

Research Article

# Processing of SPIO in macrophages and tumor tissue for MPI lymph node imaging in breast cancer

Dominique Finas<sup>a,b,\*</sup> · Janine Stegmann-Frehse<sup>b</sup> · Benjamin Sauer<sup>c</sup> · Gereon Hüttmann<sup>c</sup> · Achim Rody<sup>b</sup> · Thorsten M. Buzug<sup>d</sup> · Kerstin Lüdtke-Buzug<sup>d</sup>

<sup>a</sup>Department of Obstetrics and Gynecology, Evangelical Hospital Bielefeld, Bielefeld, Germany

<sup>b</sup>Department of Obstetrics and Gynecology, University of Lübeck, Lübeck, Germany

<sup>c</sup>Institute of Biomedical Optics, University of Lübeck, Lübeck, Germany

<sup>d</sup>Institute of Medical Engineering, University of Lübeck, Lübeck, Germany

\*Corresponding author, email: finas.d@arcor.de

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## Abstract

The most common cancer in women worldwide is breast cancer. To avoid tissue damaging while axillary surgery in breast cancer (BC), we aim to develop a new sentinel lymph node biopsy method using superparamagnetic iron oxide nanoparticles (SPIOs) and magnetic particle imaging (MPI). It is well known from intra venous SPIO application in magnetic resonance imaging that macrophages (MP) are key role player in processing of SPIOs causing a drop of signal intensity. Nevertheless, knowledge lacks concerning enrichment processes of SPIOs after injection in breast tissue, the adjacent lymphatic tissues and associated cells, especially in BC and metastatic lymph nodes. Previously we evaluated the distribution of SPIOs in an in vivo healthy and tumor mouse model. Based on these studies we investigate the processing of the SPIOs in MP. To evaluate SPIO processing we established a tumor bearing mouse model as we previously published. Tumor tissue was then analyzed by conventionally hematoxylin histology combined with Berlin blue staining and autofluorescence multiphoton microscopy. Additionally, the MP cell line J774A.1 was incubated either by Resovist<sup>®</sup> in culture medium, or culture medium only as control. This process was observed in vitro by multiphoton microscopy. Detection of SPIOs was realized by excitation at 1200 nm. Resovist<sup>®</sup> showed no toxic effects on macrophages after incubation. MP showed activity in phagocytosis of Resovist<sup>®</sup> after incubation in multiphoton microscopy. SPIOs were detectable within tumor tissue by conventional hematoxylin microscopy as well as particle processing by 3-photon microscopy. The cell associated SPIO processing signal was detected by live cell imaging. After injection of Resovist<sup>®</sup> into tumor tissue the SPIOs can be detected well distributed within the tissue by conventional histology and by a 3-photon device in a bio-medical context. System wide scanning is established (MRI, MPI), we investigate subcellular processing and localization of SPIOs in MP.

## 1. Introduction

The most common cancer in women worldwide in western industrialized countries is breast cancer (BC) [1]. Axillary lymph nodes are draining lymphatic fluids from breast tissue [2]. In case of BC therefore, exploration of

the axillary region is part of the surgical staging process in breast cancer [2]. If tumor cells are present in extracted lymph nodes, radical axillary lymph node extraction is recommended followed by high morbidity and significant loss of quality of life (QoL). This can be dramatically decreased by the concept of the so called sentinel lymph

node biopsy (SLNB). Until now, radio nuclides are used as injectable tracer substances to detect sentinel lymph nodes (SLNs), who are the first lymph nodes with tracer enrichment after peri- or intra tumoral or in breast or periareolar injection after a pre-defined waiting time. The SLNs which can then be localized intra operative as when radioactive hot spots are detected by a mobile gamma hand probe and then extracted surgically [3]. The use of radioactive substances within the operating theatre endanger the employees. Therefore we aim to develop a new SLNB method using superparamagnetic iron oxide nanoparticles (SPIOs) and magnetic particle imaging (MPI) as a three-dimensional imaging and distinct localization of SPIOs [4].

