Multispectral optoacoustic tomography resolves smart probe activation in vulnerable plaques

Daniel Razansky[1], Niels J. Harlaar[1], Jan-Luuk Hillebrands[2], Adrian Taruttis[1], Eva Herzog[1], Clark Zeebregts[2], Goitzen van Dam[2], and Vasilis Ntziachristos[1]

[1]Institute for Biological and Medical Imaging,
Technical University of Munich and Helmholtz Center Munich, Germany;

[2]University Medical Center Groningen, the Netherlands

ABSTRACT

In this work, we show, for the first time to our knowledge, that multispectral optoacoustic tomography (MSOT) can deliver high resolution images of activatable molecular probe's distribution, sensitive to matrix metalloproteinases (MMP), deep within optically scattering human carotid specimen. It is further demonstrated that this method can be used in order to provide accurate maps of vulnerable plaque formations in atherosclerotic disease. Moreover, optoacoustic images can simultaneously show the underlining plaque morphology for accurate localization of MMP activity in three dimensions. This performance directly relates to small animal screening applications and to clinical potential as well.

Keywords: Multispectral optoacoustic tomography, atherosclerosis, molecular imaging, carotid plaques

I. INTRODUCTION

It is currently generally accepted that high activity levels of certain biomarkers, such as cathepsins, integrins, and matrix metalloproteinase (MMP), are associated with atherosclerotic plaque instability [1]. However, high fidelity imaging of those markers is challenging due to intense scattering of light in biological tissues, limiting the ability to deliver accurate information on structure and molecular activity in large tissue volumes. Even though plaque anatomy and physiology can be achieved by different methods such as duplex ultrasound [2], CT-scan [3], MRI [4] or intravascular optical coherence tomography (OCT) [5], imaging of core biological processes involved in plaque formation on a cellular and molecular level, *e.g.* MMP and protease up-regulation is limited with these modalities.

Multispectral optoacoustic tomography (MSOT) has recently drawn vast attention due to its ability to resolve intrinsic optical contrast and extrinsically administered probes deep in highly scattering biological tissues while preserving high spatial resolution, characteristic of diffraction limited ultrasonic imaging. The technique illuminates the imaged object using pulsed light at multiple wavelengths and records the generated ultrasonic response in a tomographic detection setting. So far, it has demonstrated the ability to resolve three-dimensional distribution of spectrally distinct contrast agents and bio-markers, such as fluorescence molecular agents [6] and fluorescent proteins [7].

Herein, we developed an MSOT method suitable for simultaneous high-resolution visualization of morphology and molecular activity in intact tissues. Human carotid plaque sample from a symptomatic patient was incubated with a MMP-sensitive activatable fluorescent probe (MMPsense 680, VisEn, Medical, Boston, Mass) directly after endarterectomy. An intact sample was subsequently imaged in the MSOT scanner to acquire volumetric images of plaque anatomy and distribution of MMP activity with 100 micron resolution. The hot and cold spot regions, identified by MSOT of an intact specimen, had a good correspondence to planar fluorescence imaging of cryosliced plaque, and was further validated by observing macrophage and smooth muscle cells appearance in immunohistochemistry and

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immunofluorescence. Based on the achieved results, it is anticipated that in a future clinical setting, MSOT could provide new ways for highly specific visualization and staging of plaque vulnerability in atherosclerosis.

II. MATERIALS AND METHODS

A standard surgical endarterectomy was performed on an 81-year old male patient with a symptomatic, <80%, carotid stenosis. Directly after the endarterectomy the plaque was incubated for one hour in a MMP-sensitive activatable probe (MMPSense 680, VisEn Medical, Bosten, Mass) at the temperature of 37 degrees Celsius. MMPSense™ 680 is a protease activatable fluorescent in vivo imaging agent that is activated by key matrix metalloproteinases (MMPs) including MMP-2, -3, -9 and -13. In its unactivated state MMPSense does not produce fluorescence and becomes highly fluorescent following protease-mediated activation. However, from the MSOT imaging perspective, it is more important what the optical absorption characteristics of inactive versus active probe are. The extinction spectra of MMPSense is shown in Fig. 1. Since the final goal of the MSOT investigation is resolving the maps of activated MMP, one could simply subtract optoacoustic images acquired at 675 and 635 nm. Inactivated MMP has similar extinction coefficent for these two wavelengths (see gray curve in Fig. 1), while extinction of the activated one drops by at least 60% at 635 nm as compared to 675nm (blue curve in Fig. 1). This property can be utilized in order to effectively resolve MMP activity over non-specific accumulations of the probe. After the incubation the plaque was snap frozen in liquid nitrogen and stored into a -80 degrees freezer.

