ORIGINAL ARTICLES

Multimodal sentinel lymph node mapping with single-photon emission computed tomography (SPECT)/computed tomography (CT) and photoacoustic tomography

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The identification of cancer cells in the lymph nodes surrounding a tumor is important in establishing a prognosis. Optical detection techniques such as fluorescence and photoacoustic tomography (PAT) have been reported in preclinical studies for noninvasive sentinel lymph node (SLN) mapping. A method for validation of these techniques is needed for clinical trials. We report the use of a multimodal optical-radionuclear contrast agent as a validation tool for PAT in a preclinical model. Methylene blue (MB) was radiolabeled with ^{125}I for multimodal SLN mapping and used in conjunction with MB to assess the feasibility of multimodal SLN mapping in a rat model by PAT and single-photon emission computed tomography (SPECT). MB provided sufficient contrast for identifying SLNs noninvasively with a PAT system adapted from a clinical ultrasound imaging system. The signal location was corroborated by SPECT using ^{125}I labeled MB. The translation of PAT into the clinic can be facilitated by a direct comparison with established imaging methods using a clinically relevant dual SPECT and photoacoustic imaging agent. The new high-resolution PAT is a promising technology for the sensitive and accurate SLN detection in cancer patients. (Translational Research 2012;159:175–181)

Abbreviations: 3-D = three-dimensional; CT = computed tomography; MB = methylene blue; PAT = photoacoustic tomography; PET = positron emission tomography; SLN = sentinel lymph node; SPECT = single-photon emission computed tomography; US = ultrasound

Until recently, radical lymph node resection with *ex vivo* histopathology was routine for staging cancers including breast and prostate cancers. This procedure often resulted in significant morbidity from local fluid retention and consequent infection, pain, and limited limb use,[1] and it is no longer considered the standard of care.[2] The selective biopsy of lymph nodes that are first encountered by metastatic

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Supported in part by grants R01 EB008458, R01 EB007276, R01 EB008111, R01 EB000712, R01 EB008085, R33 CA123537, and U54 CA136398 from the National Institutes of Health (the Network for Translational Research) and by Grant K01RR026095 from the National Center for Research Resources (to W. J. A.).

Submitted for publication May 4, 2011; revision submitted August 8, 2011; accepted for publication September 27, 2011.

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1931-5244/$ - see front matter

doi:10.1016/j.trsl.2011.09.006

AT A GLANCE COMMENTARY

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Background

The identification of cancer cells in the lymph nodes surrounding a tumor is important in establishing a prognosis. Optical detection techniques such as fluorescence and photoacoustic tomography (PAT) have been reported in preclinical studies for noninvasive sentinel lymph node (SLN) mapping. A method for the validation of these techniques is needed for clinical trials.

Translational Significance

We report the use of a multimodal optical-radionuclear probe to validate PAT in a preclinical model. The approach will facilitate the translation of PAT into the clinic through a comparison with clinically relevant SPECT. The high-resolution PAT is sensitive and accurate for SLN detection in cancer patients.

cells is a less traumatic diagnostic method and has been found to be more useful for staging without impacting treatment in most cases. In cancer staging, the detection of tumors in the sentinel lymph nodes (SLNs), which are the first nodes that drain the area of interest, is an important prognostic factor that directs treatment planning. Lymphoscintigraphy for SLN mapping was introduced in 1977 using radiolabeled colloidal gold.[3] The tracer agents were selected by the rate of uptake in the lymph nodes and limited diffusion outside of the lymphatic system.[4] SLNs can be detected by regional subcutaneous injection of contrast agents. For example, the ^{99m}Tc sulfur colloid is widely used preoperatively and intraoperatively to detect SLNs.[5]

SLN mapping is complex because of the variety of drainage pathways from the primary tumor site.[6] The detection of lymph nodes and distinction of SLNs may be complicated by the choice of contrast agent and by lymphatic channels temporarily accumulating contrast agents that can be mistaken as lymph nodes.[7] Thus, three-dimensional (3-D) nuclear imaging methods such as single-photon emission computed tomography (SPECT) are useful for noninvasive localization of the SLN. Hybrid SPECT/CT instruments are now available and provide the ability to determine the exact anatomic location of SLNs and improve lymphatic mapping.[8]

