

TEL AVIV UNIVERSITY

The Iby and Aladar Fleischman Faculty of Engineering

The Zandman-Slaner School of Graduate Studies

**Super-Resolution Ultrasound Imaging of
Macrophages Using Blinking Nanodroplets**

A thesis submitted toward the degree of
Master of Science in Biomedical Engineering

by

Saar Gotshal Zahavi

This research was carried out in the School of Biomedical Engineering

Under the supervision of Prof. Tali Ilovitsh

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Abstract

Tracking immune cells deep within living tissue remains a fundamental challenge due to the diffraction-limited resolution of ultrasound imaging and the inability to resolve dense cellular populations. Here, we introduce an intracellular super-resolution ultrasound imaging framework based on stochastic phase-changing nanodroplets (NDs) and ultrasound localization microscopy (ULM). We engineer ~ 170 nm perfluorocarbon NDs that undergo reversible, stochastic liquid-gas transitions under acoustic excitation, generating temporally sparse “blinking” signals. Leveraging the intrinsic endocytic activity of macrophages, these NDs are internalized, enabling intracellular contrast generation independent of vascular flow. We validate this approach across imaging scales, from controlled phantoms and *in vitro* systems to *in vivo* tumor models, demonstrating robust intracellular blinking, high cell viability, and consistent super-resolution reconstruction in dense cellular environments. The stochastic blinking of internalized NDs provides the temporal separation required to localize individual sources, overcoming a central limitation of conventional ULM. Following systemic administration, ND-labeled macrophages are tracked after homing to the liver, where super-resolution ULM resolves cellular distributions with a spatial resolution of 26.3 ± 3.2 μm , corresponding to a 6.1-fold improvement over diffraction-limited imaging. This work establishes a previously unexplored paradigm for ultrasound-based intracellular super-resolution imaging, enabling non-invasive visualization of immune cell organization in deep tissue. By introducing spatiotemporally programmable intracellular contrast, this approach expands ultrasound beyond vascular imaging toward functional cellular imaging, with broad implications for immunology, diagnostics, and cell-based therapies.

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Abbreviations and symbols

ADV - Acoustic droplet vaporization

BBB - Blood-brain barrier

DLS - Dynamic light scattering

DMEM - Dulbecco's modified eagle medium

FBS - Fetal bovine serum

FWHM - Full width at half maximum

GV – Gas vesicle

MB - Microbubble

MI - Mechanical index

MIP - Maximum intensity projection

MΦ - Macrophage

NB - Nanobubble

ND - Nanodroplet

PBS - Phosphate-buffered saline

PFB - Perfluorobutane

PFC - Perfluorocarbon

PFH - Perfluorohexane

PNP - Peak negative pressure

PSF - Point spread function

TAMs - Tumor-associated macrophages

TEM - Transmission electron microscopy

TME - Tumor microenvironment

ULM - Ultrasound localization microscopy

Z - Acoustic impedance

ρ - Tissue density

c - Speed of sound

λ - Acoustic wavelength

N - Number of cycles within the transmitted pulse

z - Depth of the imaging target

D - Active aperture size of the transducer

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List of publications

S. Gotshal Zahavi, M. Bismuth, T. Bercovici, Y. Leichtmann-Bardoogo, B. M. Maoz, and T. Ilovitsh, “Super-Resolution Macrophage Imaging via Ultrasound Localization Microscopy and Blinking Nanodroplets”, under revision in ACS Nano, 2026.

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1. Introduction

Macrophages are essential components of the innate immune system, playing a role in tissue homeostasis, immune surveillance, and the regulation of inflammatory responses.^{1,2} Their infiltration into tissues is a key indicator of diverse pathologies, ranging from cancer progression to chronic inflammation.²⁻⁴ Beyond their diagnostic value, these cells have emerged as key targets and agents for therapeutic interventions, including cancer immunotherapies and targeted drug delivery.⁴⁻⁶ Therefore, the ability to track macrophages non-invasively offers vital insights for monitoring the biodistribution and therapeutic efficacy of these treatments.^{7,8} However, this remains a significant challenge. Optical microscopy is limited to superficial tissues, while whole-body methods face inherent trade-offs: magnetic resonance imaging (MRI) is often constrained by low sensitivity, and positron emission tomography (PET) lacks sufficient spatial resolution.⁸⁻¹⁰ Consequently, there is a need for an imaging modality that combines deep tissue penetration with cellular-scale resolution.

