In vivo photoacoustic mapping of lymphatic systems with plasmon-resonant nanostars†‡

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Plasmon-resonant nanostars (NSTs) provide excellent contrast enhancement for photoacoustic tomography. The high photoacoustic sensitivity of NSTs at near-infrared wavelengths enables their in vivo detection in rat sentinel lymph nodes and vessels, with direct application toward lymphangiography.

Noninvasive medical imaging systems have been widely used in clinics to improve diagnostic accuracy and treatment outcomes in cancer patients. Along with the development of imaging techniques, molecular and nanosized contrast agents have been actively explored to improve detection sensitivity and specificity in biomedical imaging. Here we demonstrate that plasmon-resonant nanostars (NSTs) are excellent contrast agents for in vivo photoacoustic tomography (PAT), with a detection limit at 1 ppm (~1 µg mL⁻¹) Au. Gold NSTs have a strong optical response at near-infrared (NIR) wavelengths, and have been recently deployed as contrast agents for optical imaging and also as substrates for SERS and other plasmon-enhanced sensing modalities. In this article, we use in vivo spectroscopic PAT to monitor the accumulation of NSTs in the sentinel lymph nodes (SLNs) of a rat specimen shortly after a footpad injection, which was later confirmed by ex vivo PAT and inductively coupled plasma mass spectrometry (ICP-MS).

PAT is a nonionizing, noninvasive, and high-resolution imaging technique that provides strong optical absorption contrast. Since the spatial resolution of this technique typically depends on ultrasound (US) parameters, the imaging depth can be extended to the optical quasidiffusive or diffusive regime while keeping high resolution. The depth-to-resolution ratio of PAT (defined as the ratio of maximum penetration depth to depth resolution) is greater than 100 for all imaging depths. PAT can provide structural information (e.g., vascular networks or melanomas) using the intrinsic PA contrast produced by hemoglobin or melanin, and also functional information based on total hemoglobin concentration, oxygen saturation, or blood flow. Exogenous materials such as nanoparticles also play an important role by enhancing PA contrast for molecular imaging and noninvasive SLN mapping. The PA sensitivities for detecting organic dyes and nanoparticles range from 10 nM at 0.3 mm resolution (~1 fmol per imaging voxel) to 10 pM at 0.06 mm resolution (~0.1 attomole per imaging voxel). Au-based nanoparticles have been approved by FDA as drug carriers or therapeutic agents for various phase-I clinical trials (e.g., http://www.nanospectra.com). From a clinical perspective, conventional US array systems can be easily adapted to perform both PA and US imaging, and hand-held, array-based probes have recently been designed.

Gold NSTs with an average span of 120 nm (Fig. 1a) were prepared by a seeded growth method as previously described, starting from either 13 nm core-shell Fe₃O₄@Au particles (sample A) or from 8 nm Au particles (sample B). Both samples were synthesized

![Figure 1](image-url)

**Fig. 1** (a) TEM image of plasmon-resonant Au nanostars (NSTs, sample A). (b) Extinction spectrum of diluted sample A. The absorbance (arbitrary unit) of the Au nanostars is ~0.75 at 767 nm. (c) A plot of photoacoustic (PA) amplitudes as a function of NST concentration (see ESI† for details).
using freshly prepared growth solution; NST growth was complete within 15 minutes after seed addition (see ESI† for details). The CTAB-stabilized NSTs were purified by treatment with a poly-styrenesulfonate solution to remove excess CTAB, then resuspended in 1% bovine serum albumin (BSA) to produce NSTs in their final, biocompatible form. The extinction spectra of BSA-stabilized NSTs with and without magnetic cores (Fig. 1b and S1, ESI†) confirm their strong NIR activities, in accord with earlier reports.3,11 PAT imaging was performed in spectroscopic mode using a developed deep-reflection modality (see ESI† for details). The PA sensitivity was calibrated using two Tygon® tubes, one filled with aqueous suspensions of NSTs at various concentrations, the other with defibrinated bovine blood. PA signals from plasmon-resonant NSTs in tissue phantoms (sample A) were detected at a concentration of 1 ppm Au with a signal-to-noise ratio of 4 ($\lambda_{\text{ex}} = 767$ nm; Fig. 1c). This detection limit corresponds roughly to NST concentrations in the femtomolar range, with $10^4$ to $10^5$ NSTs per imaging voxel (see ESI† for details).

The PA contrast was evaluated by performing a volumetric in vivo mapping of rat lymphatic systems (Fig. 2). Sprague-Dawley rats were anesthetized according to a standard protocol (see ESI† for details) with hair removed from the left axillary region in order to obtain control PA images, prior to the introduction of NSTs (Fig. 2a and d, top). The vascular networks passing through the axilla are clearly visible due to the intrinsic PA contrast of hemoglobin, however, neither the lymphatic vessels nor lymph nodes can be observed.

Rats were then inoculated with an injection of BSA-stabilized NSTs in the left footpad (0.1 mL of sample A, ~100 µg Au), and the left axilla was monitored by in vivo spectroscopic PAT. At 1.5 h post-injection, the SLN and lymphatic vessels (yellow) clearly stand out due to the exogenous contrast generated by the NSTs (Fig. 2b). The SLN is located 1.5 mm below the skin (Fig. S3, ESI†), but can be clearly resolved by subtracting the control PA image from the post-injection image (Fig. 2b). A volumetric rendering of the PA contrast reveals how the blood vessels and lymphatic vasculatures are interwoven (Fig. 2c and Movie S1, ESI†). The accumulation of NSTs in the SLN was visually confirmed after the in vivo imaging study, upon removal of the skin from the left axillary region (Fig. 2d, bottom). We note that Au NSTs without Fe$_3$O$_4$ cores produce comparable PA contrast (see below), as this is derived solely from their plasmon-resonant optical properties.

