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Ultrasound-guided Immunofunctional Photoacoustic Imaging for Diagnosis of Lymph Node Metastases

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Abstract

Metastases rather than primary tumors determine mortality in the majority of cancer patients. A non-invasive immunofunctional imaging method was developed to detect sentinel lymph node (SLN) metastases using ultrasound-guided photoacoustic (USPA) imaging combined with glycol-chitosan-coated gold nanoparticles (GC-AuNP) as an imaging contrast agent. GC-AuNPs, injected peritumorally in breast tumor-bearing mice, were uptaken by immune cells, and subsequently transported to the SLN. Two-dimensional and three-dimensional USPA imaging was used to isolate the signal from GC-AuNP-tagged cells. Volumetric analysis was used to quantify GC-AuNP accumulation in the SLN after cellular uptake and transport by immune cells. Results show that the spatio-temporal distribution of GC-AuNPs in the SLN was affected by the presence of metastases. A parameter describing the spatial distribution of GC-AuNP-tagged cells within the SLN was more than 2-fold lower in metastatic lymph nodes compared with non-metastatic controls. Histological analysis confirmed that distribution of GC-AuNP-tagged immune cells is changed by the presence of metastatic cells. The USPA immunofunctional imaging successfully distinguished metastatic from non-metastatic lymph nodes using biocompatible nanoparticles. This method could aid physicians in detection of micrometastases, thus guiding SLN biopsy and avoiding unnecessary biopsy procedures.

Graphical Abstract

A minimally invasive method to detect lymph node metastases using glycol-chitosan-coated gold nanoparticles and ultrasound-guided photoacoustic imaging.

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Conflicts of interest

The authors declare no conflict of interest.

Introduction

In many types of cancer such as breast, melanoma, prostate, and head and neck, tumor cells initially spread via the lymphatic route, lodging in the sentinel lymph node (SLN), which is the first node in the tumor drainage path. The detection of these tumor cells is fundamental in cancer staging and is critical to determine patient prognosis and suitable treatments. In fact, nodal involvement and consequent treatment strategies are significant predictors of locoregional recurrence and development of distant metastases¹. Thus, it is critical to promptly and accurately assess sentinel lymph nodes in cancer patients.

The clinical standard for the assessment of sentinel lymph nodes is SLN biopsy. However, during this procedure, locating the SLN is a challenge in itself and currently requires the use of radioactive contrast agents^{2–5}. Upon localization of the radiotracer, the SLN is surgically removed and a pathologist examines the tissue for the presence of cancer cells.

Unfortunately, the surgical procedure is associated with morbidity due to the removal of the node, and histological analysis is prone to miss small micrometastases, with false-negative rates as high as 60%^{6–7}. Thus, a non-invasive tool to assess lymph node metastases with reduced morbidity would be of benefit to clinicians and cancer patients, enabling faster, accurate diagnosis.

Ultrasound-guided photoacoustic imaging (USPA) has emerged as a synergistic imaging modality capable of anatomical, functional, and molecular visualization of pathology. Ultrasound is widely used in the clinic to study tissue morphology but suffers from poor contrast. Synergy of ultrasound with photoacoustics is based on using the anatomical information provided by ultrasound to guide photoacoustic imaging, which provides

functional and molecular information with enhanced contrast^{8–11}. Several studies have shown applications of USPA for lymphatic imaging and SLN mapping using contrast agents^{12–17}. Given the potential for anatomical, functional and molecular evaluation of the lymph node, USPA and other imaging modalities have been explored as alternatives to SLN biopsy for diagnosis of metastases, in order to avoid invasiveness and reduce cost, while maintaining clinical accuracy^{18–22}. However, these attempts still face challenges such as low sensitivity, low specificity, and potential side effects of the contrast agents.

To address these challenges, we developed an immunofunctional USPA imaging method for identifying sentinel lymph node metastases, using ultrasound-guided photoacoustic imaging augmented with glycol-chitosan-coated gold nanoparticles (GC-AuNPs). Our approach (Fig. 1) is based on the fact that GC-AuNPs are uptaken by immune cells upon peritumoral injection. After lymphatic transport by the immune cells, the presence of metastases affects the spatio-temporal distribution of GC-AuNP-tagged immune cells in the SLN. This effect can be monitored via USPA imaging and quantified to obtain a diagnostic result.

