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High-spatial-resolution x-ray fluorescence tomography with spectrally matched nanoparticles

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Abstract

PAPER

Present macroscopic biomedical imaging methods provide either morphology with high spatial resolution (e.g. CT) or functional/molecular information with lower resolution (e.g. PET). X-ray fluorescence (XRF) from targeted nanoparticles allows molecular or functional imaging but sensitivity has so far been insufficient resulting in low spatial resolution, despite long exposure times and high dose. In the present paper, we show that laboratory XRF tomography with metal-core nanoparticles (NPs) provides a path to functional/molecular biomedical imaging with ~100 μ m resolution in living rodents. The high sensitivity and resolution rely on the combination of a high-brightness liquid-metal-jet x-ray source, pencil-beam optics, photon-counting energy-dispersive detection, and spectrally matched NPs. The method is demonstrated on mice for 3D tumor imaging via passive targeting of in-house-fabricated molybdenum NPs. Exposure times, nanoparticle dose, and radiation dose agree well with *in vivo* imaging.

1. Introduction

X-ray fluorescence tomography is an emerging biomedical imaging modality. Combined with actively or passively targeted nanoparticles as contrast media it shows potential for improved functional or molecular imaging. However, to date the sensitivity is insufficient resulting in low observable spatial resolution. In the present paper we show that x-ray fluorescence tomography of spectrally matched nanoparticles provides a path to ~100 μ m resolution functional/molecular biomedical imaging in small animals.

Biomedical imaging technologies are essential for medical research and in daily medical practice (Bushberg *et al* 2002, Kagadis *et al* 2010, Weissleder and Nahrendorf 2015). In brief, the aim is to observe the studied object in its natural context with the highest possible spatial resolution and with diagnostically relevant information, preferably functional or molecular (Massoud *et al* 2003). X-rays and CT (computed tomography) delivers high resolution, sub-mm in humans and 0.1 mm in many mouse applications, but only for morphological imaging based on the classical absorption contrast. New source and detection schemes show potential for cellular-resolution x-ray CT (~10 μ m) (Lundström *et al* 2012), still, however, without functional/molecular information. Combining absorption CT with molecular contrast agents (Eck *et al* 2010, Hainfeld *et al* 2011) results in mm-to-few-mm observable resolution in mice (see below). PET and SPECT (positron emission tomography and single-photon emission tomography) provide molecular information but with low spatial resolution, typically 2 mm in humans and 1 mm in mice. Similar limitations in resolution apply to research tools based on bio-luminescence (Mezzanotte *et al* 2017) or optical fluorescence (Hong *et al* 2017). MRI (Magnetic resonance imaging) delivers certain functional information with mm resolution in humans and towards 0.1 mm in mice.

Nanoparticles (NPs) have potential as functional/molecular contrast agents in biomedical imaging, for the same reason that they are presently widely investigated as carriers for targeted drug delivery and therapy (Peer



et al 2007, Cheng *et al* 2012). Typically, the NPs are administrated via intravenous injection and then accumulate in selected tissue via passive or active targeting. For tumor applications, passive targeting exploits the enhanced permeability and retention (EPR) due to leakiness of tumor vasculature, while active targeting employs affinity ligands on the NPs that bind to, e.g. tumor-specific biomarkers or receptors.

In bioimaging, NPs are extensively used in microscopy, e.g. colloidal gold NPs in electron microscopy and quantum dots in visible fluorescence microscopy. Clinically, imaging with NPs has been limited to attempts using SPIONs (superparamagnetic iron oxide NPs) in MRI (see, e.g. Yigit *et al* (2012)). In small-animal applications, however, targeted NPs are investigated as contrast agents in many modalities. NPs based on gold and other materials have been used as alternatives to conventional contrast media for classical absorption-based x-ray imaging (Hainfeld *et al* 2006, Rabin *et al* 2006, Shilo *et al* 2012). CT in mouse has also demonstrated the 3D detection of actively targeted gold NPs against, e.g. lymph nodes (anti-CD4) (Eck *et al* 2010) or breast tumors (HER2) (Hainfeld *et al* 2011). Typically, the reachable NP dose is in the 1–3 mg Au/g tumor or organ (0.1–0.3 wt%) after intravenous injection. This corresponds to 10–100 HU (Hounsfield units), allowing the observation of mm-sized objects in micro-CT scanners. Finally, we note that Au NPs are also investigated for dose enhancement in radiation therapy, then with up to 7 mg Au/g tumor (0.7 wt%) and that mice are found to have >1 year survival after such Au NP doses (Hainfeld *et al* 2004).

