Video Article

In vivo ^{19}F MRI for Cell Tracking

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Abstract

In vivo ^{19}F MRI allows quantitative cell tracking without the use of ionizing radiation. It is a noninvasive technique that can be applied to humans. Here, we describe a general protocol for cell labeling, imaging, and image processing. The technique is applicable to various cell types and animal models, although here we focus on a typical mouse model for tracking murine immune cells. The most important issues for cell labeling are described, as these are relevant to all models. Similarly, key imaging parameters are listed, although the details will vary depending on the MRI system and the individual setup. Finally, we include an image processing protocol for quantification. Variations for this, and other parts of the protocol, are assessed in the Discussion section. Based on the detailed procedure described here, the user will need to adapt the protocol for each specific cell type, cell label, animal model, and imaging setup. Note that the protocol can also be adapted for human use, as long as clinical restrictions are met.

Video Link

The video component of this article can be found at https://www.jove.com/video/50802/

Introduction

In vivo cell tracking is essential for the optimization and monitoring of cellular therapeutics[1]. Due to its noninvasive nature, imaging offers excellent opportunities to monitor cells *in vivo*. Magnetic Resonance Imaging (MRI) is independent of ionizing radiation and allows a superior imaging resolution and intrinsic soft tissue contrast. MRI-based cell tracking has already been used clinically to follow dendritic cells in melanoma patients[2]. Conventional clinical MRI is carried out on the ^{1}H nucleus, present in mobile water in tissues. It is also possible to carry out MRI on other active nuclei, such as ^{13}C, ^{19}F and ^{23}Na. However, only ^{19}F MRI has been applied successfully to *in vivo* cell tracking as it offers the highest sensitivity after ^{1}H. The absence of MRI-detectable endogenous ^{19}F in tissues permits high signal selectivity for the detection of exogenous ^{19}F contrast agents and allows quantification of fluorine concentration directly from the image data. For a detailed discussion on ^{19}F MRI, see[3-5]. A key issue with ^{19}F MRI is the need to develop and optimize suitable ^{19}F cell labels, although several labels have been developed, with a trend towards multimodal agents[6].

The protocol we describe here is based on studies by our groups[7-9], including the first articles that described *in vivo* quantitative ^{19}F MRI-based cell tracking[10,11]. The general procedure of cell tracking using ^{19}F MRI is summarized in **Figure 1**. We describe a general protocol for labeling and imaging of dendritic cells (DCs) using a custom-made perfluorocarbon contrast agent[8]. The imaging protocol is generally applicable to different cell types, labels and animal models. The cell type and animal model described here should only be taken as an example, and thus we do not provide details on the cell isolation and labeling, but rather focus on the imaging protocol. Modifications will be necessary for each label, cell type, animal model, and imaging setup, and these can be found in the literature or may need to be optimized by the researchers. Some common modifications are included in the discussion.

Protocol

Note: All experiments and procedures involving animals must be carried out in accordance with relevant ethical guidelines and conform with standard animal care and humane requirements.

1. Cell Labeling (Standard Protocol with Coincubation)

1. Add ^{19}F label[8] to immature DCs at a concentration of 4 mg/10^{6} cells (in 2 ml medium). Swirl gently to mix.
2. Incubate the cells with the label for 3 days before maturing and harvesting the DCs.
3. Collect the DCs and remove excess label by washing at least 3x in PBS. Count the cells.

4. Resuspend in a small volume of PBS for injection or further testing.

Note: This protocol is relevant for these specific DCs and label. The procedure was optimized in another publication[8] and included here are only the steps to prepare a sample to image, since the focus of this protocol is on the imaging. Hence, the protocol for isolation, culture and labeling of a specific cell type will either need to be found in the literature or optimized by the researcher. A summary of all other ^{19}F labels that have been used in the literature is presented elsewhere[6].

2. Determination of ^{19}F/cell Using ^{19}F NMR

1. Place a known number of labeled cells (typically more than 0.5 x 10^6, or a number that results in sufficient signal) in a NMR tube.
2. Add 10 µl of 5% trifluoroacetic acid (TFA). If the resonance frequency of TFA is unsuitable due to overlap with the label, use an alternative soluble compound, preferably with a single ^{19}F resonance.
3. Place the sample in an NMR spectrometer and obtain the ^{19}F spectrum. Ensure that the bandwidth is sufficient for both the TFA (reference) and the agent.