Gold nanoparticles (AuNPs) are one of the most commonly used photoacoustic contrast agents due to their high optical absorption and versatility for various surface modifications. The use of AuNPs entails various benefits for immunofunctional imaging. Well-dispersed AuNPs have a negligible near-infrared (NIR) optical absorption and are thus invisible in photoacoustic images until they are aggregated by cellular uptake. The cellular endocytosis changes the optical absorption of AuNPs, making them visible in photoacoustic imaging with NIR wavelength excitation^{23–25}.

Glycol-chitosan enhances hydrophilicity, stability and biocompatibility of AuNPs²⁶. In previous studies, we found that GC-AuNPs could be effectively used for *in vivo* contrast-enhanced photoacoustic mapping of cervical lymph nodes in healthy mice, generating high-contrast images due to uptake by immune cells²⁷. Further *in vitro* studies have shown that, within 24 hours, GC-AuNPs are preferentially uptaken by immune cells rather than cancer cells²⁷. The nanoparticle's neutral to weak-positive surface charge facilitates protein adsorption and subsequent immune cell uptake²⁸. To enhance the delivery of agents to the lymph nodes, both chitosan and glycol-chitosan have been studied as vaccine adjuvants because they regulate immune response, thus promoting immune cell activation^{29–32}. Glycol-chitosan, however, has a much better solubility than chitosan. Furthermore, cationic particles are known to promote trafficking and retention in the lymph node^{33–35}. These properties of glycol-chitosan are advantageous for lymphatic imaging and motivated the validation of our method *in vivo*.

In the immunofunctional imaging approach, skin resident dendritic cells may play a significant role in transporting GC-AuNPs to the sentinel lymph node. Additionally, lymph node resident immune cells may also recognize and uptake passively-drained GC-AuNPs, further increasing the local photoacoustic signal. Overall, the nanoparticle-tagged cells in the lymph nodes may consist of peripheral migratory immune cells and resident immune cells.

Previous research has studied changes in immune cell transport as a method to find metastatic lymph nodes by using iron oxide nanoparticles (IONPs) and MRI. Upon

intravenous injection, healthy lymph nodes showed an increased, homogeneous presence of IONPs, after being uptaken and transported by immune cells^{18–19}. On the other hand, metastatic lymph nodes showed decreased, heterogeneous accumulation. However, this method is associated with several challenges. The intravenous administration requires a much higher dose to achieve sufficient agent delivery and imaging sensitivity. Additionally, an intravenous delivery offers no distinction between sentinel and non-sentinel nodes. This, together with negative contrast of IONPs, makes the method not time-effective nor easily compatible with the current diagnostic paradigm.

Another study suggested using IONPs similarly for *ex vivo* photoacoustic detection of metastases³⁶. However, IONPs are poor NIR absorbers thus affecting the imaging sensitivity at depth. As such, *in vivo* attempts failed to detect differences between metastatic and non-metastatic groups³⁷.

In this study, we intend to overcome these challenges by using a contrast agent that is highly lymphotropic, promotes immune cell interaction, and generates high-contrast photoacoustic images, providing highly sensitive diagnosis.

Results and discussion

Glycol-chitosan and polyethylene glycol (PEG) coated gold nanoparticles were synthesized by reduction of chloroauric acid. The UV-Vis absorbance spectra of nanoparticles (Fig. 2(a)) showed surface plasmon resonance (SPR) peaks at 528 nm for GC-AuNPs and at 523 nm for PEG-AuNPs. The difference in locations of SPR peaks is due to the surface properties caused by different coating materials. Transmission electron microscopy (TEM) images (Fig. 2(b)) showed the ~20-nm size and spherical morphology of both GC-AuNPs and PEG-AuNPs. In addition, the TEM image of GC-AuNPs revealed the coating layer of glycol chitosan around GC-AuNPs. Dynamic light scattering measurement of the particles indicated that the hydrodynamic diameter of GC-AuNPs was 94.5 ± 46.5 nm while that of PEG-AuNPs was 27.8 ± 12.1 nm. Even though the sizes of both particles were similar to each other in TEM images, their hydrodynamic diameters were drastically different because of the hydrophilicity of glycol chitosan. Two distinct zeta potential values also reflected the difference between GC-AuNPs (37.4 ± 4.4 mV) and PEG-AuNPs (-3.3 ± 0.8 mV). The reason that GC-AuNPs had more a positive value was due to the existence of amine groups in glycol chitosan.