To aid intraoperative visualization of the lymph drainage into SLNs, commonly vital dyes are employed. One dye used to guide SLN biopsy in humans is methylene blue (MB). SLN localization using vital dyes alone is limited to wide-field exposure of the peritumoral area for visualization of the distinct blue color with unaided human eye.[9] This approach is not efficient because of the need to use large amounts of the dye, which can only be visualized invasively. This limitation of detection has led several groups to postulate that radioisotopes are superior to dye contrast agents for identifying SLNs in cancer patients.[10,11]

Recent studies, however, showed that a new hybrid imaging method known as photoacoustic tomography (PAT) may alter the existing paradigm of SLN mapping. PAT combines the sensitivity of optical imaging with the spatial resolution of ultrasound. This imaging technology can detect light-absorbing compounds several centimeters below the skin surface.[12] The sensitivity of PAT to contrast agents is much greater than ultrasound,[13] and the ability of PAT to interrogate deep tissue noninvasively allows its use for 3-D imaging and noninvasive lymph node identification for SLN biopsy. The success of this technology in small animal imaging has motivated current efforts to translate it into the clinical arena.[14] A primary focus of these efforts is the validation of PAT as a viable method for imaging SLN in human patients. Because PAT does not use ionizing radiation, it is amenable to use in surgical suites. However, there is an urgent need to validate this new technology with an established imaging method. Considering the important role of SPECT in SLN mapping, 1 strategy to validate PAT is to use a common contrast agent for both PAT and a more established imaging modality such as SPECT. Here, we report our initial efforts in multimodal imaging with methylene blue (MB) and ^{125}I-MB for validating PAT of SLN with SPECT/CT.

METHODS

Radiolabeling. Methylene blue (Sigma, St. Louis, Mo) was radiolabeled with ^{125}I sodium iodide (American Radiolabeled Chemicals, Inc, St. Louis, Mo) as described previously.[15] ^{125}I-NaI (37 MBq) was added to freshly prepared potassium iodide/iodate solution (585 μg KI and 3.85 mg KIO_3). The mixture was acidified (0.25 mL, 0.18 mol/L HCl) and heated (60 min, 100°C.) The reaction progress was monitored by reversed-phase, radio-high-performance liquid chromatography (Altima, C18, 3 μm, 7 mm $\times$ 53 mm, 2.5 mL/min, A = 0.1% TFA, water, B = 0.1% TFA, acetonitrile, 30% to 90% B, 10 m).

***In vivo* imaging.** All animal studies were conducted according to guidelines on the humane care and use of laboratory animals under protocols approved by the

Animal Studies Committee at Washington University School of Medicine.

A combined handheld PAT and ultrasound (US) imaging system adapted from a clinical US array system (iU22; Philips Healthcare, Amsterdam, the Netherlands) was used for *in vivo* mapping of lymph nodes in rats.[14] An optically tunable dye laser (Precision-Scan-P; Sirah, Kaarst, Germany), pumped by a Q-switched Nd:YAG laser (PRO-350-10; Newport Corporation, Irvine, Calif), produced pulsed lasers with a duration of 6.5 ns at a repetition rate of 10 Hz. The light was coupled to bifurcated fiber bundles (CB18043; Fiberguide Industries Inc, Stirling, NJ) that were integrated with a handheld US array probe (L8-4, Philips Healthcare). An optical wavelength of 650 nm, close to the peak optical absorption wavelength of MB (667 nm), was used. The fight fluence on the surface was ~3 mJ/cm^2, only 1/7 of the ANSI safety limit (20 mJ/cm^2).[16] Generated PAT waves were detected by the linear array US probe with a nominal bandwidth of 4–8 MHz. The PAT images were displayed at ~1 frame per second (fps). The imaging depth was increased by layering ~2-cm-thick chicken tissue on top of the rat to demonstrate the deep-imaging capabilities of PAT at clinically relevant depths. MB (0.1 mL of 1%) was injected in the left front forepaw of 200–250 g female Sprague Dawley rats (HSD, Indianapolis, Ind) (n = 3), and *in vivo* PAT imaging was performed. Coregistered PAT and US images identify the MB-enhanced SLNs (from PAT) in rats and the surrounding anatomic structures (from US imaging).