There are SPIOs approved specifically for MR imaging (e.g. ferucarbotran, Resovist<sup>®</sup>, Bayer Schering Pharma). After intravenous (i.v.) bolus administration, uptake can be observed in lymph nodes [5]. It is well known from i.v. SPIO application in magnetic resonance imaging (MRI) that macrophages (MPs) are key role player in processing of SPIOs (e.g. in liver) causing a drop of signal intensity [6–11]. So far, few local and systemic adverse effects were noted when SPIOs like Resovist<sup>®</sup> were used within MRI procedures. Nevertheless, knowledge lacks concerning enrichment processes of SPIOs after injection in breast tissue, the adjacent lymphatic tissues and associated cells, especially in BC and metastatic lymph nodes. Therefore, tissue and cell associated processing of SPIOs is of major interest for this SLNB concept. Previously we evaluated the distribution of SPIOs in an in vivo healthy and tumor mouse model [12]. Based on these studies we investigate the processing of the SPIOs in MP [13].

## II. Material and Methods

To evaluate SPIO processing we established a tumor bearing mouse model as we previously published [13]. Tumor tissue was then analyzed by conventionally hematoxylin histology combined with Berlin blue staining and autofluorescence multiphoton microscopy. Life imaging by multiphoton laser microscopy seems to be a feasible method to investigate those SPIO associated processes in active MPs. Due to the development Ti:sapphire fs lasers in the last 10 years, multiphoton-microscopy has become a popular method in life sciences. Its ability to excite a broad variety of intrinsic fluorescent proteins such as FAD [14, 15], NADH [16] or serotonin [17] without additional staining and without extensive tissue damage is ideal for in vivo applications. It was also shown that multiphoton microscopy is able to track quantum dots in the colon to investigate the uptake of nanoparticles by the intestines. Therefore, the MP cell line J774A.1 was incubated either by dextran-coated SPIOs (Resovist<sup>®</sup>) in culture medium (RPMI, FBS), or culture medium only

as control. This process was observed in vitro by multiphoton microscopy [18]. Detection of SPIOs was realized by excitation at 1200 nm.

### II.I. Macrophages

A commercially available mouse monocyte macrophage cell line J774A.1 (BALB/c) was attached to thermanox<sup>TM</sup> coverslips to growth, than incubated in culture medium (RPMI 1640 (Roswell Park Memorial Institute), 20 % fetal bovine serum (FBS), 1 % Penicillin/Streptomycin) and further processed, as described follows. Pre-incubated MPs were washed and further processed in heated 6 well plates with  $10^5$  cells per well for immediate in vitro live cell imaging laser procedure. Cells were analyzed directly after incubation either by 100  $\mu$ l/mg Resovist-Fe (0.5 mmol/l) in culture medium (RPMI 1640, 20 % FBS, 1 % Penicillin/Streptomycin), or culture medium only as control with live cell imaging.

### II.II. Multiphoton microscopy and fluorescence lifetime imaging

The multiphoton microscope used is an upright TriM Scope 2 (LaVision Biotec, Bielefeld, Germany). The detection is realized with H7422-40 photo multiplier tubes (Hamamatsu, Hamamatsu, Japan). The collected signal from the sample is separated from the excitation light by a 700 nm beam splitter. Excitation is realized with two Mai Tai tuneable Ti:sapphire lasers (SpectraPhysics). The MaiTai are combined with a 850 nm beam splitter before entering a beam shaper to provide a pre compensation for the dispersion of the fs pulses in the optics of the microscope. As a third laser source a Insight Deep See (DS, Spectra Physics) broad bandwidth tuneable laser is used. Three pockels cells control laser power at the sample. Exact confocality for all lasers is checked with fluorescent poly styrole beads of 50 nm size.

FLIM is a method that allows distinguishing of different fluorescent species by their fluorescence lifetime. The FLIM data was recorded with 100 frames (at 0.6 frames per second with 1.6  $\mu$ s pixel dwell time) summed up, to get the necessary threshold of photons for fluorescence lifetime fitting. The whole collected fluorescence was directed to the detectors. A 50/50 beam splitter allowed simultaneous image acquisition with the GaAsP PMT of the TriM Scope 2.

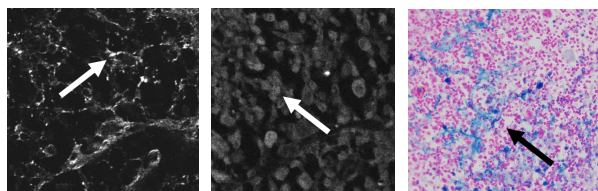
### II.III. Data collection

The scanning is done at a speed of 1000 lines per second with a pixel dwell time depending on the chosen pixel size of the images taken. The laser power for the Insight DS during measurements is about 50 mW at the sample for 1200 nm. For most measurements one MaiTai tuned to 740 nm is used to excited intrinsic fluorescent proteins.