To characterize the interaction of the nanoparticles with immune cells, macrophages were incubated with either GC-AuNPs or PEG-AuNPs. Each nanoparticle type showed different behavior in cellular uptake by macrophages (Fig. 2(c)). Dark-field images (Fig. 2(d)) revealed that more GC-AuNPs were endocytosed by macrophage cells while PEG-AuNPs were not: the enhanced cellular uptake and aggregation GC-AuNPs caused the intense signals in the dark-field microscopy images.

For *in vivo* experiments, breast tumor-bearing mice (non-metastatic group) were injected peritumorally with GC-AuNPs for drainage into the sentinel lymph node. USPA images of the SLN were acquired before and immediately after injection, and then after drainage to the

inguinal lymph node (sentinel node). Conventional B-mode ultrasound identified the location of the SLN. The accumulation of GC-AuNP in the SLN after uptake by immune cells was visualized using PA imaging. Upon injection and cell endocytosis, GC-AuNPs exhibited substantial enhancement of imaging contrast. USPA images before and 24 hours after injection demonstrate a 10-fold increase of PA signal in a non-metastatic lymph node (Fig. 3). Prior to injection, low background PA signals were present due to endogenous absorbers, i.e. hemoglobin (Fig. 3(a)). Upon uptake and transport of the injected GC-AuNPs, the PA contrast was enhanced at the sentinel lymph node as well as in the lymphatic vessel. A homogeneous distribution of GC-AuNP signal was observed in the SLN (Fig. 3(b)). Multiwavelength PA was used to characterize the absorption spectrum of the lymph node, showing the 10-fold increase in signal at 700 nm (Fig. 3(c)). This increase enables single-wavelength imaging of GC-AuNPs, therefore simplifying image acquisition and analysis. Single-wavelength imaging leads to shorter imaging time, simpler hardware, and computational effectiveness, but may suffer from poor signal-to-background ratio. Alternatively, to locate GC-AuNPs, a more time-consuming multiwavelength imaging can be used.

Following the findings from non-metastatic mice, GC-AuNPs were injected in animals with metastatic breast cancer, and both non-metastatic and metastatic groups were compared. The inguinal lymph nodes, i.e. sentinel lymph nodes, were identified as a hypoechoic bean-shaped region in the ultrasound images (yellow contour on Fig. 4(a,d)). The overlaid two-dimensional ultrasound and photoacoustic images visualized the endocytosed GC-AuNPs in the tumor-draining lymphatic vessel and the inguinal lymph nodes (Fig. 4(b,e)). Visualization of the lymphatic vessel is critical to confirm that nanoparticles indeed traveled to the SLN in cases where the lymph nodes display low or no accumulation of GC-AuNP. However, to better evaluate the distribution of GC-AuNPs in the SLN, the combination of ultrasound and photoacoustic imaging was used to segment the image and visualize only the signals within the sentinel lymph node (Fig. 4(c,f)). The images of the segmented SLN after drainage showed a different distribution pattern of the GC-AuNP-tagged cells in the SLN in the metastatic compared to non-metastatic animal. Two-dimensional PA images effectively demonstrated a decrease in the SLN area associated with GC-AuNP signal. No significant changes in imaging contrast were observed between 24 h and 48 h drainage.