For multimodal imaging, ^{125}I-MB (~200 μCi) was added to 1% MB (0.1 mL, PBS). An aliquot of 0.1 mL was drawn into a 0.5 mL insulin syringe and activity measured with dose calibrator before injecting subcutaneously in the left forepaw as for PAT (n = 3). The activity remaining in the syringe was measured and the injected dose was calculated. SPECT/CT imaging was performed immediately after injection of ^{125}I-MB and at 1 h after injection with the NanoSPECT/CT preclinical imaging system (Bioscan, Inc, Washington, DC). The scanning regions were selected by side-view topogram, extending from the xyphoid to the base of the jaw. CT was performed on a 45 KVP, 177 mA, and 180 400 ms projections helical CT with pitch of 1. The CT scan was followed by helical SPECT with 16 projections and 60 s each. The energy windows were set to 28 KeV and 30% width. CT and SPECT projections were reconstructed using InvivoScope software (Bioscan, Inc).

After SPECT/CT imaging, planar scintigraphy was performed using the IS4000MM multimodal imaging system (Carestream Health, New Haven, Conn). White light, fluorescence, and X-ray and scintigraphy images were acquired. Following SPECT/CT, the rats were euthanized with pentobarbital solution (150 mg/kg, intraperitoneally). The skin overlying the axillary region was removed for visualization of the axillary lymph nodes. Planar scintigraphy was performed and the color images were acquired with a digital camera. The lymph nodes were resected and the images were acquired for a comparison of contralateral, control lymph nodes to confirm ^{125}I-MB uptake in lymph nodes using the optical imaging and planar scintigraphy. Activity in the lymph nodes was measured using the dose calibrator and percent injected dose per gram tissue (%ID/g) calculated.

RESULTS

Initial attempts to prepare iodo-methylene blue using the standard iodogen method resulted in the appearance of a major impurity that was not characterized any more. To overcome this problem, we used KI/KIO$_3$ under acidic conditions instead of the iodogen method.[15] Purification of the mixture yielded ^{125}I-MB [17] (Fig 1), which has a retention time of 4.86 min and 97% purity based on the peak area. The unlabeled MB eluted approximately 2 min before the iodinated species. A minor component that eluted at 4.95 min was the putative di-iodinated species that has been previously described.[15] Incorporation of ^{125}I in I-MB was quantitative and complete in 10 min. Because specific activity was not an issue in this study, the isolated ^{125}I-MB was not purified any more.

PAT images show clearly localized lymph nodes containing blue dye (Fig 2). A control PAT image was acquired before injection of MB (Fig 2, *A*). Soon after injection, MB accumulation in the SLN was detected photoacoustically. Fig 2, *B* shows the PAT image of the MB-dyed SLN acquired at 10 min postinjection. An overlaid PAT and US image (Fig 2, *C*) provides both functional (MB uptake in the SLN) and structural information. Postmortem photographs taken after PAT imaging confirmed MB uptake in the SLN (*vide infra*).

SPECT imaging showed localized signal from ^{125}I-MB in the axillary region within 1 h after injection (Fig 3). The high-intensity regions at the injection site, in the forelimb and at the location of the axillary lymph nodes were distinguished easily, allowing accurate mapping of the lymph fields. Approximately 9% (17.7 ± 0.48 μCi, n = 3) of the injected dose was detected in the axillary lymph node within 1 h after injection, whereas almost no signal was detected in the region of the contralateral lymph node (Fig 3). High uptake was confirmed by planar scintigraphy and postmortem optical imaging (*vide infra*).

We validated that both PAT and SPECT signals originated from the same lymph node. Because our PAT and SPECT/CT imaging systems are not colocalized in

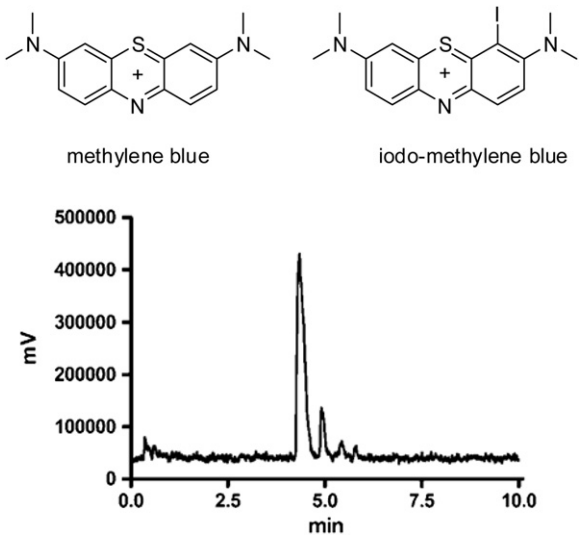

Fig 1. Structures of MB and iodo-MB[17] (top). Reversed-phase radiochromatogram of ^{125}I-iodo-MB (bottom).

