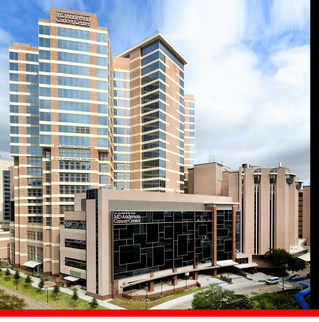

Optimization of Molecular Ultrasound Imaging using Nanodroplet Contrast Agents for *In Vivo* Translation

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Introduction

Diagnostic Molecular Ultrasound (US) Imaging

- A non-invasive imaging technique to visualize and characterize biomarkers within the tumor microenvironment.[1]
- Combine anatomical and physiological information to help inform treatment plans.[2]
- The use of microbubble contrast agents (MCAs) to probe tissue properties does not allow for cellular uptake in extravascular space.[3-5]

Phase-changing Perfluorocarbon Nanodroplet Contrast Agents (PNCAs)

- Imaged and activated by high-clinical-frequency US (Fig. 1).
- PNCA size before activation enables access to tumor cell surface receptors, followed by a phase (and size) change to provide US contrast.[6-7]
- However, localization of PNCAs *in vivo* is desired prior to activation.

Objectives

- Investigate the addition of Cy7 dye to PNCA coating for optical tracking in both phantom and *in vivo* models.
- Translate US imaging and activation of optimized PNCAs to *in vivo* models through activation and imaging of PNCAs in phantoms and murine models.

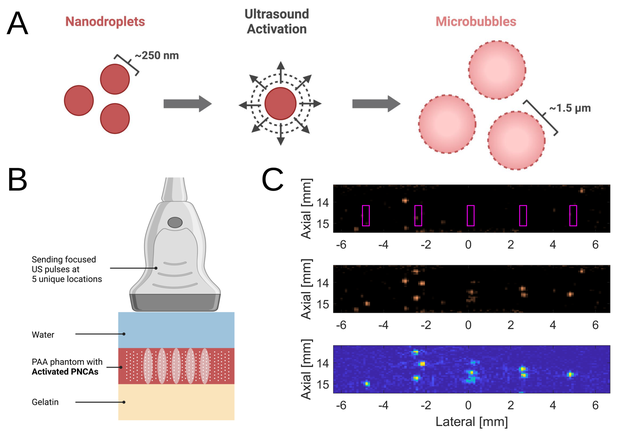

Figure 1. (A) Depiction of PNCA activation. Liquid nanodroplets (ø ~ 250 nm) are exposed to high amplitude negative pressure waves from focused US transmission and undergo a phase change to gaseous microbubbles (ø ~ 1.5 µm). (B) Depiction of focused US transmission to induce phase change of non-activated PNCAs embedded in the PAA phantom. Upon exposure to spatially targeted US activation pulses, PNCAs are activated and generate targeted contrast. (C) Representative pre- (top) and post-activation (middle) pulse-inversion images and a difference (post-activation – pre-activation, bottom) image in a PAA phantom containing PNCAs without Cy7 dye.

Materials and Methods

Nanoparticle Composition

- Core: C6-core perfluorocarbon (C_6F_{14}, 58°C boiling point).
- Lipid Coating: 97.5% DSPC, 2.5% DSPE-PEG-2000.
- Cy7 dye conjugated with PEG (for Cy7-labeled PNCAs).

Imaging Setup

- Imaging Platform: Verasonics Vantage 128 system with a Kolo Medical L22-8v CMUT transducer (15 MHz).
- Data Acquisition: Co-registered B-Mode and Pulse-Inversion Harmonic Imaging (PIHI) with high-frequency US sequences were used to image (9 MHz, 1 cycle).
- Focused US pulses were used to activate PNCAs (12 MHz, 6 cycles).
- Imaging and Activation were performed at body temperature per setup in Fig. 2A, B.
- Contrast from activation was calculated as followed:

$$\text{Contrast} = \frac{\text{Signal} - \text{Background}}{\text{Background}}$$

Experimental Design

Polyacrylamide (PAA) Phantom Imaging

- PNCAs with and without Cy7 dye in the coating were activated and imaged when embedded in a PAA phantom per standard protocol.

Injection in PAA and Meat Phantoms:

- PNCAs w/o Cy7 injected using 25-G and 27-G needles.
- Contrast Enhancement Post-injection – Pre-injection.

