 \text{ and } m_s = \pm 1)\) ground and excited states as well as the pair of singlet metastable states [1, 31]. NV\(^{-}\) centers can be optically excited over a broad range of wavelengths (450-650 nm) as long as the \(\Delta m_s = 0\) condition is satisfied between the two states (green arrows). NV\(^{-}\) centers in the \(m_s = \pm 1\) sublevels of the excited states have a higher probability to decay via the metastable states [31] (grey dashed arrows) than to the \(m_s = \pm 1\) sublevels of the ground state. From the metastable state pathway, NV\(^{-}\) centers predominantly transition to the \(m_s = 0\) sublevel of the ground state without emitting visible light. Therefore, in the absence of a magnetic field, NV\(^{-}\) centers are rapidly pumped into the \(m_s = 0\) sublevel of the ground state when excited. This results in an initial increase in fluorescence emission intensity as steady state is reached. In the presence of a magnetic field, the \(m_s = 0\) and \(m_s = \pm 1\) states are mixed, making the decay pathway through the metastable singlet state accessible and therefore decreasing the fluorescence emission intensity. (b) A scanning confocal image of FNDs with an average diameter of 40 nm containing \(\sim 15\) NV\(^{-}\) excited with \(\sim 100\) W/cm\(^2\) at 532 nm. (c) Intensity modulation of the FND enclosed in the box in Fig. 1(b) from application of a \(\sim 100\) G magnetic field with 0.1 Hz square wave (0-100 G amplitude) modulation.

The most straightforward method to exploit magnetic field modulation of FND fluorescence to improve the image signal-to-background ratio is to subtract images with the magnet field on from images with the magnet field off. The small difference in FND emission
will result in a positive signal in the subtracted image. The background fluorescence will not be affected by the magnetic field, thus background will be effectively eliminated in the subtracted image. Averaging over several subtracted images will further reduce background fluctuations that nominally have a zero mean. To demonstrate this process, we first imaged FNDs affixed to a slide (Fig. 2(a)). A ~1 µM Alexa647 dye solution was then introduced into the flow-cell, which generated sufficient background to obscure the FNDs (Fig. 2(b)). 1000 pairs of images with and without a ~100 Gauss magnetic field were then captured and pairwise subtracted. The FNDs are clearly visible in the processed image representing the average of 1000 subtracted images (Fig. 2(c)). To quantify the degree of background rejection, we computed an effective signal-to-background ratio in each image as the ratio of the total diamond intensity in a defined area (2x2 pixels) to the total background intensity in the same area at randomly chosen locations in the image (Methods). The signal-to-background ratio defined in this manner was 9 in the original image, decreased to 1 in the presence of the dye solution, and increased to 12 after processing of the magnetically modulated images collected in the presence of dye.

![Image](image_url)

**Fig. 2.** Background-free imaging via magnetic modulation of FND emission. (a) A field of view with ~40 nm FNDs each containing ~15 NV− imaged as in Fig. 1(b). (b) Image of the same field of view after introducing ~1 µM Alexa647 dye solution into the flow cell. (c) Image of the same field of view after processing images in the presence of the Alexa647 background signal (as in part b). The difference between pairs of images collected with and without the magnetic field was computed and 1000 of these difference images were averaged together to generate the processed image. Through this processing, images of the diamonds shown in Fig. 2(a) are recovered from images like Fig. 2(b) with high background.