Fig. 2 In vivo photoacoustic (PA) mapping of rat lymphatic systems, enhanced using NSTs (sample A). (a) Control PA image acquired before NST injection, displaying only blood vessels (BV). (b) PA difference image acquired post-injection ($t = 1.5$ h), revealing the sentinel lymph node (SLN) and lymphatic vessels (LV). A separate intensity scale (yellow) was applied to the lymphatic system after subtraction of image (a). (c) 3D rendering of (b). (d) Photographs of the left axillary region before inoculation with NSTs (top) and after in vivo PA imaging (bottom), revealing the accumulation of NSTs in the SLN. (e) Photograph (top) and ex vivo PA image (bottom) of excised SLNs; the left axillary region (closest to the injection site) contains a visibly high concentration of NSTs, whereas the SLN from the right axillary region does not. (f) In vivo and ex vivo spectroscopic PA amplitudes measured within the SLNs, 1.5 h after inoculation with NSTs.
The SLNs from the left and right axillae were harvested for *ex vivo* spectroscopic PAT. Only the left SLN (closest to injection site) accumulated an appreciable amount of NSTs, as indicated by both visual inspection and PA imaging (Fig. 2e). The spectral amplitudes measured by *in vivo* and *ex vivo* imaging produced similar trends ($r^2 = 0.99$), confirming the SLN as the source of PA signal.

*Ex vivo* PA imaging was also performed at visible wavelengths ($\lambda_{ex} = 570$ nm) on lymph nodes containing gold NSTs (sample B) using optical-resolution PA microscopy (see Fig. 3 and ESIF). In addition to confirming their strong PA contrast at shorter wavelengths, the microscopic PA image revealed the relative distribution and density of NSTs throughout the SLN. The NSTs appear to accumulate in both the subcapsular and trabecular sinuses, implying their diffusion into successive lymph nodes.

The PA image contrasts from *in vitro*, *in vivo*, and *ex vivo* studies are compared in Table 1, using an excitation wavelength of 767 nm. The image contrast is defined simply as $(S - B)/B$, where $S$ is the mean PA signal from objects of interest (e.g., SLNs or blood vessels) and $B$ is the mean background signal. For *in vitro* imaging, the PA contrast of NSTs at 1000 ppm Au is $\sim$10 times stronger than that of whole blood, whereas *in vivo* studies indicate the mean PA contrast of NSTs accumulated in SLNs (1.5 h after injection) to be $\sim$3 times stronger than that of nearby blood vessels. The *ex vivo* PA contrast produced from NSTs in excised SLNs (an average weight of $\sim$50 mg) was similar to that above; ICP-MS analysis indicated a loading of 16 ppm Au, corresponding to a mean injected dose percentage $\text{NTs (1.5 h after injection) to be} \sim 3\times$ times stronger than that of nearby blood vessels.

Although *in vivo* and *ex vivo* spectroscopic PA imaging confirm the uptake of NSTs in the SLNs, it is worth mentioning that their excitation spectra do not coincide with the initial optical (extinction) spectrum of the NSTs in solution (Fig. 1b). Reasons for this discrepancy may be attributed as follows: (i) the NST extinction spectrum is defined in part by plasmon-resonant scattering, whereas PA imaging is mostly sensitive to optical absorption; (ii) the NSTs are likely to be aggregated upon accumulation in SLNs, causing significant changes in their collective optical response. We note similar changes in the optical response of NSTs when dispersed into tissue phantoms (not shown).

Lymph node mapping has become an important objective in the detection and subsequent prevention of early stage metastases. The image contrast is defined simply as $(S - B)/B$, where $S$ is the mean PA signal from objects of interest (e.g., SLNs or blood vessels) and $B$ is the mean background signal. For *in vitro* imaging, the PA contrast of NSTs at 1000 ppm Au is $\sim$10 times stronger than that of whole blood, whereas *in vivo* studies indicate the mean PA contrast of NSTs accumulated in SLNs (1.5 h after injection) to be $\sim$3 times stronger than that of nearby blood vessels. The *ex vivo* PA contrast produced from NSTs in excised SLNs (an average weight of $\sim$50 mg) was similar to that above; ICP-MS analysis indicated a loading of 16 ppm Au, corresponding to a mean injected dose percentage $\text{NTs (1.5 h after injection) to be} \sim 3\times$ times stronger than that of nearby blood vessels.

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![Fig. 3](https://example.com/fig3.png)  
(a) Brightfield optical microscopy of an excised SLN, harvested from a rat specimen 1.5 h after inoculation with solid Au NSTs (sample B). (b) *Ex vivo* PA image of the excised SLN, using a micro-PAT system operating at shorter wavelengths ($\lambda_{ex} = 570$ nm).

### Table 1  
Photoacoustic (PA) image contrast of NSTs derived from *in vitro*, *in vivo*, and *ex vivo* studies (sample A, N = 3)

<table>
<thead>
<tr>
<th></th>
<th>Blood</th>
<th>NSTs (ppm Au)</th>
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<tbody>
<tr>
<td><em>In vitro</em></td>
<td>5 ± 1</td>
<td>51 ± 3\text{a}</td>
</tr>
<tr>
<td><em>In vivo</em></td>
<td>18 ± 4</td>
<td>59 ± 22\text{b}</td>
</tr>
<tr>
<td><em>Ex vivo</em></td>
<td>—</td>
<td>44 ± 21\text{b}</td>
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* a [NST] = 1000 ppm; mean and standard deviation.  
* b NSTs in SLN; mean and standard error.

### Notes and references