Note: The TR should be long enough for full relaxation of the reference and the sample spins (around 5 times T_1 relaxation time of the longest T_1 component in the tube, typically TR ~5-8 sec). Some spectrometers require D_2O for a frequency lock signal. A standard ^{19}F spectrum is sufficient. Extensive averaging or higher cell numbers will be required if cell uptake of the label is poor or if cell numbers are low. The spectrum should be centered between the reference and relevant label peaks, unless corrections are made to account for partial excitation.

4. Calculate the relative areas under the peaks of the reference and the sample (or the main peak of the sample), and then calculate the average number of ^{19}F's/cell in the sample.

Note: The entire process must be repeated and averaged extensively enough to reach statistical significance. This can also be done using spectroscopy directly at the MRI scanner. However, note that this procedure reveals only the average ^{19}F loading for a large number of cells, not the variability between cells. Checking uniformity of cell uptake requires the presence of a fluorescent component at the ^{19}F agent, so that cell uptake can be analyzed using microscopy or flow cytometry.

3. Considerations for Experimental Design - Cell Injection

Due to the relatively poor sensitivity of ^{19}F MRI for cell tracking, an animal model where the cells will localize in large numbers is necessary for successful *in vivo* imaging. Typical sensitivity values range from 1,000-100,000 cells/voxel/1 hr scan time[6], with a cell loading in the range of 10^{11}-10^{13} fluorine atoms.

The number and frequency of imaging sessions must be carefully planned, as this influences the choice of anesthetic. Note that isoflurane may not be suitable for ^{19}F MRI if the ^{19}F resonance frequency of isoflurane is close to that of the label compound used. isoflurane may still be suitable if the imaging sessions are short enough to prevent significant build-up. Several alternative anesthetics have been used in the literature[10,11]; an example is included in the imaging protocol. Anesthetics may need to be optimized for different mouse strains and disease models.

4. Design of an External ^{19}F Reference

1. Use an external reference containing a known amount of ^{19}F signal for quantification[12]. Choose the signal intensity of the reference to be roughly comparable to that expected from the subject.

Note: Generally, it is simplest if the reference compound is the same as the cell label, to match the relaxation parameters. If spectroscopic sequences or sequences without spatial localization are used, then a compound with a different chemical shift may be necessary.

2. Modify the placement, size and shape of the reference as necessary, dependent on the region of interest (ROI). Note: It is generally easiest to use tubes with a uniform cross-section for the reference.

5. Imaging and Mouse Preparation

1. Dilute commercially available ketamine and xylazine 1:10 v/v with physiological saline to yield stock solutions of 10 mg/ml ketamine and 2 mg/ml xylazine.
2. Turn on the heating system in the scanner (typically warm air) to ensure the bore will be warm before the mouse is imaged.
3. Inject 100 mg/kg ketamine and 10 mg/kg xylazine intraperitoneal to initiate anesthesia. Note: These doses have been tested for CD1(ICR) and NU-Foxn1^{nu} 8-week old male mice. Other strains may need slightly different doses, which should be optimized in a separate experiment. This dose will keep the mouse anesthetized for about 45 min. Note: The depth of anesthesia is normally sufficient if the animal shows no reaction to a foot pinch.
4. Once anesthetized, place the animal in an animal cradle and immobilize to prevent motion during MRI. If required, position the external reference next to the animal. Both the reference and the ROI (expected location of the injected cells) should be in the center of the coil. The animal should be connected to temperature and breathing control for physiological monitoring during scanning.
5. Cover the eyes of the mouse with sterile eye ointment to prevent drying out during long imaging sessions.
6. If the imaging time exceeds 40 min, prepare additional catheters for injection of additional ketamine/xylazine before the animal may wake up. Position two separate subcutaneous catheters with the working solutions of ketamine and xylazine. Use this catheter to give the mouse an additional 2.5 mg of ketamine every 20 min after the first 40 min. If necessary, further prolong anesthesia by injection of additional 0.5 mg

xylazine through the catheter, 100 min after the initial injection. In all cases, experimental time should not exceed 3 hr, except possibly under terminal anesthesia.