X-ray fluorescence (XRF) can provide sensitive and quantitative detection of NPs and is extensively used for nanoimaging in the 10 keV regime at synchrotron radiation sources (see, e.g. Paunesku *et al* (2003)). However, the low-divergence beam and limited access to these large facilities make them less applicable for small-animal biomedical imaging. The last few years laboratory (x-ray-tube-based) fluorescence tomography (Ahmad *et al* 2014) from gold NPs has been investigated experimentally and theoretically in phantoms (Cheong *et al* 2010, Bazalova *et al* 2012, Jones *et al* 2012, Ren *et al* 2014, Ahmad *et al* 2015), and in a mouse (Manohar *et al* 2016). The goal is to provide functional or molecular imaging capability. Typically these experiments show few-to-several mm spatial resolution with 0.5–4 wt% metal NP concentration despite long exposure times (hours per slice) and high dose (several hundred mGy or more). The major constraints are the low useful x-ray flux from the source and the high Compton background, resulting in low signal-to-noise ratio (SNR) (Hertz *et al* 2014). Imaging phosphor NPs instead (x-rays in, visible out) (Pratx *et al* 2010) show similar SNR and resolution limitations as in the x-ray fluorescence.

In the present paper we demonstrate quantitative tomographic XRF imaging of NPs in rodents with 200 μ m spatial resolution at exposure times, NP dose, and radiation dose agreeable with *in vivo* experiments. The method is demonstrated in mice for 3D tumor imaging via passive targeting of in-house fabricated molybdenum-based (Mo) NPs injected in the tail vein. The XRF system is based on a liquid-metal-jet micro-focus source and photon-counting spectrally selective detection. The high brightness of the source and spectral matching of the 24 keV source emission to the Mo-based NP x-ray absorption profile provides short exposure times, acceptable dose, and the elimination of the Compton background (Hertz *et al* 2014). Our experiments suggests a path towards functional/molecular biomedical imaging with ~100 μ m spatial resolution. Such high-resolution imaging would be of significant interest for both research and diagnostics, e.g. for increased detail when following molecular processes or for early tumor detection (Massoud and Gambhir 2003, Weissleder and Nahrendorf 2015).

2. Methods

2.1. Laboratory nanoparticle x-ray fluorescence (XRF) tomography

2.1.1. Experimental arrangement

Figure 1 describes the experimental procedure. The arrangement (figure 1(a)) includes a liquid-metal-jet microfocus source, multilayer optics, the mouse with injected Mo NPs, and two energy-dispersive detectors, one for XRF detection and one for absorption CT. Figure 1(b) shows the emitted spectrum from the indium-alloy-target high-brightness source (D2, Excillum AB, Sweden), including the 24.1 keV In K_{α} line and the broadband bremsstrahlung background. The source operates at 170 W and 120 kVp, producing a flux of 6 × 10¹¹ ph/(s × sr) in the 24.14 keV In K_{α} line. The tube voltage was chosen in order to maximize the yield of the characteristic In K_{α} line emission.

The W-C-coated multilayer Montel mirror (ELM61, Incoatec GmbH, Germany) selects the 24.1 keV emission from the source (see figure 1(c)) and produces a semi-monochromatic high-brightness pencil beam with ~100 × 100 μ m² FWHM focus 56 cm from the x-ray source (figure S6). The beam divergence is 1.83 mrad which makes the beam <130 μ m wide over the full 3 cm diameter object. The brilliance and flux in the focus was measured to 8 × 10⁸ ph/(s × mrad² × mm² × line) and 3 × 10⁷ ph s⁻¹, respectively.

The NP-injected and sacrificed mouse (see below) was placed upside-down in a 30 mm diameter plastic tube and positioned in the focus of the x-ray pencil beam. No other form of immobilization was used. Molybdenum has its K absorption edge at 20.0 keV, which nicely matches the pencil beam energy of 24.1 keV. Upon excitation by the pencil beam the Mo NPs emit K_{α} XRF at 17.4 keV.