Lock-in detection is a sensitive phase-sensitive technique to recover a weak modulated signal from background that can exceed the signal by orders of magnitude [16]. Typically, lock-in detection is performed in real-time on the output of a point detector using a lock-in amplifier. Motivated by lock-in amplification techniques and the high background rejection they afford, we developed a detection scheme for wide-field images that incorporates the same principles in a software-based approach. The scheme relies on post-processing to computationally perform the equivalent of lock-in amplification on the intensity time series for each pixel in a movie (Methods). To demonstrate this process, we recorded a 1000-frame movie at 0.25 s per frame with a laser scanning confocal microscope (Fig. 3(a)). A ~100 G magnetic field with 0.1 Hz square wave modulation was applied during acquisition of
Fig. 3. Background-free imaging using wide-field lock-in detection. (a) One frame of a 1000 frame movie recorded at 0.25 s per frame with a scanning confocal microscope. A ~100 G magnetic field modulated at 0.1 Hz was applied during acquisition of the movie. (b) Time series of the pixel values corresponding to a FND (Red arrow in a) shows fluorescence modulation. (c) Time series of the pixel values corresponding to background (blue arrow in a) shows no fluorescence modulation. (d) Magnitude as a function of frequency of the fast Fourier transform (FFT) of the pixel value time series in b. Note the peaks at 0.1 and 0.3 Hz. (e) The magnitude as a function of frequency of the FFT of the pixel value time series in d. (f) FFT magnitude of the pixel value time series in b multiplied by a reference sine wave, $1 + \sin(2\pi \cdot 0.1 \cdot t)$. Note the prominent peak at 0.2 Hz, corresponding to twice the modulation frequency. (g) FFT magnitude of the pixel value time series in c multiplied by a reference sine wave, $1 + \sin(2\pi \cdot 0.1 \cdot t)$. Grey vertical lines at 0.2 Hz indicate the values at twice the reference frequency. This process was repeated by multiplying the pixel values by a reference cosine, $1 + \cos(2\pi \cdot 0.1 \cdot t)$. The mean of three points around 0.2 Hz in the sine and cosine FFT amplitudes was calculated for each pixel and added in quadrature, i.e., $I = \sqrt{I_{\sin}^2 + I_{\cos}^2}$. (h) The resulting wide-field background-free image. Means of the pixel values over 1000 frames before applying the lock-in algorithm are 173 and 18 for the pixels corresponding to b and c respectively, corresponding to a signal-to-background ratio of ~10. Means of the FFT amplitudes for three points around 0.2 Hz are 21.42 and 0.28 for the pixels corresponding to b and c after applying the lock-in algorithm corresponding to a signal-to-background ratio of ~77. The movie. Pixels within the point spread function of an FND showed intensity modulation with the same frequency as the applied magnetic field (Fig. 3(b)), whereas pixels outside the point spread function of an FND showed no intensity modulation (Fig. 3(c)). Fourier transforms of the of pixel intensity time series confirmed these results (Figs. 3(d), 3(e)). Pixels corresponding to FNDs exhibited peaks at the fundamental (0.1 Hz) and odd harmonics (0.3 Hz) of the modulation frequency, as expected for square wave modulation, whereas other pixels did not exhibit these peaks (Fig. 3(e)). To reduce background noise in the image we performed computational lock-in detection. For each pixel, the intensity time series was multiplied with a reference sine or cosine wave with the same frequency as the applied magnetic field. Periodograms (magnitude of the FFT) of the resultant time series are shown in Figs. 3(f) and 3(g). The amplitudes of the periodograms at 0.2 Hz for both sine and cosine reference waves were used to calculate the square root of the sum of the squares of the intensities at each pixel, $I = \sqrt{I_{\sin}^2 + I_{\cos}^2}$, to eliminate the effects of possible phase offsets.
between the modulation and reference signals. The resulting wide-field background-free image is shown in Fig. 3(h). The signal-to-background ratios before (Fig. 3(a)) and after (Fig. 3(h)) lock-in processing are 12 and 50 respectively.