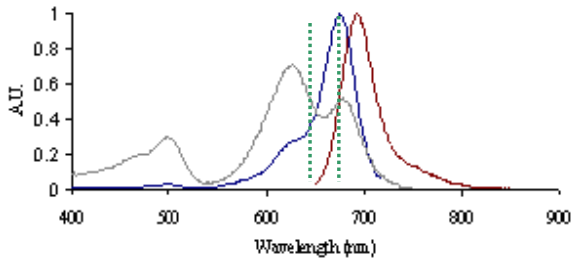

Fig. 1. Extinction coefficient of activated (shown in blue) versus inactive MMPSense 680 probe.

The agar-embedded sample was transported into the multispectral optoacoustic tomography (MSOT) scanner [8]. It consisted of a tunable MOPO laser (Quanta-Ray MOPO-700, Spectra Physics), pumped by a Q-switched Nd:YAG laser (Quanta-Ray Lab-Series 190-30, Spectra Physics) operating at its third harmonic (355nm). The pulse duration of the laser is less than 10ns and the repetition rate is 30Hz. The output laser beam size is adjusted to fit the size of the specimen. The beam splitter splits the beam into two equal-intensity parts being guided from two opposite directions onto the object's surface through two transparent windows in the imaging tank filled with water. In this way, optimal excitation conditions close to uniform illumination are achieved. Wideband piezoelectric PZT transducer (V319, 3.5Mhz central frequency, Panametrics-NDT UT Transducers, Olympus) was used to detect the optoacoustic signals of the illuminated sample. The transducer is cylindrically focused in the imaging plane (38 mm focal distance) to allow 3D data acquisition via vertical scanning. Two 45° tilted mirrors are used to change the beam height by moving the bottom mirror with a vertical translation stage. The beam splitter and the ultrasonic transducer are translated by the same stage, thus both the illumination and detection planes are translated simultaneously to allow for 3D image acquisition via vertical scanning. The sample is mounted on a rotational stage so that in-plane tomographic data acquisition is done by 360 degrees rotation of the sample. A 14-bit resolution PCI digitizer with a sampling rate of 100 MS/s (NI PCI-5122, National Instruments Corp., Austin) is used to record the time-resolved acoustic signals detected by the transducer. We place a photodiode (FDS010, 200-1100 nm, 1 ns Rise Time, Thorlabs GmbH) in the vicinity of the laser output window to record the intensity change of each pulse and to normalize the detected signals for laser output instabilities. This continuous power monitoring is of critical importance for multispectral reconstructions since some of important biomarkers may present only a small variation of the optical absorption over highly absorbing background, in which case even small quantification inaccuracies may lead to uninterpretable results. The laser, stage controllers, and data acquisition and all normalized via Labview-based interface (National Instruments Corp., Austin, TX). Single wavelength two-dimensional data acquisition took 30 s using continuous acquisition method with 900 projections [R. Ma et al., Med

Phys, in review]. Full 3D data set with 3 wavelengths and 10 vertical slices took about 20 minutes. Image reconstruction for each vertical slice required 3sec and was performed by using a two-dimensional filtered back-projection (cylindrical Radon) algorithm [Nat Phot, 2009], [Phys Med Bio, 2009].

Immediately following the MSOT imaging session, the agar-embedded specimen was placed into 30 mm ∅ syringe (Falcon, Becton Dickinson, Franklin Lakes, USA) and OCT was added. The syringe and its content were snap-frozen to -80^{0}C. After freezing, cryosections through the entire sample were made with 50 μm steps. Every 1mm, the thickness of the sections was refined to 10μm for histopathology. Selective 50 μm slices (approx. every 1mm) were imaged in the epi-fluorescent setup described above to attain scattering-free images of fluorescence activity in each slice.

In order to confirm presence of active MMPs in area's with increased fluorescence as identified by MSOT, a cryosection (10 μm) from the corresponding area was subjected to *in situ* zymography to visualize gelatinase activity. To this end the section was incubated with DQ^{TM}-gelatin (100 μg/ml, EnzCheck® Gelatinase/Collagenase Assay Kit, Invitrogen-Molecular Probes, Breda, the Netherlands) dissolved in 1% agarose (in PBS, Type IX, Ultra-low Gelling Temperature, Sigma-Aldrich Chemie B.V., Zwijndrecht, the Netherlands) overnight at 37°C. 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) was added to the 1% agarose solution to stain nuclei. To control for autofluorescence, a serial section was incubated with 1% agarose/PBS/DAPI in the absence of DQ^{TM}-gelatin. Sections were analyzed on a Leica DMLB fluoresensce microscope (Leica Microsystems, Rijswijk, the Netherlands) equipped with a Leica DC300F camera and LeicaQWin 2.8 software.