Figure 1. Laboratory x-ray fluorescence (XRF) tomography for high-spatial-resolution nanoparticle (NP) imaging in rodents. (a) The XRF tomography of Mo NPs and absorption CT is performed simultaneously on tumor-bearing mice. The NPs are intravenously injected prior to the scan and passively enhanced in the tumor by the EPR effect. A focused x-ray pencil beam scans the mouse to obtain both the spatial distribution and local concentration of the NPs. (b) Source emission spectrum. The In K_{α} line is selected by the multi-layer mirror and focused into a pencil beam. (c) Total reflectivity of the multi-layer mirror. (d) The x-ray fluorescence spectrum with its 17 keV Mo peaks. (e) TEM micrograph of the Mo NPs.

The 17 mm² silicon-drift detector (X123-SDD, Amptek Inc., MA) provides spectrally dispersed single-photon detection of the Mo K_{α} XRF at 17.4 keV and the higher-energy Compton scattering. Its energy resolution was measured to 230 eV at the Mo K_{α} line. Figure 1(d) shows the detected x-ray spectrum. In order to maximize the signal, the XRF detector is placed as close as possible to the object (3 mm from the plastic tube) and mounted on the horizontal stage. The CT detector is a 25 mm² CdTe detector (X123-CdTe, Amptek Inc., MA). It was chosen since it uses the same read-out electronics as the XRF detector, making it easy to synchronize the two detectors. Its settings are optimized for high throughput rather than good energy resolution since the pencil beam is semimonochromatic. To comply with the maximum count rate of the absorption detector, the flux from the pencil beam is reduced using an 11 mm Al filter in front of the detector.

The high sensitivity of the arrangement relies on the matching of the excitation energy to the Mo absorption and the negligible influence of the Compton background due to the single-line excitation and single-line detection.

2.1.2. Data acquisition

Data is acquired using an in-house built 'on-the-fly' scan software. Here the sample is scanned continuously in the horizontal direction but acquisition is paused during vertical and rotational movements. In each step, the full spectrum of both the fluorescence and the absorption detectors is read out. The acquisition time and stage velocity are matched to give an effective step size of 200 μ m in the horizontal direction. The step size in the vertical direction was set to 200–500 μ m depending on the type of measurement and the angular resolution was 3.6°. In order to maximize the signal, only the half of the object closest to the fluorescence detector is scanned, but as a 360° tomography instead of the typical 180° in order to get data from the whole object.

As for dose, three different data acquisition modes are used; low dose (~50 mGy), medium dose (~100 mGy) and high dose (~200 mGy), corresponding to an acquisition time of 25 ms, 50 ms and 100 ms per step, respectively. For a tomography series with 100 projections over 360° this corresponds to a total acquisition time (incl. overhead) of 4:00 min, 7:30 min and 14:30 min per slice. These numbers can be reduced 2 orders of magnitude (see discussion).

2.1.3. Data processing and image reconstruction

The image reconstruction is done in three steps. First, the absorption and fluorescence signals are extracted from the measured spectra. Secondly, the absorption image is reconstructed using a standard filtered back-projection algorithm. Finally, the fluorescence image is reconstructed using an iterative algorithm that accounts for attenuation of the pencil beam and self-absorption of the fluorescence signal.

In the first step the spectra from the absorption and fluorescence detectors are analyzed. First, all spectra are corrected for errors in acquisition time and dead time. Secondly, the relevant signals are extracted. From the fluorescence detector the signal is taken as the integrated signal under the FWHM of the Mo K $_{\alpha}$ peak at 17.4 keV. The background to be subtracted is mainly due to multiple Compton scattering. However, this signal is very weak making a direct estimate of the background from the vicinity of the Mo K $_{\alpha}$ peak inaccurate. Therefore, the background signal is assumed to be directly proportional to the orders-of-magnitude larger primary Compton scattering, which can be extracted from the same spectrum. This procedure is graphically illustrated in figure S7. The absorption signal is determined directly from the absorption detector as the integrated signal under the FWHM of the 24.1 keV peak without any background correction. Finally, the extracted signals are stored in two separate datasets containing the sinograms for the absorption and fluorescence signal, respectively. Each sinogram is corrected for errors in stage movement, object movement and tilt of the rotation axis. In the second step the absorption image is reconstructed using a standard filtered-back-projection algorithm, giving the linear attenuation coefficients at 24.1 keV for the whole object.