Fig. 4. *In vivo* imaging of sentinel lymph nodes. (a) FNDs were injected into the front footpad of a mouse and imaged using spectral unmixing methods to separate the emission of the FNDs (top right, red in overlay) from background fluorescence (top left, white in overlay). For comparison, a raw unprocessed image from the Maestro imager is shown in the inset in the overlay. FNDs were not detected through the skin in the draining axillary lymph node. (b) The same mouse imaged by averaging 475 images obtained by pairwise subtracting images with and without the magnetic field. The processed image (top right inset, red in overlay) was overlaid on an unprocessed image obtained with the magnetic field off (top left inset, white in overlay). The white arrows point to the injection site in the footpad and the location of the auxiliary lymph node. Signal from the FNDs in the lymph node is clearly detected. (c) Lock-in detection of emission from FNDs from the same images used to generate Fig. 4(b) using the lock-in algorithm described in Fig. 3 (top right inset, red in overlay) overlaid on the unprocessed image obtained with the magnetic field off (top left inset, white in overlay). (d) Magnetic modulation image of the same mouse’s open chest cavity by averaging images obtained by pairwise subtracting images with and without the magnetic field. The processed image (bottom inset, red in overlay) was overlaid on an unprocessed image obtained with the magnetic field off (top inset, white in overlay). The white arrows point to the injection site in the footpad and the location of the auxiliary lymph node. (e) Lock-in detection of emission from FNDs from the same images used to generate Fig. 4(d). The lock-in signal (bottom inset, red in overlay) is overlaid on the unprocessed image obtained with the magnetic field off (top inset from panel d, white in overlay). The top inset is a contrast enhanced version of the unprocessed image in panel d. The gross anatomy of the mouse (front footpad on the right) and open chest cavity can be discerned. In the partially dissected mouse, localization of the FNDs to the lymph node can be clearly seen. (f) The pixel values as a function of time corresponding to the selected points in b and d. The pixels selected were over 1) the axillary lymph node and 2) a negative control on the skin in Fig. 4b; 3) the axillary lymph node and 4) a negative control on a rib in Fig. d. Signal modulation as a result of the applied magnetic field is clearly visible in the lymph node through the skin, as well as when the chest cavity was opened. Meanwhile, the skin and rib showed random signal as expected for the negative control.
To demonstrate the application of background-free FND magnetic modulation imaging in vivo, we imaged sentinel lymph nodes in intact mice. Mice were injected in the front foot pad with silica-coated FNDs and imaged to determine if the particles drained to the proximal axillary SLNs. Spectral unmixing is commonly used to extract the signal from autofluorescence and other background fluorescence [32, 33]. Through this method, the nanodiamond signal was detected at the injection site, but the signal was too weak to be detected through the skin in the region near the lymph node (Fig. 4(a)). The same mouse was imaged with magnetic modulation and a strong signal was detected at the lymph node through the skin both with the pairwise subtraction method (Fig. 4(b)), and the computational lock-in method (Fig. 4(c)). Imaging the open chest cavity confirmed that the FNDs had accumulated in the lymph nodes (Fig. 4(d)-4(e)). Magnetic modulation of tissue containing FNDs showed distinct modulation of the fluorescence signal (Fig. 4(f) – 1 and 3), whereas tissue without FNDs did not (Fig. 4(f) – 3 and 4). These results demonstrate significant improvement over current optical in vivo imaging techniques in recovering the FND fluorescence single from the background. The lymph node that was undetectable using spectral unmixing methods was visible through the skin by both magnetic modulation-based methods. The signal-to-background ratios were 3 in the spectrally unmixed images (Fig. 4(a) red), 1 in the unprocessed magnet off images, 105 using pairwise subtraction of images with and without magnetic field (Fig. 4(b) red), and 89 using computational lock-in detection of FND emission (Fig. 4(c) red), an increase of 38- and 31-fold for Figs. 4(b) and 4(c) over Fig. 4(a), and an increase of ~100-fold in comparison to the unprocessed images collected in the absence of the magnetic field. There is a notable difference between the images generated by pairwise subtraction versus computational lock-in detection. Both methods, though the lock-in method in particular, are sensitive to motion or drift of the sample during the acquisition of the movies. This drift likely accounts for the reduced signal in the lock-in images, particularly at the injection site in the mouse forepaw, which is likely more prone to move during the measurement, in Figs. 4(c) and 4(e). Whereas the results presented demonstrate the principle of these methods, improvements in image quality and signal-to-background ratios can be made with faster image collection, higher field strength, higher dynamic range cameras, and a greater number of images collected per movie.

Background-free imaging achieved by selective magnetic modulation of FND fluorescence (Fig. 1) combined with computational phase sensitive detection processing permits sensitive in vivo imaging that overcomes many of the challenges and limitations arising from background fluorescence. Background-free imaging of FNDs can be implemented by subtracting an image acquired in the presence of a magnetic field from one without a magnetic field (Fig. 2). The signal-to-background ratio can be further improved by averaging subtracted images. A similar approach was recently demonstrated for isolated FNDs [22] in agreement with our in vitro results. Sample drift would degrade the average over many pairwise subtracted images, but these artifacts could, in principle, be removed through drift correction. One drawback of this approach is that it requires the excitation illumination intensity and emission collection efficiency to remain constant over pairs of images. Changes in either parameter and, more generally, any artifactual differences between image pairs would lead to spurious signals in the subtracted images. The discrete nature of the subtraction and averaging process suggests that on the fly processing and averaging could be implemented. In this scenario, pairs of images could be subtracted and used to generate a running average image that could be updated in real-time. This implementation would permit dynamic and immediate evaluation of the imaging performance that would be relatively simple to implement.