Injection into athymic nude mice:

- Intra-tumoral injection of Cy7-labeled PNCAs to track drainage kinetics through lymphatic system (Fig. 2C).
  - PA Imaging with an iThera MSOT inVision system.
  - Post-mortem fluorescence imaging with Emit Xerra.
- Activate PNCAs w/o Cy7 *in vivo* using US pulses.
  - Intra-muscular injection in the hind limb (Fig. 2C).
  - Multiple activation locations based on the injection site.

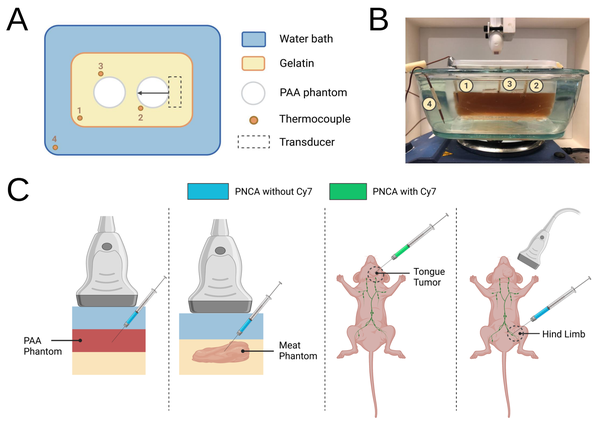

Figure 2. (A) Diagram of phantom setup for imaging, including PAA phantoms (white), gelatin-standoff (yellow), water bath (blue), thermocouples (orange) and transducer footprint (black dashes). (B) Photograph of phantom setup showing the water bath on the hot plate and depth of thermocouple probes. (C) Depiction of injection experiments, including the injection into PAA and Meat Phantoms, followed by the injection into the tongue tumor of an athymic nude mouse to track particle drainage, and the intramuscular injection into the hind limb of an athymic nude mouse to activate the particles *in vivo*.

Results

US Activation of PNCAs with and without Cy7 dye

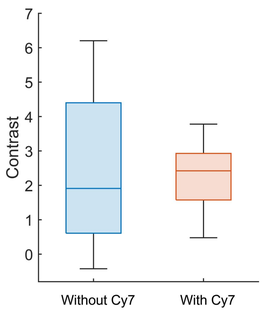

Figure 3. Contrast in PAA phantoms containing C6-core PNCAs without and with Cy7 dye.

- Neither particle experienced spontaneous activation at body temperature (Fig. 1C).
- Median Contrast (Fig. 3):
  - Without Cy7: 1.9
  - With Cy7: 2.4
- PNCAs without Cy7 showed greater variance but higher maximum contrast values.

Injection of PNCAs into Phantom Models

- Smaller needle size (27-G) introduced more spontaneous activation upon injection in both PAA and tissue-containing models.
- Contrast enhancement between pre- and post-injection (Fig. 4):

	25-G	27-G
PAA Phantom	8.7	12.9
Meat Phantom	0.8	1.2

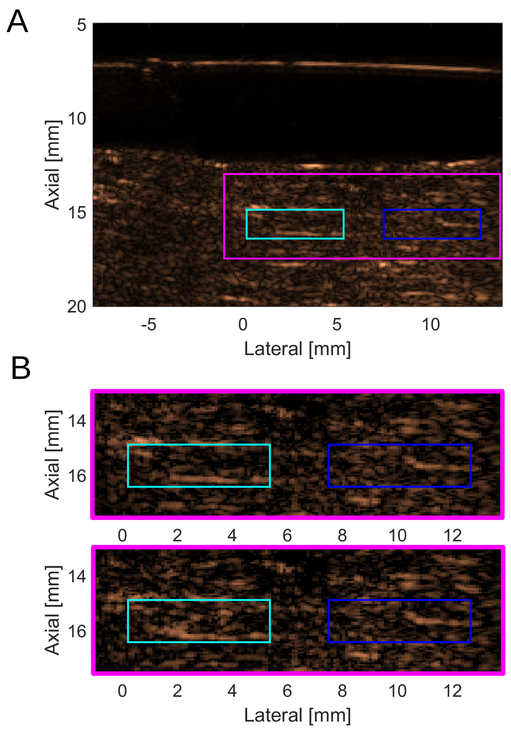

Figure 4. (A) PIHI image of chicken breast before injection of PNCAs without Cy7 dye at the center of imaging area using 25-G needle. The cyan box represents Signal ROI, and the matched blue box laterally adjacent to the cyan box represents Background ROI. (B) Cropped ROI of the magenta box from (A) showing contrast enhancement upon injection: pre-injection (top) and post-injection (bottom).