The non-uniform distribution pattern of GC-AuNP-tagged cells in metastatic lymph nodes was further confirmed in three-dimensional USPA images (Fig. 5(a–c)). The volume of the sentinel lymph nodes was calculated using ultrasound, and PA was used to quantify the volume of the SLN containing GC-AuNP-tagged cells (Fig. 5(d)). The percent volume of the lymph node occupied by nanoparticle-tagged cells was referred to as nanoparticle-to-tissue (NT) ratio. Naïve mice injected with GC-AuNPs subcutaneously in the mammary fat pad did not show any statistically significant difference in NT ratio compared to non-metastatic tumor bearing mice. In the metastatic study group, the NT ratio of the SLN had a statistically significant reduction of more than 2-fold compared with non-metastatic controls.

PEGylated gold nanoparticles (PEG-AuNPs) were injected in naïve mice as an additional control to confirm the immunogenicity of GC-AuNPs. Upon injection, and 24 hours after, no significant changes in PA signal associated with PEG-AuNPs were seen (Fig. 6(a,b)),

because PEGylation prevents opsonization and thus phagocytosis by immune cells³⁸. With no cell interaction, there is no aggregation and, therefore, no plasmon coupling of the nanoparticles occurs. Consequently, PEG-AuNPs did not show significant NIR absorption (Fig. 6(c)). This confirms that the surface characteristics of GC-AuNPs play a significant role and are essential to generate high PA contrast from interaction with immune cells upon injection.

At the end of the imaging studies, the animals were euthanized, and the sentinel lymph nodes were resected and fixed for histological analysis. Histology results (Fig. 7) were consistent with the imaging findings. H&E staining (Fig. 7(a)) confirmed the presence of metastases. However, the metastatic cells also exhibited GFP fluorescence, which gave a more evident visualization. In dark-field microscopy, signals from gold nanoparticles were mapped in red. The overlaid images (Fig. 7(b,c)) showed no overlap between scattered light from AuNPs and fluorescence signals from cancer cells. The orange and blue colors correspond to residual red blood cells, and an imaging artifact from the microscope, respectively. A close-up dark-field image of tissue with gold nanoparticles and its corresponding H&E section are shown in Fig. 7(d,f). Figures 7(e,g) show a close-up GFP fluorescence image of metastatic cells and the corresponding H&E section. The results in Figure 7 confirm that metastatic cells inside the SLN change the distribution of nanoparticle-tagged immune cells, as identified by USPA imaging (Figs. 3–6). In addition, the microscopy images indicate that the nanoparticles did not coincide with cancer cells. This corroborates that the GC-AuNPs did not interact with cancer cells, but with the immune cells, as was expected from *in vitro* uptake experiments described elsewhere²⁷. Furthermore, previous *in vivo* study also showed that in non-metastatic mice, the injected GC-AuNPs are found in subcapsular sinuses across the SLN²⁷.

Results likely indicate that the metastatic foci lodged in the afferent region of the node affect the natural inflow and accumulation of nanoparticles and nanoparticle-tagged cells. Additionally, it is known that metastatic and pre-metastatic tumors induce morphological and functional remodeling in sentinel lymph nodes, including changes in the lymphatic sinuses and immune cell trafficking, as well as modulation of immune cells and immune suppression^{39–41}. This may also explain why naïve mice showed slightly lower accumulation than pre-metastatic nodes although the differences were not statistically significant. Further investigations of immunofunctional imaging of naïve and pre-metastatic lymph nodes may be needed.

The optimal delivery of agents to lymph nodes has been the subject of numerous studies. The delivery can occur via passive flow and subsequent uptake by lymph node resident immune cells, or via cell trafficking after uptake at the injection site. In general, delivery efficiency can be affected by nanoparticle size, shape, and surface properties, such as charge, hydrophobicity and chemical composition⁴². It is thus noted that the lymphatic drainage and cellular uptake mechanisms of GC-AuNPs should be further studied. While a portion of the GC-AuNPs may be phagocytosed by dendritic cells at the injection site and transported to the sentinel lymph node, it is possible that some nanoparticles passively flow to the SLN where they are uptaken by lymph node resident immune cells. Some of these free-flowing particles may even be endocytosed by endothelial cells on the lymphatic vessel walls,

contributing to their intense PA signal. Although the specific aspects and proportion of each mechanism are of interest, they are out of the scope of the current study.