the same laboratory area, we explored a multimodal imaging platform. In principle, ^{125}I-MB can provide photoacoustic, fluorescence, and gamma imaging signals. However, it was difficult to detect MB fluorescence through the skin by planar reflectance optical imaging. After removing the skin in the MB injection area, the lymph node and vessels were detected visually by dark blue stains (Fig 4, *A*). The lymph tracts leading to the lymph node can be viewed as well. Even with the exposed skin, only a low fluorescence signal was detected in the forelimb and in the lymphatic vasculature, but it was particularly dim from the area of the lymph node. This low nodal fluorescence could be attributed to the low fluorescence quantum yield of MB and likely quenching within the highly stained lymph nodes. The strength of the PAT signal suggests that much lower concentrations of MB can be used in future studies.

Unlike fluorescence imaging, planar gamma scintigraphy clearly showed high intensity from the left paw, lymphatic vessel, and lymph node (Fig 4, *C*), corresponding with the dark blue color observed in Fig 4, *A*. The animal was euthanized, and the dissected lymph nodes from the left axillary region were removed. After dissection, the first lymph node is distinctly blue from dye accumulation, whereas the following nodes are free of the dye (Fig 4, *B*). Thus, the postmortem photograph taken after PAT imaging confirmed MB uptake in the SLN. Similarly, the *ex vivo* planar scintigraphy confirmed that the first lymph node is the source of the SPECT signal, demonstrating that ^{125}I-MB could serve as a reliable molecular imaging probe for *in vivo* validation of PAT in clinical trials.

DISCUSSION

Central to the current SLN mapping is the administration of 2 complementary detection agents, ^{99m}Tc-labeled colloids and MB, to guide SLN identification and biopsy. Although the goal of the procedure is to localize the SLN, significant differences in the size, physical properties, and chemical properties of these 2 agents result in differential distributions and uptakes of the agents in the lymph nodes. These differences are not problematic when used for SLN detection and biopsy. However, validation of PAT for human use will benefit from a direct comparison of the nascent PAT method with established SPECT. Although PAT has been shown to image objects several centimeters in animal tissue, the optical properties of human tissue are different. Thus, it is important to demonstrate that PAT will not miss critical SLNs in humans.

To facilitate this validation, we explored the use of a single imaging agent for both PAT and SPECT. Previous studies have shown that MB can be radioiodinated with different radioisotopes of iodine for imaging cancer in humans and animals.[18] These studies demonstrated that the combination of radiotracer and MB increases the rate of correct SLN detection. In particular, ^{125}I-MB was reported previously as an alternative to the mixture of ^{99m}Tc colloid and MB for the intraoperative guidance of SLN biopsy. A phase I/II clinical trial was performed in which a handheld gamma detector was used to locate hotspots followed by skin incision and excision of SLNs that were stained blue.[19]

We prepared ^{125}I-MB and used it in a different context from previously reported applications. Here, MB served as a contrast agent for PAT, which relies on light absorption by chromophores such as exogenous dyes or endogenous targets like hemoglobin. This absorption process generates local thermal expansion of tissue that is captured as an acoustic signal for PAT image reconstruction. By radiolabeling MB with ^{125}I, it also yields SPECT signals, thus providing a direct approach to validate the application of the nascent PAT for lymph node mapping in humans. This is particularly important if PAT will be used as a standalone noninvasive imaging modality in the surgical room. We used a small animal model of lymph node mapping to explore the validity of the aforementioned strategy. Our study showed that the multimodal optical-nuclear contrast agent can be used to confirm the location of SLNs detected by noninvasive PAT in deep tissue. This strategy of validation is effective for preclinical and clinical studies using PAT.