Finally, in the last step the fluorescence image is reconstructed using an iterative reconstruction method. First, a map of the pencil beam intensity in each voxel is created using the reconstructed absorption image and the pencil beam flux, unique for each rotation angle. Secondly, the linear attenuation coefficients at 17.4 keV are estimated from the reconstructed 24.1 keV absorption image by using the ratio of the linear attenuation coefficient between 17.4 keV and 24.1 keV obtained from NIST (Berger et al 2010). Since the ratio is different for bone and soft tissue, the reconstructed absorption image is segmented into two parts, bone and soft tissue, and different ratios are used for each segmented subset. For each voxel, the 17.4 keV attenuation values are then used to calculate the fluorescence self-absorption along the path to the fluorescence detector. Finally, the pencil beam intensity map and the fluorescence self-absorption map, together with a priori information about the imaging system (detector efficiency, Mo K_{α} fluorescence yield (Thompson *et al* 2009) and the solid angle to the XRF detector from each point in the object) are used to create a forward projection matrix. This is entered into a multiplicative SIRT reconstruction algorithm together with the fluorescence projection data to reconstruct the final fluorescence images. The number of iterations is set dynamically based on the RMS difference between the measured and reconstructed sinograms. Surface renderings of the reconstructed images are performed in Amira 6.3 (FEI Visualization Sciences Group, Bordeaux, France). The analysis of individual organs and tumors was done by manually segmenting the reconstructed images in Amira.

2.1.4. Quantitative XRF

In order to obtain quantitative Mo concentrations, the XRF tomography system is calibrated with a reference object with known ionic Mo standard concentration. The reference object is a 20 mm diameter polyethylene terephthalate phantom with three 2 mm diameter holes filled with 1 mg ml⁻¹ of Mo ICP standard. The reference signal is measured under the same imaging geometry and reconstructed using the same iterative algorithm as the mouse data.

2.1.5. Radiation dose

The absorbed radiation dose is calculated as the average dose in soft tissue. First, the reconstructed absorption data is segmented into two parts, soft tissue and bone, and the mass of each voxel, M_i , is estimated from the voxel size and density (1060 kg m⁻³ and 1920 kg m⁻³ for soft tissue and bone, respectively). Next, the energy absorbed in each voxel, ΔE_i , is computed from the pencil beam intensity map (see 'Data processing and image reconstruction'). The dose in each voxel, D_i , could then be determined as $D_i = \Delta E_i/M_i$. Finally, the average volume dose is calculated as $D = (\Delta y_{\text{beam}}/\Delta y_{\text{step}}) N^{-1} \Sigma D_i$, where Δy_{beam} is the pencil beam height, Δy_{step} is the step size in *y* and *N* is the number of voxels.

2.2. Nanoparticle synthesis and characterization

2.2.1. Synthesis of Mo nanoparticles

The NPs are synthesized by a method adapted from Dou and Zeng (2012). Briefly, 300 mg ammonium heptamolybdate (AHM, $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$, Sigma-Aldrich) is dissolved in 44 ml deionized water with 18 M Ω resistivity. After the addition of 20 ml ethanol (99.7%, Solveco) 1 g of polyvinylpyrrolidone (PVP; (C₆H₉NO) n, 55 kDa, Sigma-Aldrich) is dissolved in this solution and stirred for 30 min at room temperature. When all the components are dissolved, 2 ml of 1 M HCl (Sigma-Aldrich) is added and the resulting solution is transferred to a Teflon lined stainless steel autoclave (100 ml capacity). A hydrothermal reaction is performed at 180 °C for 18 h in the autoclave. The autoclave is cooled to room temperature and the black precipitate is collected by successive centrifugation and re-dispersion with ultrapure water (Milli-Q[®] Reference, Merck Millipore).