7. Ensure that the mouse is warmed directly after the first injection and maintained at 37 °C body temperature. With this protocol, <80% blood oxygenation was detected under atmosphere air breathing. To prevent side effects from the lowered blood oxygenation, use 100% oxygen (0.3 L/min). Body temperature and breathing should be controlled throughout the whole experiment and until full recovery of the animal. Note that the spontaneous breathing rate under ketamine/xylazine is very high (200-250/min). Irregularities in the breathing pattern, rather than the breathing rate, can indicate that a higher dose is necessary to maintain a suitable state of anesthesia. The mouse will awaken spontaneously within 20-30 min after the last dose of anesthetic.

Note: For cell tracking, it is necessary to image the same animal more than once. In that case, the imaging sessions should be scheduled with consideration to the time necessary for clearance of anesthesia from the system and animal use guidelines of the institute.

6. Imaging

^{1}H adjustments and imaging

1. Position the animal inside the scanner so that the ROI is in the isocenter using a low-resolution, anatomical scan with different slice orientations (a localizer).
2. Use the regular shimming protocol on ^{1}H, *e.g.* a global shim on the whole volume inside the coil.
3. Adjust the reference pulse gain (RG), *i.e.* the transmitter power measured in dB for a 1 msec Hermite 90° RF pulse, using the adjustment scan provided by the vendor.

Note: This adjustment will vary with the type of RF coil and is essential for ^{19}F MRI since the signal on the ^{19}F frequency is usually too low for direct adjustments. As a consequence, an estimate of the RG must be made from measurements on the ^{1}H signal. For RF transmission with a surface coil, the ^{1}H RG should be adjusted to a plane parallel to the coil through the part of interest, for a volume coil with homogenous B_1 profile, the exact settings of the adjustment are less important.

4. The following protocol of ^{19}F reference pulse gain adjustment works only if both ^{1}H and ^{19}F RF transmission is carried out with the same (single or double-tuned) RF coil. For such an imaging setup, the coil profile (B_1 transmit field) on ^{1}H should be proportional to the ^{19}F coil profile, when a fixed RG is applied, *i.e.* $B_{1,1H}=C*B_{1,19F}$ with a proportionality constant C.
    1. To confirm that coil profiles are proportional for a specific setup, acquire a ^{1}H and a ^{19}F flip angle map (see following section on B_1 correction) on a tube with highly concentrated ^{19}F, *e.g.* TFA in water (1:1 v/v) using identical fields-of-view (FOVs), beforehand (**Figure 3**).
    2. Using the same RG, check that both maps are identical except for the global factor C.
    3. Once the ^{1}H RG is determined, note this number down.
5. Carry out a conventional ^{1}H MRI scan as an anatomical reference for the ^{19}F imaging. Tune the system to the ^{19}F frequency of the cell marker and carry out a ^{19}F MR scan. Ideally the ^{19}F MRI scan will have the same field-of-view and slice selection as the ^{1}H MRI, although usually with lower resolution. The animal can be revived as soon as the imaging is complete. Image processing can then be carried out offline.
6. Determine the ^{19}F RG via the relation $dB_{19F}=dB_{1H}-20*\log_{10}(C)$. Estimate the ^{19}F agent frequency in a separate *in vitro* experiment.

Note: *In vivo*, the exact frequency can vary slightly from experiment to experiment due to different shimming conditions. To prevent chemical shift artifacts the exact frequency should therefore be determined in each experiment by ^{19}F MRS, if possible. A typical scan for very low ^{19}F signal would be global (pulse-acquire) spectroscopy (TR=200 msec, NEX=3,000, TA=10 min, BW=50 kHz). NEX, thus TA, can be dramatically reduced for high ^{19}F signal. The ^{19}F agent frequency is determined from the spectrum after Fourier transformation and phase correction. NEX refers to the number of excitations, TA is the acquisition time and BW is the bandwidth.

Note: Typical imaging parameters[9] on a 11.7T/16 cm dedicated animal scanner are: An anatomic ^{1}H imaging scan using a turbo spin echo (TSE) sequence with TR/effective echo time (TE_{eff}) = 2,200 msec/42.8 msec, 8 echoes per excitation, NEX = 2, 10 consecutive, 1 mm thick slices, FOV = 1.92 x 1.92 cm^2, 128 x 128 matrix, *i.e.* a resolution of 150 x 150 x 1,000 µm^3, TA = 1 min, BW = 50 kHz and a linear phase encoding scheme. ^{19}F images were acquired with the same sequence and matching geometry, but at lower in-plane resolution and lower BW (NEX = 256, 48 x 48 matrix, *i.e.* a resolution of 400 x 400 x 1,000 µm^3, TA = 57 min, BW = 10 kHz).