Mapping the lymphatic vasculature and nodes by high resolution PAT for SLN biopsy demonstrated great potential. The validation of this technique by SPECT/CT will improve whole-field identification of lymph nodes

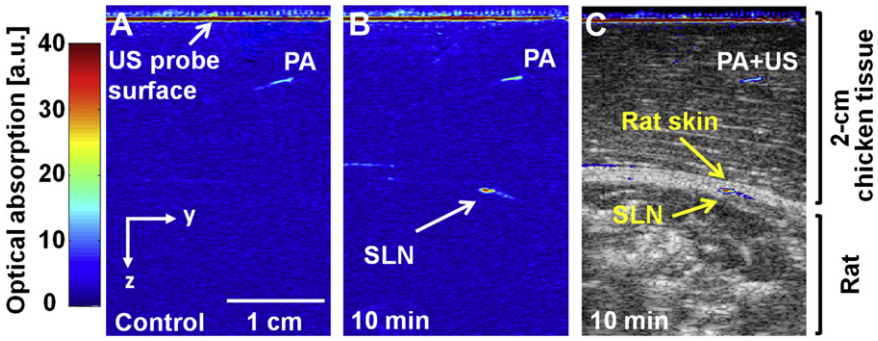

Fig 2. PAT imaging of lymph nodes after injection of MB. (**A**) Control PAT image obtained before MB injection. (**B**) PAT image taken at 10 min post-injection. (**C**) Overlaid PAT (pseudocolor) and US (grayscale) image. (Color version of figure is available online.)

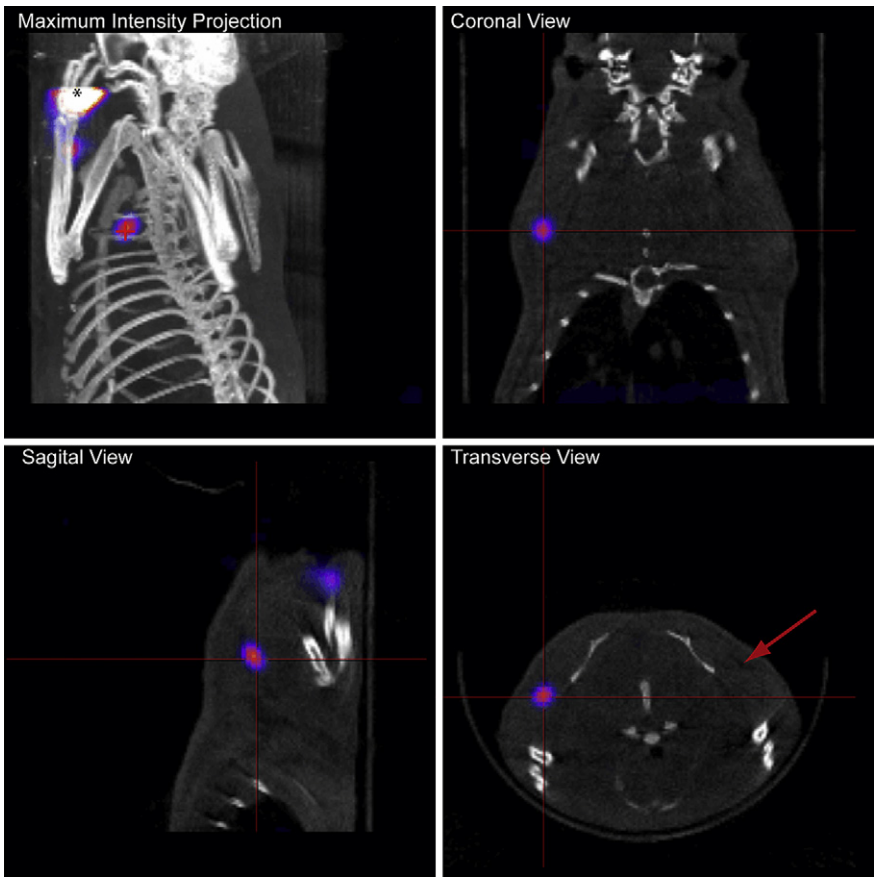

Fig 3. SPECT/CT projection images of rat acquired 1 h after subcutaneous injection of ^{125}I-MB in the left forepaw. *The location of injection and crosshairs indicate location of signal from axillary lymph node. The red arrow indicates the contralateral lymph node in the transverse view. The lymph node was detected about 3 mm below the surface of the skin, correlating well with the PAT finding. (Color version of figure is available online.)

draining the tumor area. The successful demonstration of lymph node localization using the clinically compatible ^{125}I-MB imaging agent in small animals and the ability of PAT to interrogate deep tissue suggest that this strategy will speed clinical translation of PAT for SLN biopsy.