Background-free imaging based on the magnetic modulation of FND fluorescence can also be implemented with techniques based on lock-in detection. For wide-field imaging, the lock-in method can only be implemented in a post-processing stage after the movie has been acquired (Fig. 3). For a point detector such as a photomultiplier tube or avalanche photo...
detector used in a confocal imaging mode, the lock-in method could be implemented at each pixel in real time with a lock-in amplifier. Implementing this would require much faster magnetic modulation, which previous measurements suggest is feasible [21, 34]. Despite the nominal superior performance of lock-in methods, the pairwise detection method gave slightly better results in our hands (Fig. 4). As mentioned above, the lock-in technique is likely more sensitive to motion and drift of the sample, which may have degraded the images somewhat, particularly during the acquisition of long movies comprising long exposure times. Degradation of the lock-in signal by motion artifact during imaging is likely the cause of the significantly lower apparent intensity at the point of injection in the lock-in images (Fig. 4(c) and 4(e)). The forepaw where the FNDs were injected is less rigid than the chest cavity of the mouse where the SNLs are located. Over the course of imaging small uncorrected motion of the paw would lead to a significicant degradation of the lock-in signal, but a more modest degradation of the pairwise subtraction signal. Faster modulation and image acquisition will likely result in further gains in the signal-to-background ratio achieved with the lock-in method.

Despite the slightly better performance of the pairwise subtraction method, the lock-in technique offers several potential advantages. Since the lock-in process is sensitive to the frequency of the signal, it is relatively robust to changes and inhomogeneities in the illumination or detection efficiency. Furthermore, since the lock-in technique relies on modulation of the magnetic field, rather than switching it on and off, as a practical matter it is easier to increase the sinusoidal modulation frequency of the magnetic field suitable for lock-in detection than the square wave modulation required for pairwise subtraction.

The background free methodology we developed relies on mixing the \( m_s = 0 \) and \( m_s = \pm 1 \) spin states by applying a magnetic field. In principle, any method that transiently mixes the 0 and ± 1 spin states, thereby allowing excited NV\(^-\) centers to decay through the non-radiative pathway via the metastable states, can be used to modulate the FND emission. For example, the ground state spin sublevels \( m_s = 0 \) and \( m_s = \pm 1 \) can be resonantly driven with radio frequency (RF) radiation. The resulting oscillations between the spin sublevels effectively mixes them resulting in excited state decay via the non-radiative pathway and the concomitant decrease in fluorescence intensity. This approach has been used for background-free imaging [21] but requires a microwave source that complicates the experimental design and results in potentially damaging heating effects, limiting in vivo applications. Furthermore, it is difficult to produce a wide-field RF signal with enough power to produce sufficient modulation of the FNDs, particularly deep in tissue. In contrast, magnetic fields of several Tesla are routinely used in whole body MRI imaging. The magnetic modulation approach that we developed here is an alternative means of background-free FND imaging that is more straightforward to implement, particularly for wide-field imaging.

4. Conclusion

We have reported two methods to achieve background-free imaging of magnetically modulated FNDs. One method relies on simple subtraction of images with and without a magnetic field. The other is phase sensitive lock-in detection of FNDs emission modulation for which smaller magnetic fields and faster modulation rates can be used. These simple and relatively straightforward approaches to achieve background-free imaging of FNDs described can be readily incorporated into existing microscopes or animal imaging systems. The efficient penetration of magnetic fields through tissues and the relatively small magnetic fields required for the background-free imaging make it suitable for biomedical imaging. For many applications such as animal imaging or histology, the acquisition time is less important than sensitive background-free imaging. The dramatic increase in background rejection afforded by this technique has the potential to extend existing FND based measurements and possibly open up new applications that would benefit from improved signal-to-background measurements. In particular, the application of background-free imaging can likely extend the
tissue depth at which FNDs can be usefully imaged \textit{in vivo} by improving the rejection of background fluorescence and improving the detection of weak FND fluorescence. Similar considerations would enable imaging of smaller, and therefore less bright, FNDs that are potentially more easily dispersed and compatible \textit{in vivo}. More generally, these background free techniques could be applied to many of the FND based imaging modalities that have already been developed.

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