7. B_1 correction
   Using an RF coil with inhomogeneous B_1 profile, *e.g.* a surface coil, can hamper quantification of ^{19}F data since the signal depends not only on ^{19}F concentration but also on the distance from the coil. Acquire a B_1 map on the ^{1}H channel in order to retrospectively correct ^{19}F data. This requires that ^{1}H and ^{19}F coil profiles are identical, except for the proportionality factor C, and that sufficient ^{1}H background signal is provided in regions of ^{19}F. An example protocol for correction of a ^{19}F spin echo sequence is presented, using a single-tuned Tx/Rx surface coil tunable to both ^{1}H and ^{19}F and with proportional B_1 profiles[13].
    1. Acquire a ^{1}H flip angle map with the two flip angle method [14]. Acquire a gradient echo scan with very long TR (>3 times ^{1}H T_1) with an estimated flip angle of just below 90°. Close to the coil. Acquire a second gradient echo scan with a $RG=dB_{1H}-6$, *i.e.* twice the flip angle of the first scan.
    2. Calculate the ^{1}H flip angle, α, at voxel (x,y,z) via the signal intensities (SI) of the first and second gradient echo scans: $\alpha(x,y,z)=\arccos(SI_{GE,1}(x,y,z)/2SI_{GE,2}(x,y,z))$[14]. The ^{19}F flip angle is identical (except for the factor 1/C) due to proportional B_1 profiles. According to Faraday's principle of reciprocity[15], the attenuation of ^{19}F signal intensity from a spin echo sequence (excitation flip angle α, refocusing flip angle 2α) during signal reception is $att_{Rx}(x,y,z)\sim\alpha(x,y,z)$. The attenuation due to imperfect excitation is $att_{Tx}(x,y,z)\sim\sin^3(\alpha(x,y,z))$[16]. The overall attenuation is written as $att=attRx*attTx=\alpha*\sin^3(\alpha)/90°$. Note that it is normalized to 1 in case of perfect 90°/180° excitation/refocusing flip angle. The B_1 corrected ^{19}F image signal intensities $SI_{19F,corr}$ are calculated via $SI_{19F,corr}(x,y,z)= SI_{19F}(x,y,z)/att(x,y,z)$.

3. In order to compare signal intensities from different experiments, place a reference tube with defined ^{19}F concentration in the field of view, and normalize the $SI_{19F,corr}$ to the mean signal in the reference.

Note: Other ^{1}H B_1/flip angle mapping methods can be used and different ^{19}F pulse sequences require modifications of $att_{Tx,19F}$. Any kind of B1 correction scheme will introduce additional error in the cell quantification procedure. If accurate cell quantification is the main premise, an RF coil with homogenous B1 profile can be used to circumvent this part of the protocol.

7. Image Processing

1. Making ^{1}H and ^{19}F overlay images
    1. Export the image data for the final ^{1}H and ^{19}F magnitude images from the scanner.
    2. Open the files in an image processing program, such as ImageJ (freeware, NIH).
    3. Carry out image adjustments as necessary (cropping, brightness, contrast) keeping the adjustments the same for all images. The ^{19}F images will typically need to be upscaled to a higher resolution to match the corresponding ^{1}H images.
    4. Render the ^{19}F images in false color (select a different look-up table, under the "image" menu in Image J) and then overlay on the corresponding ^{1}H images in order to localize the signal.
2. ^{19}F Quantification
    1. Select a ^{19}F image (magnitude image) with the relevant cells and the reference visible.
    2. In a program such as Image J, draw ROIs over the relevant cells, the reference and a region outside the subject (noise).
    3. Use the command Analyze and then Measure (or simply Ctrl+M) to calculate the mean pixel intensity within these regions, and their area. Let S(cells) refer to the mean pixel intensity in the ROI over the cells. Multiply that by the volume (= area x slice thickness) to calculate the total signal.
    4. Calculate the SNR, for example using the formula $SNR = 0.65 \times S(cells) / \sigma$, where σ is the standard deviation of pixel intensity in an ROI outside the sample which is free of any chemical shift artifact (typically in the background air)[17]. If the SNR is under 5, a correction for the signal due to the noise may be necessary and can be carried out as described elsewhere[11 10].
    5. Otherwise, calculate the total amount of ^{19}F responsible for the signal in the ROI over the reference by multiplying the area of the reference in the slice by slice thickness (to calculate the volume) by the concentration of ^{19}F in the reference, which is known and assumed to be homogeneous.
    6. Multiply that value by the total signal in the ROI over the cells, divided by the total signal over the reference to calculate the amount of ^{19}F in the cells.
    7. Calculate the number of cells by dividing that number by the value for ^{19}F/cell, which was calculated in section 2.