III. RESULTS

In Figure 2, the results of optoacoustic imaging of an intact sample show good correspondence to the color photographs of a sliced plaque. The differences can be attributed to the fact that it has been difficult to exactly match the location of cryo-slices to the heights at which the MSOT images of an intact sample were made. The optoacoustic data is shown at a single wavelength of 635nm where MMPSense has low excitation efficiency so that only morphological information can be extracted from the images. The various details of internal plaque anatomy are readily recognizable in Fig. 2(a) with in-plane resolution on the order of 200 μm, limited by the useful bandwidth of the ultrasonic detector (~4Mhz).

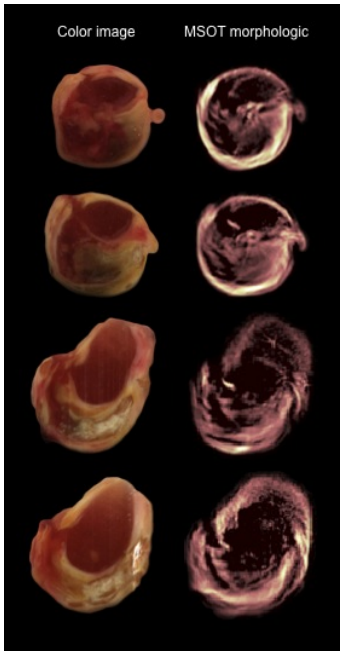

Figure 2. Morphologic characterisation compared with optoacoustic images of an intact plaque sample (left column) and RGB images of a dissected plaque (right column).

As previously described, in order to resolve MMP activity over non-specific intrinsic tissue background and inactivated probe accumulations, we perform and subtract MSOT images at two wavelengths, namely 635 nm and 675 nm. The results are shown in Fig. 3(c-d), revealing the locations with high activity level of MMP, the so called hot spot regions. In order to validate the MSOT findings, we compare them to cryo-sections of the plaque, shown in Fig. 3(a-b). Both methods reveal the highest level of MMP activity close to the bifurcation area of the carotid artery.

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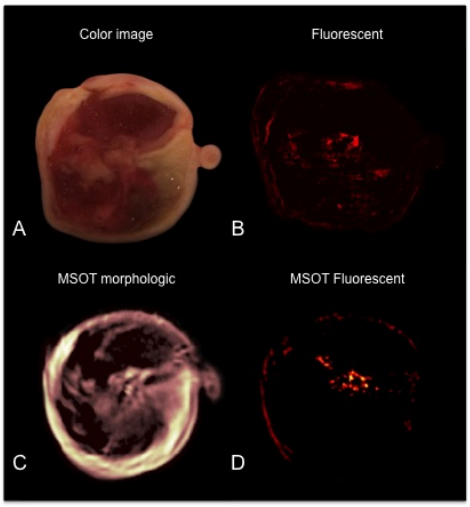

Figure 3. Localization of MMP activity. A)color image of cryosection in the birfucation area; B)Epi-fluorescent image of the cryosection ; C)Corresponding morphological optoacoustic cross-sectional image of an intact plaque sample using single 635 nm wavelength; D) Result from MSOT reconstruction of an intact plaque sample revealing location of MMPSense 680 activity in the corresponding slice.

In order to confirm the MSOT data showing differential MMP activity within an atherosclerotic plaque area, additional histological analyses were performed. *In situ* zymography was performed on 10 µm cryosections to visualize presence of active gelatinases, *i.e.* MMP's able to degrade gelatin (Figure 4). Based on the MSOT and epi-fluorescence data, two regions of interest were selected based on the presence (denominated as a 'hot-spot') or relative absence (denominated as 'cold-spot') of the signal (A). Incubation with dey-quenched (DQ) gelatin as a substrate for MMP's revealed increased gelatinase activity in the 'hot-spot' area (B) compared with the 'cold-spot' area (C). Within the 'hot-spot' active MMP's were primarily extracellularly located. Within the 'cold-spot' area active MMP's were detected which were, however, primarily located within the vascular media (C). Although some fluorescence was detected within the plaque area of the 'cold-spot', this is most likely the result of autofluorescence since after incubation in the absence of DQ-gelatin fluorescence was still detected (E). The autofluorescence is probably derived from necrotic cell debris and calcified fragments as also suggested by the aberrant nuclear staining pattern with DAPI and absence of nuclei with normal morphology (C, E). In the 'hot-spot' area autofluorescence was virtually absent (D).