2.2.2. Nanoparticle characterization

The crystallographic phase of the NPs was investigated using x-ray powder diffraction (XRPD) (Panalytical Xpert Pro alpha powder, PANalytical) with Cu K_{α} radiation, 1.5406 Å wavelength and scanning rate of 0.13° min⁻¹, and

with electron diffraction in transmission electron microscopy (TEM) (JEM-2100F, 200 kV, JEOL). The size and morphology of the NPs were analyzed in the same TEM. Here 20 μ l NP suspension was dispensed on the TEM grid and air dried. For the size analysis, at least 500 NPs/clusters are measured in different field of view. Surface-chemical species was studied by Fourier-transform infrared spectroscopy (FT-IR) (Thermo Fisher Scientific) with an Attenuated total reflection unit. Thermogravimetric analysis (TGA) (TGA550, TA instruments) was used for the quantification of PVP adsorbed on the surface of the NPs. The volume-averaged hydrodynamic diameter and the surface charge of the NPs dispersed in both deionized water (DIW) and phosphate-buffered saline (PBS) were measured using dynamic light scattering (DLS) and zeta potential (Zetasizer Nano ZS90, Malvern) analyses techniques. The Mo concentrations in the MoO₂ NP stock solution was determined by inductively coupled plasma optical emission spectroscopy (iCAP 6000 series, Thermo Scientific).

2.3. Measurements on mice

2.3.1. Animals

All animal experiments were performed on NOD SCID gamma mice from the Karolinska Institutet, MTC Breeding Unit, and from Taconic Biosciences. The mice were kept in a specific-pathogen-free environment with controlled temperature (21 °C), humidity (50%–60% RH) and light (12 h day/night cycles) with ad libitum access to water and food. All animal experiments were carried out in accordance with the principles and guidelines of Karolinska Institutet and Swedish law, and were approved by the Stockholm regional ethics committee for animal research (N50/16).

2.3.2. Experiments in mice without tumors

To determine appropriate experimental parameters such as NP dose, signal levels, temporal and spatial distribution of the Mo-based NPs, as well as the long-term clearance of the NPs, experiments were first performed on mice without tumors. For these experiments 100 μ l of NPs suspended in PBS are injected in the tail vein. Mo concentration ranged between 1 and 3 wt%. The age of the animals at the time of injection ranged from 8–12 weeks and the weight from 19 to 32 g. The mice were sacrificed and imaged 15 min to 4 months after injection.

2.3.3. Experiments in mice with a tumor xenograft

For the tumor experiment, SK-N-BE(2) neuroblastoma cells were cultured in a MEME (Sigma Aldrich) and nutrient mixture F-12 (Thermo Scientific) mix (1:1) supplemented with 0.5% Glutmax (Thermo Scientific), 1% penicillin/streptomycin and 10% fetal bovine serum (FBS, all from Hyclone). $3-10 \times 10^6$ cells were injected subcutaneously into the groin fat pad of 4-week old mouse.

The size of the tumors was estimated daily to follow tumor growth (volume = width × height × length × 0.52). At an estimated tumor volume varying between 10–369 mm³, 100 μ l of 15 mg ml⁻¹ Mo NP suspended in PBS (1.5 wt% Mo) were injected intravenously in the tail vein. This corresponded to 6–8 week old mice with a weight between 23–28 g. 24 h after the NP injection the mice were sacrificed and imaged. Actual tumor volumes were assessed post mortem by weighing the extracted tumors as well as from segmentation in XRF tomography. In total six tumor-bearing mice were imaged, all with similar result as in figure 3.

2.3.4. Mo concentration in organs by ICP-MS

After the XRF analysis, the mice were frozen and the organs (lungs, liver, spleen, kidney) were extracted at a later point. The elemental analysis was performed at ALS Scandinavia, Luleå, by high-resolution inductively coupled plasma mass spectrometry (ICP-MS) (Element 2, Thermo Fisher) following standardized procedure (Ecke *et al* 2017, Genuis *et al* 2011). The elemental concentrations are reported as an average of triplicate measurements.