CONCLUSION

SLN biopsy has become the standard of care for the staging of many types of cancer. The nodal status is an important prognostic indicator after the diagnosis of cancer. PAT has demonstrated great potential for the noninvasive detection of SLN after the injection of an

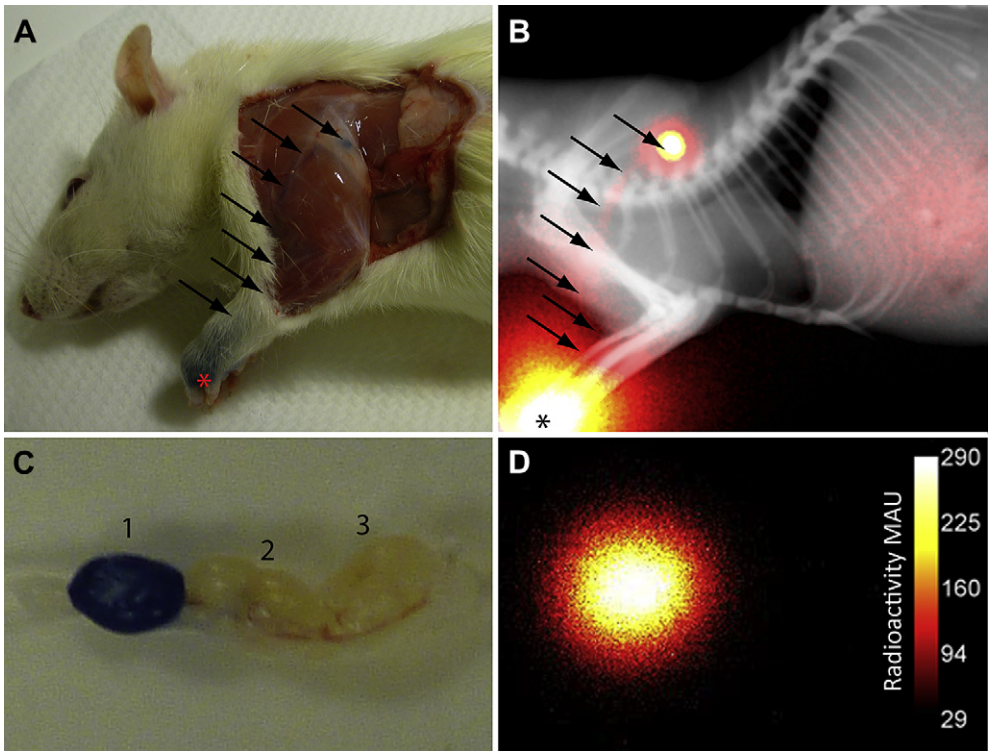

Fig 4. Postmortem optical and planar scintigraphy of rat 2 h after subcutaneous injection of ^{125}I-MB in the left forepaw. (**A**) The MB can be observed clearly as blue coloring in the forepaw near the site of injection (*) and at the first lymph node after removal of overlying skin and hair. The lymph tracts leading to the lymph node can be viewed as well (arrows). (**B**) Planar scintigraphy corresponds with blue color with high intensity from the left paw and lymph node. (**C**) After dissection, the first lymph node is distinctly blue from dye accumulation, whereas the following nodes are free of the dye. (**D**) *Ex vivo* scintigraphy confirmed that the first lymph node was the source of the signal. (Color version of figure is available online.)

optically absorbing contrast agent. Validation of PAT for SLN biopsy is needed for clinical translation. The multimodal imaging approach presented in this report is a good strategy for the validation of optical imaging techniques. This multimodal technique will be useful for validating the signal location determined by noninvasive PAT using the handheld device. SLN mapping for subsequent fine-needle aspirate, core-needle biopsy, or minimally invasive resection can be achieved with high confidence. The next stage is to use the dual reporter imaging probe in human studies; this process will help validate the future role of PAT in breast cancer staging.

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