The immunofunctional imaging approach has several advantages. One of its strengths is that the statistically-significant reduction of NT ratio is based on the volume of the SLN containing GC-AuNP signal, as calculated via USPA, but it is independent of the average signal intensity. Thus, the method is unaffected by changes in laser fluence, differences in skin absorption, or degree of nanoparticle aggregation. As metastases further invade the lymph node, it is possible for the reduction in NT ratio to become more pronounced to the point where no PA contrast would be seen inside the node. Although a fully-invaded SLN could ultimately show no GC-AuNP PA signal, i.e., NT ratio is equal to zero, immunofunctional imaging would still detect lymphatic transport of the nanoparticles. The advantage of our method is that GC-AuNPs, being aggregated inside of cells, can be visualized at the lymphatic vessels on their way to the lymph node (Fig. 4(e)), thus indicating that the drainage occurred, even if the SLN is fully invaded.

Because the nanoparticles are not in tumor cells, the immunofunctional imaging described here is independent of the molecular characteristics of the tumor and, as such, does not require expensive antibodies or sophisticated surface modifications of the contrast agent. This can be particularly useful with triple-negative tumors (such as the model used in this study) which are challenging to target due to the lack of over-expressed tumor-specific cell membrane receptors.

However, non-specificity could be caused by several potential sources including other lymphatic ailments, such as slow lymph flow or infections. In cases where immunofunctional imaging is inconclusive but indicative of metastases, further molecular imaging could be performed²¹. Thus, the immunofunctional imaging is expected to be highly sensitive, with high but limited specificity in patients with ongoing lymphatic ailments. Nevertheless, many other alternative diagnostic approaches would likely be affected by such ailments as well.

The minimum number of metastatic cells to cause a distinguishable change in signal distribution is yet to be determined. In the breast oncology field, it is up to debate whether lesions smaller than 2 mm warrant treatment strategies any different from those in patients with no nodal involvement^{43–48}. In this study, the diameter of the whole organ was close to 2 mm, demonstrating that the spatial resolution of USPA would be sufficient to identify changes at submillimeter scales. The pattern of these changes is consistent with those found using intravenous iron oxide contrast agents^{18–19}. These patterns can be characterized in future trials with larger sample numbers, to create tables such as those obtained using MRI and iron oxide contrast agents to identify the degree of nodal involvement⁴⁹. Our technique, however, would add versatility and cost-effectiveness, enhanced by a superior imaging sensitivity and spatial resolution.

The GC-AuNPs used in this study were 20 nm and, as such, do not readily clear via renal excretion. However, as a proof of concept the study was successful in identifying SLN metastases. The same approach may be applied using different types of contrast agents, such

as smaller (~ 5 nm) or biodegradable nanoparticles that can be cleared by the renal system^{50–52}.

Conclusions

The immunofunctional imaging approach presented here was used to localize immune cells with endocytosed GC-AuNPs, allowing for evaluation of their spatio-temporal distribution within the SLN. This technique goes beyond merely detecting SLN location by providing functional information that can be correlated to the presence of metastases. The results are consistent with previous literature and *in vitro* studies. Additionally, this method is cost-effective, because it does not require expensive imaging modalities, antibodies or sophisticated molecular targets. From a research perspective, the developed USPA imaging tool could also advance research in basic science and areas such as immunology or immunotherapy. From a clinical perspective, the tool can aid physicians in the detection of sentinel lymph node metastases, thus guiding and potentially avoiding unnecessary SLN biopsy.

Materials and Methods

Synthesis of gold nanoparticles

GC-AuNP were synthesized through chlorauric acid reduction as described elsewhere⁵³. Briefly, glycol chitosan solution (300 mL, 1 mg/mL, Sigma-Aldrich Corp., St. Louis, MO) was boiled to 70°C and mixed with H₂AuCl₄·3H₂O solution (1 mM, 100 mL) under stirring for 24 hours until the solution turned red. Glycol-chitosan acted as a reducing and stabilizing agent. The resulting nanoparticle size was 20 nm, with an optical absorption peak of 520 nm. Nanoparticles were washed and concentrated via centrifugation, and diluted to a concentration of 0.1 mg/mL of gold.