Representative Results

Here we show the typical results for a protocol involving the transfer of ^{19}F-labeled cells homing to a draining lymph node. **Figure 2** shows a ^{19}F NMR spectrum of 10^6 labeled cells using a TFA reference. The imaging setup was carried out as described in the protocol (**Figure 3**). For the *in vivo* imaging, we used a reference consisting of a sealed cylinder of the same label used for the cells placed between the feet of the mouse. A representative processed image is shown in **Figure 4**. By applying the protocol described in section 7.2, we calculated a cell number of approx. 1.2×10^6 based on the raw ^{19}F magnitude image.

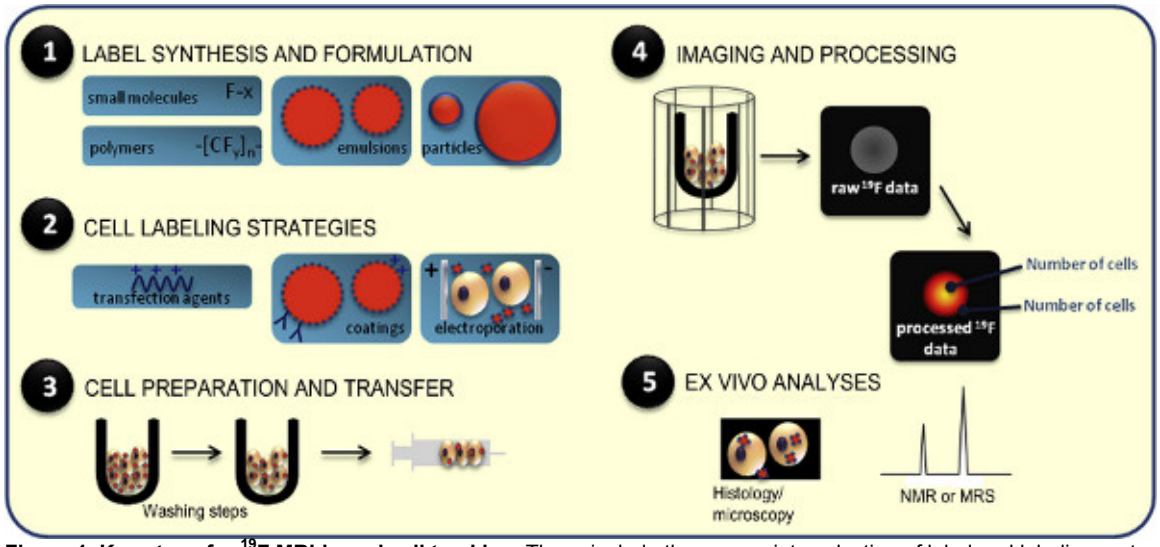

Figure 1. Key steps for ^{19}F MRI-based cell tracking. These include the appropriate selection of label and labeling protocol is crucial for success of the experiment (Step 1). Cell labeling (Step 2), may require enhancement through the use of coatings or transfection agents. After suitable preparation, including removal of any excess label, (Step 3) the cells can be imaged. Post-processing can be used to generate quantitative data from the images, if acquired suitably (Step 4). Finally, various ex vivo analysis can be carried out (Step 5) to corroborate the *in vivo* data. Figure reproduced with permission[6]. Click here to view larger figure.