3. Results

3.1. Nanoparticles

The Mo NPs are synthesized by wet-chemistry for properties tailored for the experiments. XRPD and electron diffraction show that the MoO₂ crystalline phase dominates (figure S1 in the supplementary material (stacks.iop. org/PMB/63/164001/mmedia)). This is the most stable phase in water of the molybdenum oxides (Rumble 2017) and therefore suitable for the present task. TEM show spherical MoO₂ particles of 6 (±1.4) nm agglomerated in clusters with average diameter 50 (±16) nm (figures 1(e) and S2), an appropriate size for EPR-based NP accumulation (Blanco *et al* 2015). The clusters are coated by a thin PVP layer, visible in TEM (figure S2) and confirmed by FT-IR (figure S3). TGA results in a PVP/MoO₂ ratio of 1:4–1:3 (figure S4). PVP is a common coating in NP synthesis for its stabilizing (Koczkur *et al* 2015) and toxicity-reducing properties (Bühler 2005). DLS confirms the presence of NP clusters, with an average hydrodynamic diameter of 54 ± 3 nm in DIW and 51 ± 3 nm in PBS media. Finally, surface-charge measurements show that the PVP-MoO₂ clusters are strongly negatively charged both in DIW (-34 mV) and PBS (-26 mV), providing colloidal stability in both dispersion media (table S1).

3.2. XRF tomography in mouse

Figure 2(a) shows a full-body 3D XRF tomography (color) with CT overlay (grey). NPs were injected 24 h prior to the scan and the 'low dose' mode was used. The reconstructed volume consists of 160 axial slices with a spacing of 500 μ m. Each slice consists of 163 × 163 pixels with a pixel size of 200 μ m. The majority of the injected NPs accumulate in the lungs and liver but smaller amounts are also found in the spleen, abdominal area and in the nose sinuses. Figure 2(b) shows selective areas of the lungs, liver, spleen, and heart with enhanced contrast. Figures 2(c) and (d) shows quantitative data on the NP update (id%, Mo uptake per injected Mo dose) and average Mo concentration (wt%, Mo weight per organ weight) in the organs of the mouse in figure 2(a), as measured both with the XRF tomography (blue) and with Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (yellow). The ICP-MS was performed following the XRF experiments on extracted organs from the same mouse. We estimate the 1 σ error in the XRF measurements to be 25%–35% where major factors include photon noise and reconstruction errors. The errors in the ICP-MS measurements are assumed to be very small. Finally, we note that the kidneys show close to zero Mo concentration, both in XRF and ICP-MS, and that ICP-MS was not performed on the heart due to the difficulty in obtaining an appropriate numbers given the large volume of blood.

The two independent quantitative measurements of NP distribution and concentration agree well. From figure 2(c) it is obvious that the majority of the NPs accumulate in the lungs and the liver with minor amounts in the heart and spleen. Figure 2(d) shows concentration in the major organs is on the order of 0.1 wt%. We note that in this 'low-dose' mode it was not possible to detect and localize the tumor with XRF. However, the ICP-MS showed an average Mo NP concentration of 0.0020 wt% in the tumor. Finally, we conclude that the total amount of Mo NPs as detected by XRF adds up to 89% of the injected dose, indicating that ~11% of NPs were either below the detection limit or cleared during the first 24 h. The supplementary material also includes an animated movie of figure 2(a) (movie S1 (stacks.iop.org/PMB/63/164001/mmedia)).

3.3. XRF xenograft tumor detection

Figure 3 shows the 3D XRF tomography (color) and CT overlays (grey) of two mice with tumors. The scans were performed 24 h after the NP injection and in both cases the 'high dose' mode was used. To reduce the exposure time only a smaller region over the tumor (30–35 axial slices, 400 μ m spacing) was scanned. Figure 3(a) shows a 22 mm³ tumor (approx. $4 \times 3 \times 3$ mm) located next to the right leg and with the urinary bladder in the center. The average concentration and total amount of Mo in the tumor was measured to 0.003 wt% and 0.06 id%, respectively. This is in good agreement with the ICP-MS analysis showing 0.0036 wt% and 0.056 id%. Figure 3(b) shows a 151 mm³ tumor (approx. $9 \times 6 \times 5$ mm). The average Mo concentration is 0.002 wt% and total amount of Mo 0.2 id%. This is also in good agreement with the ICP-MS analysis which gave 0.0027 wt% and 0.29 id%. The XRF tomography was performed on in total six tumor-bearing mice, all with the same or similar results as shown above. Needless to say, none of the tumors were detectable by the absorption CT. Movies of figures 3(a) and (b) are available in the supplementary material (Movie S2 and S3).