As a control, PEG-AuNPs were synthesized. A hydrogen trichloroaurate (III) solution (6 mL, 25 mM) was diluted with 279 mL of distilled water and heated to boil under stirring. Then, a trisodium citrate solution (15 mL, 1 wt%) was added to produce gold nanoparticles with a diameter of approximately 20 nm. After cooling down to room temperature, the nanoparticle colloid (5 mL) was mixed with mPEG-SH solution (Laysan Bio, Inc., MW=5000, 2 mg/mL, 5 mL) under stirring for 24 hours. Before injection, PEG-AuNPs were washed via centrifugation, and concentrated to 0.1 mg Au/mL.

Nanoparticle characterization

The optical absorption properties of PEG-AuNPs and GC-AuNPs were characterized using a microplate reader (SynergyTM HT, BioTek Instruments, Winooski, VT) to measure the optical absorbance from 350 nm to 850 nm. A JEOL 2010-F TEM with 200 kV operating voltage was used for transmission electron microscopy (TEM) images of nanoparticles. For TEM samples, 10 µL of nanoparticle colloid was dropped on carbon-coated copper grids. Particle size and zeta potentials were obtained using a Zetasizer Nano ZS (Malvern Instruments, UK).

Cellular uptake characterization

For cellular uptake of nanoparticles, J774A.1 macrophage cells were cultured on 6-well plates with gelatin-coated cover slips in each well. After incubation of the cells with nanoparticles (0.05 mg Au/ml) for 4 h, cells were washed twice with phosphate buffer saline (pH 7.4) and fixed with paraformaldehyde solution (4%). Then, cover slips with particle-endocytosed cells were mounted on a slide glass and observed in a Leica DMI3000B microscope (Leica Microsystems, Wetzlar, Germany).

Ultrasound-guided photoacoustic (USPA) imaging

USPA imaging was performed using a Vevo LAZR system (Visualsonics Inc.). Laser irradiation was delivered through a fiber optic bundle that was integrated with a 40 MHz ultrasound transducer (LZ-550). Photoacoustic imaging was performed in the 680 to 970 nm wavelength range using 4–6 ns laser pulses at a 20 Hz pulse repetition frequency. The laser fluence was kept below the American National Standard Institute (ANSI) safety limits. For 3D scans, the transducer was attached to a translational motor and moved perpendicular with respect to the imaging plane while acquiring USPA imaging slices. Images were post processed and analyzed using Amira (Thermo Fisher Scientific) and MATLAB (MathWorks).

Animal model

All animal procedures were performed in accordance with federal guidelines for the care and use of laboratory animals, and were approved by the Institutional Animal Care and Use Committee (IACUC) at the Georgia Institute of Technology. Five-week old female nude mice (Nu/Nu, Charles River) were inoculated in the right caudal mammary fat pad with 2×10^6 human breast adenocarcinoma cells in 50% Matrigel (MDA-MB231-Red-FLuc-GFP, PerkinElmer) for the metastatic group. The MDA-MB231-Red-FLuc-GFP are transfected with green fluorescence protein (GFP) to be detected in histology. For the non-metastatic control group, mice were inoculated similarly with non-metastatic human ductal carcinoma cells (BT474, ATCC). Tumors were allowed to grow up to a 10 mm diameter prior to imaging studies. Naïve mice of the same strain and age were used as an additional control group.

Imaging protocol

Mice were injected with 100 μ L of GC-AuNP colloid peritumorally. USPA imaging was performed before, and immediately after injection, and after drainage to the inguinal lymph node. Conventional B-mode ultrasound was used to identify the inguinal lymph node anatomy. Accumulation of GC-AuNP in the SLN after cellular uptake by immune cells was visualized using PA imaging. At the conclusion of the imaging studies, the animals were sacrificed and tissue was harvested for histological analysis.