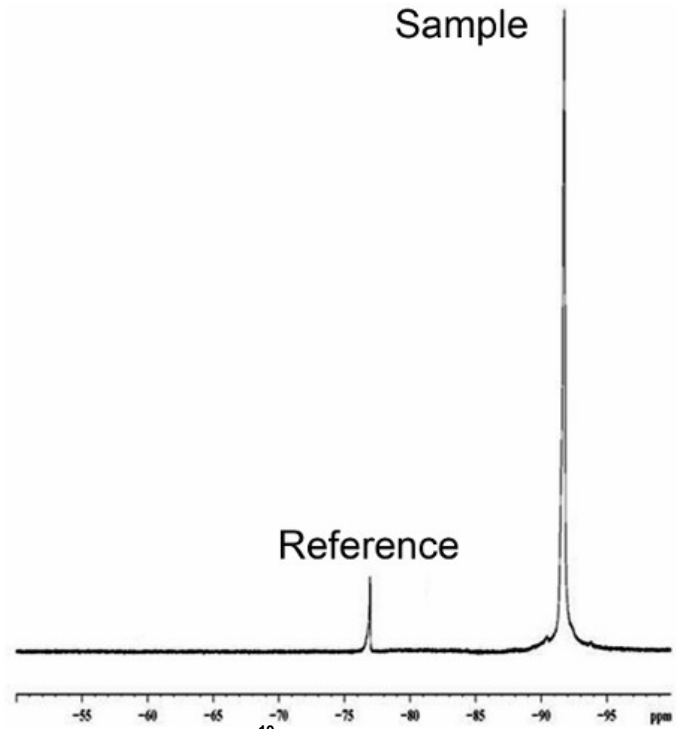

Figure 2. Representative ^{19}F NMR spectrum. The spectrum shows the ^{19}F signal obtained due to the known amount of reference, in this case TFA, and the known number of cells, labeled "sample". This can be used to calculate the amount of fluorine per cell, which is necessary for *in vivo* quantification from MR image data.

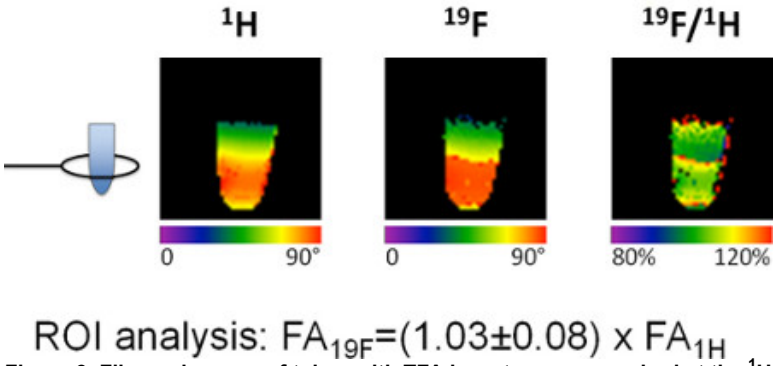

Figure 3. Flip angle maps of tubes with TFA in water were acquired at the ^{1}H and ^{19}F channel. The proportionality factor was calculated to be 1.03±0.08 for this setup. FA: flip angle.

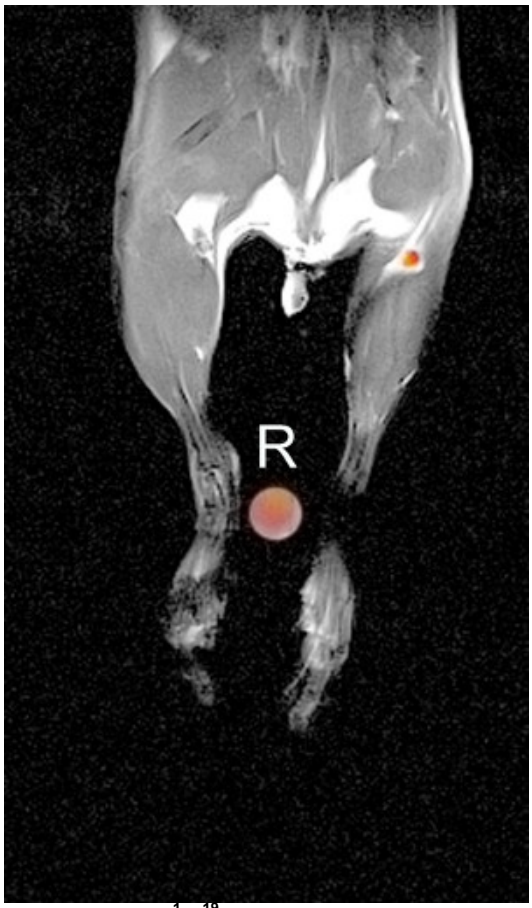

Figure 4. *In vivo* ^{1}H/^{19}F image of dendritic cells homing to a draining lymph node. The figure shows a representative image of a mouse, with ^{19}F signal visible, in false color, in the reference (R) and the labeled cells. Only the legs of the mouse were imaged here. In this example, the injected labeled cells migrated to the draining lymph node. The imaging parameters are summarized in the main text. The figure is for illustration purposes only.