Tracking Cy7-Labeled PNCAs *In Vivo*

- Photoacoustic imaging validated lymphatic drainage of Cy7-labeled PNCAs after intra-tumoral injection (Fig. 5A, top).
- Particles were localized in the tongue tumor immediately after imaging with Xerra and detected within the sentinel lymph node 2-hours post-injection (Fig. 5A, middle/bottom).
- No PNCAs were detected in the contralateral lymph node (Fig. 5A, bottom).

In Vivo US Activation and Imaging

- Spontaneous activation of PNCAs was detected upon injection with 25-G needle (Fig. 5, bottom left).
- Despite this, significant contrast enhancement from US activation was observed at 2 locations. (Fig. 5, bottom middle/right).
- 1.8 mean image contrast of the activated regions.
- Enhancement locations were spatio-temporally aligned with US activation and particle location.

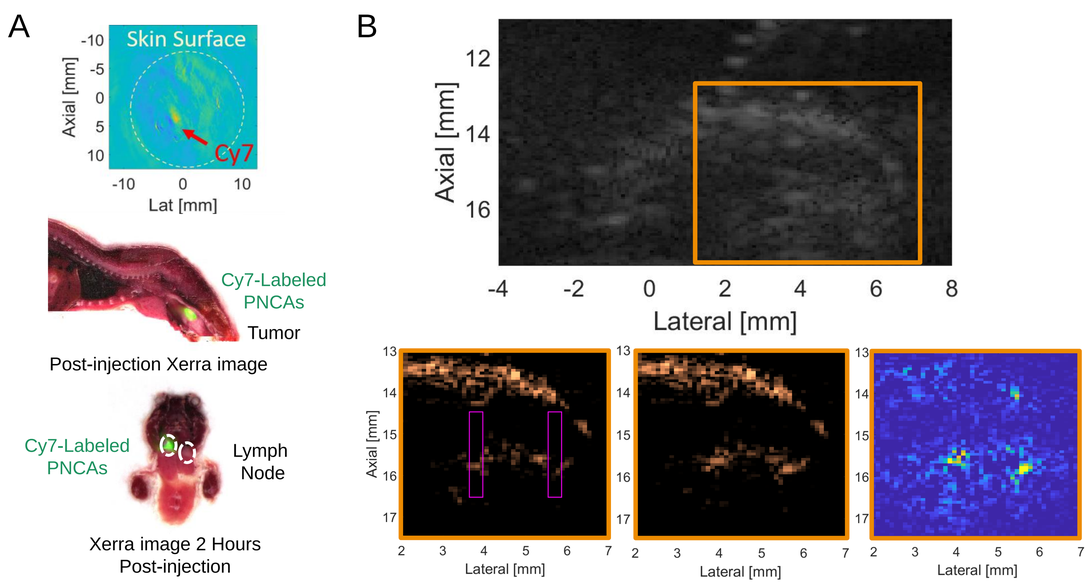

Figure 5. (A) Photoacoustic imaging showing Cy7-labeled PNCAs drain from the primary tumor after injection (top), as validated post-mortem with white light and fluorescent imaging (middle and bottom). (B) B-Mode image of Murine hind limb after injection but before activation of PNCAs without Cy7 (top). PIHI images of the cropped ROI of the yellow box from B-Mode image showing post-activation enhancement (bottom): pre-activation (left), post-activation (middle) and difference (post-activation – pre-activation, right).

Discussion

- PNCAs with and without Cy7 generate similar contrast upon US activation in PAA phantoms, indicating successful Cy7 integration.
- PNCAs without Cy7 demonstrated higher variation across activation locations, likely due to particle age.
- The size of needle used for injection could affect PNCA contrast from US activation due to increased spontaneous activation with smaller gauge needles.
- Cy7 can be incorporated into PNCA coatings to allow for optical tracking in multimodality imaging scans. Additional investigation is needed to determine if Cy7-tagged PNCAs are activated with laser irradiation in PA imaging.
- *Limited* spontaneous activation of PNCAs can prove beneficial to localize the particles prior to selecting activation locations. However, it is important to ensure that not all PNCAs activate upon injection.
- *In vivo* activation of optimized PNCAs suggested that PNCAs are promising molecular imaging targets for US-mediated multimodality imaging.
- Successful injection and activation of PNCAs *in vivo* with US-mediated imaging modalities could provide additional molecular information to clinicians during intraoperative procedures, such as detection of micro-metastases during lymph node biopsy.

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