Histological analysis

The inguinal lymph nodes were resected after imaging studies and kept in 10% buffered formalin. Samples were then embedded in paraffin and sliced for H&E staining. Histology slides were visualized on a Leica DMI3000B microscope. Cancer cells were identified by

the presence of GFP fluorescence, and GC-AuNP were identified using dark-field microscopy.

Image analysis

Three-dimensional B-mode ultrasound images were used to segment the lymph node volume of each mouse, creating a lymph node mask. Photoacoustic images were filtered using a 3D median filter followed by a 3D averaging window. A low threshold was applied to remove pixels under the noise floor. Then, adaptive thresholding⁵⁴ was performed using a moving window 1/8 the size of the image, to avoid overestimation of the threshold in cases where strong skin signals were present. The lymph node mask was applied to the thresholded images. The ratio of bright pixels over the total pixels in the mask, i.e. the percent volume of the lymph node occupied by nanoparticle-tagged cells, was referred to as the nanoparticle-to-tissue (NT) volume ratio.

Statistical analysis

A one-way analysis of variance (ANOVA) test was performed for the healthy, non-metastatic, and metastatic study groups. A post-hoc Tukey's honest significant difference (HSD) test was performed to determine the statistically significant differences among study group means.

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Figure 1. Immunofunctional imaging paradigm. (a) GC-AuNP contrast agent is injected peritumorally. The contrast agent becomes NIR-absorbing upon uptake by immune cells and is transported to the sentinel lymph node via afferent lymphatic vessels. (b) A reduction of GC-AuNP-tagged cell accumulation in the lymph node is an indicator of metastasis.

Figure 2.

Characterization of GC-AuNPs and PEG-AuNPs. (a) UV-Vis spectrum of GC-AuNPs and PEG-AuNPs. GC-AuNPs have a surface plasmon resonance peak at 528 nm while PEG-AuNPs have a peak at 523 nm. (b) TEM images show the size and spherical morphology of GC-AuNPs and PEG-AuNPs. Bright-field (c) and dark-field (d) images of J774.A1 macrophage cells after 4 h-cellular uptake show high interaction of GC-AuNPs with immune cells vs. low interaction of PEG-AuNPs.

Figure 3. Ultrasound-guided photoacoustic (USPA) images of a non-metastatic sentinel lymph node (yellow contour) (a) before and (b) 24 hours after injection of GC-AuNP. (c) After 24 hours, the photoacoustic signal spectrum shows a 10-fold increase in absorption at 700 nm.

Non-metastatic**Metastatic** **Figure 4.**

Two-dimensional USPA immunofunctional imaging. (a, d) B-mode ultrasound was used to localize the lymph nodes. (b, e) Overlaid photoacoustic imaging shows GC-AuNP-tagged cells in the lymph node and afferent lymphatic vessel. (c, f) Ultrasound-masked photoacoustic images show the effect of metastases in nanoparticle-tagged cell accumulation.

Figure 5.

3D-rendered ultrasound-guided photoacoustic (USPA) images of the sentinel lymph node 24 hours after GC-AuNP injection in (a) a naïve mouse, (b) a tumor bearing mouse without metastasis, and (c) a tumor bearing mouse with metastasis. (d) The bar graph shows an over 2-fold decrease in the nanoparticle-to-tissue (NT) ratio, i.e. the fraction of the lymph node volume occupied by nanoparticle-tagged cells. Data are shown as means $\pm$ SD ($n = 3$). ** $p = 0.002$; *** $p = 0.001$

Figure 6. Ultrasound-guided photoacoustic (USPA) images of a healthy sentinel lymph node in a naïve mouse (a) before and (b) 24 hours after injection of PEG-AuNPs. (c) After 24 hours, there are no significant changes in the photoacoustic signal spectrum.

Figure 7. Histological analysis of a metastatic lymph node shows (a) general tissue morphology with H&E staining. (b, c) GC-AuNPs were visualized using dark-field microscopy, and cancer cells using GFP fluorescence. The merged images show that GC-AuNPs and cancer cells do not spatially coincide. Zoomed images show areas with (d) GC-AuNPs or (e) cancer cells, with their respective H&E sections (f,g). Scale bars are (b) 300 μm , (c) 150 μm , and (d-g) 30 μm .