Discussion

The protocol outlined here describes the general procedure for *in vivo* ^{19}F MRI cell tracking. Despite the described co-incubation method, there are several different protocols for labeling cells with a ^{19}F agent. However, the co-incubation is most often used and can be further optimized *e.g.* by addition of transfection agents[6]. The actual cell labeling protocol will depend on the cell type. Only key labeling steps are described in the protocol here. Generally, the label is prepared and added to the cells for a selected time ranging from a few hours to a few days. Finally, the cells must be washed carefully to remove any excess of label, especially if quantification from the image data will be carried out[3]. If the ^{19}F agent includes a fluorescence tag, the presence of label outside (or not bound to) the cells can be detected using microscopy. After labeling, it is necessary to study the cell viability and functionality, in relation to nonlabeled cells (*e.g.* by trypan blue exclusion assay). Cell-specific

functionality tests, such as the ability to activate T cells in the case of DCs, must also be carried out. Finally, as some non-^{19}F MRI labels have an effect on cell migration[18], such tests should also be included for ^{19}F labels, especially with high cell loading.

The protocol for the animal model is also dependent on the user´s needs. However, it is important to remember the strict sensitivity limits of ^{19}F MRI when planning experiments. Published sensitivity values range from 1,000-00,000 cells/voxel/1 hr time[6], with a cell loading in the range of 10^{11}-10^{13} ^{19}F. Sensitivity and expected label concentration in the region of interest also impact the imaging parameters. For example, the slice thickness used for the ^{19}F image can be adjusted to match that of the ^{1}H images or to cover a much thicker area (such as the entire lymph node or region of interest in a single slice). Typically, the ^{19}F imaging is carried out at lower resolution to maximize signal detection, especially since high resolution is not usually necessary to distinguish structures such as lymph nodes. If the signal is sufficient, a larger number of thinner slices can be acquired and the total signal over the volume of interest calculated from these. However, the optimal parameters will need to be derived for each application individually.

We describe an injection anesthesia protocol with ketamine/xylazine avoiding fluorinated inhalation gases like isoflurane. Other protocols have been used in the literature[10,11]. However, in all cases, these protocols will need to be adjusted for the mouse strain, age, gender, health and the length and frequency of the imaging sessions.

Several ^{19}F MRI imaging sequences have been used for cell tracking, for a review see[6]. Our imaging protocol can be readily modified to include other imaging sequences. However, the quantification method described here does not take into account any partial volume effects, discrepancies in selection of ROIs, changes in relaxation parameters of the label *in vivo*, or changes in cellular ^{19}F loading. These issues are described elsewhere[3]. In brief, the error has been found to be within tolerable limits for longitudinal cell tracking[10]. Compared to these earlier works we additionally propose an image correction scheme for the use of surface RF coils by mapping the B1 field. Depending on the specific RF coil setup it is important to be aware of limitations of B1 mapping techniques, which have been well-studied in the context of ^{1}H MRI[16]. For example, for the double angle method presented here the B1 map is most accurate for flip angle pairs just below 90°-180° [14] and significant error can be introduced in other regions. Still, after proper validation *in vitro*, such retrospective correction can further improve cell quantification. In general, we recommend corroboration of the data with more established techniques, at least initially. This is typically done in combination with other *in vivo* imaging techniques, *e.g.* optical imaging, to help exclude large errors. In most cases, histological evaluation is necessary to assess ^{19}F label location precisely and to allow correlation with the imaging data. This may require the addition of a fluorescent dye to the ^{19}F agent.

Finally, although ^{19}F MRI has not yet been applied to clinical cell tracking, it would be possible to modify this protocol for human use. The main modifications necessary would be to carry out as much of the optimization and setup beforehand as possible (to minimize the length of time the subject is in the scanner), and to adjust imaging parameters to keep within clinical specific absorption rate (SAR) restrictions.

Disclosures

The authors have no conflicts of interests to disclose.

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