3.4. Small feature, low contrast detection

The ability to detect a certain feature is dependent on both the size and local NP concentration of the feature (specifically, the total mass of the NPs). To investigate our method's potential for low contrast, small feature observability (of, e.g. a small tumor), a test target was inserted into a mouse. The test target consists of two glass tubes, 200 μ m and 500 μ m inner diam., filled with Mo NPs with 0.15 wt% Mo. The NP concentration was chosen to match that of previously observed concentrations in tumors, >0.35 wt% after intravenous injection using affinity ligands (Hainfeld *et al* 2011) and 0.2–0.7 wt% with EPR (Hainfeld *et al* 2004, 2006, Manohar *et al* 2016). Figure 4 shows the reconstructed 3D XRF tomography and CT overlay resulting from a 'high dose' scan. Both tubes are clearly visible but since the pixel size is 200 μ m the signal is spread out over several pixels. Accounting for this, the reconstructed Mo concentration in the tubes are 0.10 wt% and 0.17 wt% for the 200 μ m and 500 μ m tubes, respectively.

3.5. NP biocompatibility and clearance

All experiments were performed with 1–2 wt% Mo in the injected 100 μ l NP suspension. For higher concentrations (>2.5 wt%) the animals exhibit time-limited (typically minutes) passive behavior after the injection or in rare cases even death. With 1–2 wt% the animals show normal growth curves. Figure S5 shows the growth in weight of six mice from the time of injection to 14 weeks after the injection. Three mice were injected with 1 wt% and three with 2 wt%, and both groups have normal and similar growth curves. As for the clearance of the Mo NPs, figure 5 shows the XRF projection images and corresponding data for healthy mice injected with 2 wt% from 15 min to 14 weeks. Each image is 34×83 mm with a pixel size of $200 \times 200 \,\mu$ m and the acquisition time per step is 50 ms. The 15 min–24 h mice are male and the 4 and 14 weeks mice are female. The integrated fluorescence signal for each mouse (relative to 15 min after injection) is displayed in the bottom of the image.



Figure 2. Full-body 3D reconstruction overlay of XRF and CT, and NP bio distribution. (a) A full-body 3D XRF tomography and CT overlay. The image consists of 160 axial slices with a spacing of 500 μ m and a pixel size of 200 μ m. (b) Enhanced contrast of some regions from figure 2(a). (c) and (d) The quantitative Mo NP bio-distribution and average concentration in different organs for the mouse in (a), as measured with XRF and ICP-MS.







This measurement is closely related to total amount of Mo NPs in the body so the relative number gives an indication of the NP clearance with time. We observe a gradual clearance of the NPs with only a small fraction of the Mo remaining after 14 weeks.

4. Discussion

We have shown *ex vivo* tumor imaging in mice using a laboratory dual CT and XRF tomography setup and inhouse fabricated Mo NPs. Below we discuss the necessary steps for a successful *in vivo* implementation of this new imaging modality.

For *in vivo* rodent imaging the exposure time needs to be reduced while still keeping the radiation dose at the present level or lower. Assuming a maximum practical scan time of ~ 1 h, the exposure time per slice needs to be reduced by a factor of ~ 50 in order allow full-body imaging with present tumor sensitivity. As for the radiation dose, values similar or below to those of the present experiment (200 mGy) are acceptable and comparable with state-of-the-art micro-CT imaging (few 100 mGy)(Badea *et al* 2008). Increasing the source power is feasible (Larsson *et al* 2016) and this would shorten exposure times but, unfortunately, this also increases the radiation dose. Instead, focus should be on detector efficiency and/or NP uptake since both parameters reduce exposure times as well as radiation dose.

Both dose and exposure times are inversely proportional to the fluorescence-detector solid angle. Here several technological alternatives exist. Many large area/multi-element detectors are already in place at synchrotron facilities that could be included in the experimental arrangement (Ryan *et al* 2007). More elegant, however, would be a ring detector as suggested by Bazalova *et al* (2012). A 10 mm wide 360 degree ring would increase the solid angle from the present 0.075 sr to >4 sr, i.e. $>50 \times$ reduction in exposure time and dose. Thus, improving the detector alone would provide a platform for whole-body *in vivo* imaging with scan times in the 10 min to 1 h range, depending on imaging parameters. As a positive side effect, the dose reduces to less than 10 mGy, a very low level for mice experiments and approaching clinically acceptable numbers.