The software to collect the data and control the microscope as well as the MaiTai lasers is the Inspector application (32bit version for Windows XP) provided by LaVision Biotec. The Insight DS is controlled by a separate Laptop with Spectra Physics control software.

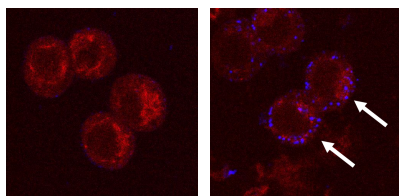
### III. Results

We are presenting our results of SPIO processing in tumor tissue and by MPs, which showed activity in phagocytosis of Resovist<sup>®</sup> after incubation in multiphoton microscopy. After injection of Resovist<sup>®</sup> into tumor tissue and extracting the tissue, SPIOs were detectable within the tissue by conventional hematoxylin microscopy as well as particle processing by 3-photon microscopy (see Fig. 1).



**Figure 1:** Tumor tissue with Resovist<sup>®</sup> (arrows); left: 3-photon microscopy (1200 nm Insight), middle: Autofluoreszenz (MaiTai 740 nm), right: Berlin blue and hematoxylin staining.

MPs showed activity in phagocytosis of Resovist<sup>®</sup> after incubation in vitro in multiphoton microscopy at an excitation of 1200 nm. Recordings were done for 760 nm and 1200 nm excitation separately because the laser pulses from MaiTai and Insight DS are not synchronous. The cell associated SPIO processing signal was detected by live imaging. Resovist<sup>®</sup> showed no toxic effects on macrophages after incubation in live cell imaging, where cell migration and healthy cellular aspect of MPs persists after incubation. Nanoparticle amount within cells increases over time which shows phagocytotic processes (see Fig. 2).



**Figure 2:** In vitro life cell imaging of MPs incubated with Resovist<sup>®</sup> (3-photon microscopy: 0.55 s and 31.10 s: SPIOs within the cells (arrows)).

### IV. Discussion

Radical axillary lymph node detection and extraction are still part of the standard surgery process in BC. To avoid further surgically caused damage of the axillary region, an intra operative tracer targeted imaging and surgically extraction of the SLNs is needed to improve patients QoL. This could be achieved by the use of SPIOs as tracer and MPI as finder [19, 20], may be by using a MPI hand probe later on [21]. The tracer for MPI is easy to obtain and this would make the method accessible to all patients [22]. SPIOs can be detected after injection of Resovist<sup>®</sup> into tumor tissue. They are well distributed within the tissue, confirmed by conventional histology and by a 3-photon device in a bio-medical context. Our own previous findings showed that SPIO detection within tissue is feasible and may be performed with MPI in future scenario [13]. Furthermore, some investigators showed distribution of SPIOs in axillary lymph nodes clinically after i.v. injection within a MRI based SLNB concept in BC [23, 24]. This approach differs to ours, were SPIOs should be locally injected [12]. A new magnetometer device (Sentimag<sup>®</sup>) showed efficacy in detecting SLNs after locally administered SPIOs [25–29]. This procedure is similar to conventional SLNB using an gamma hand probe, and therefore not comparable to the MPI methodology [26, 27]. But, Nanoparticles are widely discussed as environmental toxins [30]. Knowledge lacks concerning enrichment processes of SPIOs after injection in breast tissue, the adjacent lymphatic tissues and associated cells, especially in BC and metastatic lymph nodes. We already evaluated the distribution of SPIOs in an in vivo healthy and tumor mouse model [18, 31]. Cellular uptake of dextran-coated SPIOs without cytotoxic effects, e.g. negative influence on proliferation or promotion of cell death, is demonstrated in vitro within this work and also in experiments on human adult stem cells and others [10, 32, 33]. Other investigators showed similar results in other diseases (e.g. inflammatory diseases, stroke) and tumors than BC [34, 35]. Whether the affinity and magnetic properties of SPIOs changes in cells, e.g. MPs and if so, how, is still under investigation. Therefore, further investigation with MPI is required.

This project is part of a comprehensive test program to develop a new SLNB technique. This might be less complex and incriminating for the patient and the staff, especially if the new MPI hand probe with unilateral solenoid arrangement will be ready for use in the operating theater [19, 36, 37].

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