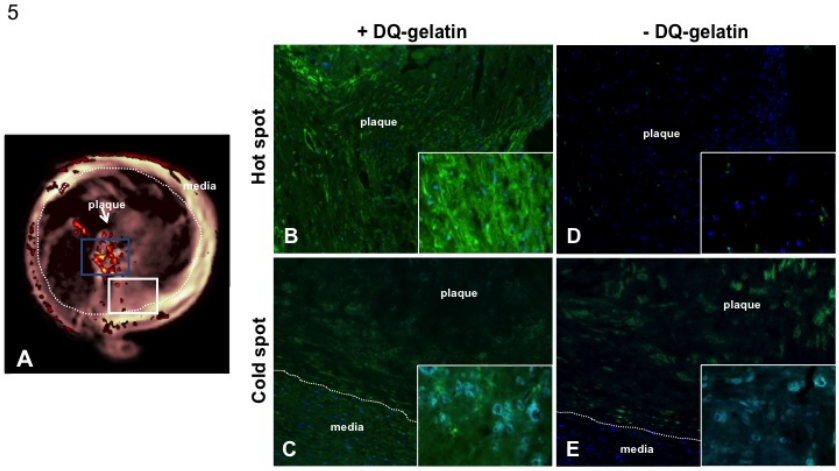

Figure 4. 'Hot spot' area identified by MSOT is characterized by increased gelatinase activity. *In situ* zymography was performed on 10 μm cryosections corresponding to the area that revealed enhanced MMP activity as identified by MSOT (A). Incubation with dye-quenched (DQ) gelatin on the 'hot spot' (B) and 'cold spot' (C) area. To check for the presence of autofluorescence, cryosections were incubated in the absence of DQ-gelatin and 'hot spot' (D) and 'cold spot' (E) area's were analyzed. Nuclei were stained with DAPI. Original magnifications: B-E: 160x; insets in B-E: 640x.

IV. DISCUSSSION AND CONCLUSIONS

The indication for a carotid endarterectomy is nowadays mainly based on symptomatology or, alternatively, on the degree of stenosis in carotid arteries (>80%). Those indications however do not provide an accurate assessment of plaque vulnerability and therefore only a small percentage of patients do actually benefit from the surgical intervention by preventing a major cerebro-vascular event especially in the asymptomatic group[12, 13].

Indeed, introduction of fluorescent molecular probes, sensitive to plaque vulnerability, has proven to provide a generally good indication of the existence of inflammatory processes. Nonetheless, volumetric fluorescence imaging of plaque activity was so far limited by high degree of scattering in large tissue volumes, which makes it difficult to accurately localize, quantify and characterize the morphology of the plaque and its vulnerability.

Herein, we developed a multispectral optoacoustic tomography (MSOT) method suitable for simultaneous high-resolution visualization of morphology and molecular activity in intact tissues. Human carotid plaque sample from a symptomatic patient was incubated with a MMP-sensitive activatable fluorescent probe (MMPsense 680, VisEn, Medical, Boston, Mass) directly after endarterectomy. An intact sample was subsequently imaged in the MSOT scanner to acquire volumetric images of plaque anatomy and distribution of MMP activity with 100 micron resolution.

We further validated the MSOT studies by performing epi-fluorescence imaging of cryo-sections and *in situ* zymography. The results clearly show a correspondence from the detected "suspected" area containing a high level of inflammatory activity, macrophage influx and MMP activity. Results from both MSOT investigations and histological sections confirmed that most of the vulnerable plaque activity occurs, as expected, close to the bifurcation area of the carotid artery.

Based on the achieved results, it is anticipated that in a future clinical setting, MSOT could provide new ways for highly specific visualization and staging of plaque vulnerability in atherosclerosis. Furthermore, optoacoustics is an inherently three-dimensional visualization tool that has already proven to be capable of penetrating several centimeters into biological tissues [9]. The spatial resolution of the method is not affected by degree of scattering, thus, it can deliver high

quality data from large diffuse tissue volumes, not accessible by other optical imaging techniques. We therefore foresee our MSOT method to be implemented as a noncontact diagnostic tool for accurate detection and characterization of cardiovascular disease and biomarker activity.

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