Exposure times and radiation dose is reduced even further if the local NP concentration in the studied object (e.g. the tumor) can be increased. With our present NP formulation, the highest Mo concentration achieved in tumors was 0.0036 wt% (2.43 id%). However, there are several papers demonstrating $60-120 \times$ higher concentrations (wt%) in tumors when Au NPs are used, both with active (Hainfeld *et al* 2011) and passive targeting (Hainfeld *et al* 2004, 2006, Manohar *et al* 2016), indicating that there is significant room for improvement. These papers typically inject $15-40 \times$ higher NP dose. In order to increase the injected Mo NP dose and the tumor uptake, we will investigate different Mo NP sizes and coatings with the goal to increase circulation time and uptake in targeted tissue, and decrease toxic effects (Ecke *et al* 2017). Here coating with inert materials like SiO₂ (Vogt *et al* 2015) and Au (Silva *et al* 2016) for surface passivation should provide protection of the MoO₂ core for increased bio-compatibility as well as a platform for grafting various active targeting agents. More dense layers of polymers like, e.g. polyethylene glycol (PEG), PVP, and polyvinyl alcohol (PVA) (Ishihara *et al* 2010) may offer the advantage of increased bio-compatibility and antifouling properties besides good chemical and colloidal stability.

Employing active targeting of the NPs, instead of the passive targeting demonstrated here, could enable molecular information in x-ray tomography. Such active targeting involves attaching appropriate affinity ligands



to the NPs. With Au NPs active targeting has demonstrated $100 \times$ higher local NP concentrations in breast tumors (Hainfeld *et al* 2011) and 25× in lymph nodes (Eck *et al* 2010) compared to the passively obtained concentrations demonstrated here. Consequently, active targeting has potential for early detection schemes of very small tumors via binding to tumor-specific biomarkers (Weissleder and Nahrendorf 2015). For the neuroblastoma tumors used here, they express high levels of GD₂ on their surfaces which has been exploited for active NP targeting (Tivnan *et al* 2012). As for the more generic biomedical imaging outlook, the combination of actively targeted NPs with the high detection efficiency of the improved XRF tomography system outlined here appears to provide a path towards molecular imaging of tumors or other features with much higher spatial resolution that present methods (e.g. PET, SPECT) allow. From the experimental data and SNR extrapolations, we estimate 100 μ m resolution or better for *in vivo* imaging.

Finally, we note that molecular imaging with higher spatial resolution than present methods would be of significant clinical interest, e.g. for early tumor detection. However, it is much too early to speculate if the concept presented here can be extended to humans. From an x-ray-properties, x-ray-technology, and radiation-dose perspective, the improved system described above could be directly applicable for thin objects (breast, hand, etc), while full-body imaging would involve harder x-rays, new NPs, new detectors and more difficult SNR issues. In addition, the biocompatibility, uptake, and clearance of the NPs is of key importance and a challenging optimization problem. We therefore prefer to refrain from speculating about future clinical use at the present time.

5. Conclusion

We have demonstrated that laboratory x-ray fluorescence tomography provides 3D imaging of nanoparticles in rodents with 200 μ m spatial resolution at exposure times, nanoparticle dose, and radiation dose suitable for *in vivo* experiments. The system relies on spectral matching between the x-ray excitation and the NP absorption, and is demonstrated on mice for tumor localization by passive targeting of the NPs. The unique high detection sensitivity in combination with the pencil-beam optics enables functional or molecular imaging at significantly higher spatial resolution than existing biomedical imaging modalities allow. Furthermore, we note that our method removes the classical limitation of x-ray imaging to morphological diagnostics and extends x-ray imaging providing also functional and molecular information. Thus, our experiments pave the way for *in vivo*

whole-body 3D NP-based functional/molecular imaging of rodents with high spatial resolution (100 μ m range). This would be of considerable interest for research as well as diagnostics, e.g. for increased detail when following molecular or physiological processes or for the detection of smaller cancers (Massoud and Gambhir 2003, Weissleder and Nahrendorf 2015).

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