Ultrasound imaging offers substantial penetration depth for non-invasive body imaging, along with advantages in safety, cost-effectiveness, and accessibility.¹¹ However, it is inherently incapable of resolving individual cells due to the diffraction limit.^{12,13} Furthermore, the minimal acoustic impedance mismatch between cells and surrounding tissue results in poor intrinsic image contrast. Therefore, there is a need to develop ultrasound-based methods capable of imaging macrophages *in vivo*. One possible strategy is to exploit the intrinsic endocytic activity of macrophages to internalize ultrasound contrast agents. In ultrasound imaging, microbubbles (MBs) are the most widely used contrast agents.¹⁴ They consist of a gas core encapsulated by a stabilizing lipid shell and typically have diameters in the range of 1-10 μm . Owing to their strong acoustic scattering, MBs provide sufficient sensitivity to enable detection of single agents *in vivo*. However, due to the diffraction limit, each MB is not resolved as a point object but instead appears as a blurred signal defined by the system's point spread function (PSF).^{15,16} Previous studies have leveraged MB uptake by macrophages and other endocytic immune cells to enable high-contrast cell tracking using conventional ultrasound.¹⁷⁻²⁰ A similar concept has also been explored using smaller contrast agents such as gas vesicles and nanodroplets (NDs).²¹⁻²⁵ Despite these advances, fundamental spatial resolution constraints remain. Macrophages have a typical diameter of $\sim 15 \mu\text{m}$ and often accumulate in dense clusters within tissue.²⁶ In contrast, the MB-derived PSF

extends over hundreds of microns. As a result, signals from multiple labeled cells spatially overlap, making it infeasible to resolve or isolate individual macrophages based on the ultrasound contrast signal. Ultrasound localization microscopy (ULM) offers a powerful strategy to overcome diffraction-limited resolution, enabling super-resolution imaging by localizing individual MB contrast agents.¹⁶ In ULM, each MB appears as a diffraction-limited PSF, and its center of mass can be estimated with subwavelength precision. By accumulating localizations over multiple frames, a super-resolved image is reconstructed with spatial resolution improved by an order of magnitude. In conventional implementations, this approach relies on MB motion within the vasculature, where blood flow provides the temporal sparsity and spatial separation required between individual PSFs.^{16,27–31} Extending this concept to cellular imaging, internalization of MBs by macrophages could, in principle, enable their super-resolution visualization. However, this approach faces fundamental limitations. Internalized MBs are confined within cells and do not exhibit sufficient motion to generate the temporal sparsity required for ULM. In addition, macrophages often accumulate in dense clusters, while the acoustic signal from MBs remains continuously “on,” leading to overlapping PSFs from multiple nearby cells.^{32,33} Under these conditions, individual MBs become difficult to reliably isolate or localize, thereby limiting the application of ULM for *in vivo* super-resolution imaging of macrophages. NDs serve as an alternative class of ultrasound contrast agents, typically composed of a liquid perfluorocarbon (PFC) core stabilized by a lipid shell. Ranging from 100 to 1000 nm in diameter, their liquid state and nanoscale size offer distinct advantages for ultrasound imaging.^{27,34} Upon exposure to an ultrasound pulse, the liquid core of the ND undergoes acoustic droplet vaporization (ADV), transitioning into a highly echogenic gaseous state. The acoustic pressure threshold required for ADV is tunable and depends on parameters such as the ND's size and the boiling point of the encapsulated PFC.^{34–36} Although utilized for ULM, conventional NDs form relatively stable MBs upon vaporization. Consequently, achieving the spatial sparsity needed for ULM relies on high-intensity, destructive acoustic pulses, a “vaporize-and-destroy” approach that rapidly depletes the contrast agent.^{27,37} To overcome the reliance on destructive pulses or hemodynamics, blinking NDs have emerged as a unique class of contrast agents.²⁷ Unlike standard NDs that undergo a permanent liquid-to-gas transition, blinking NDs undergo reversible, stochastic phase changes between liquid and gas states under continuous acoustic exposure or pulse laser irradiation, thereby

acting as blinking sources.^{38–42} This stochastic activity provides a mechanism for the temporal separation of overlapping PSFs, allowing ULM methods to localize and reconstruct super-resolution images of dense and stationary agents. However, to date, blinking ND-based ULM has been demonstrated primarily for freely circulating or extracellular agents following systemic administration.^{27,38} The application of this approach to intracellular environments, and in particular for resolving contrast agents internalized within cells, has not been established.

In this thesis, we develop a method for intracellular super-resolution imaging of macrophages *in vivo* using ULM and blinking NDs (Figure 1). By leveraging the natural endocytic activity of macrophages, we labeled these cells with ~ 170 nm blinking NDs prior to systemic administration. We show that the stochastic phase-transition dynamics of internalized NDs generate sufficient spatiotemporal sparsity to enable localization and tracking of individual macrophages within dense cellular environments.⁴³ This approach establishes a previously unexplored paradigm for ultrasound-based intracellular super-resolution imaging, enabling non-invasive visualization of immune cell dynamics deep within living tissues.

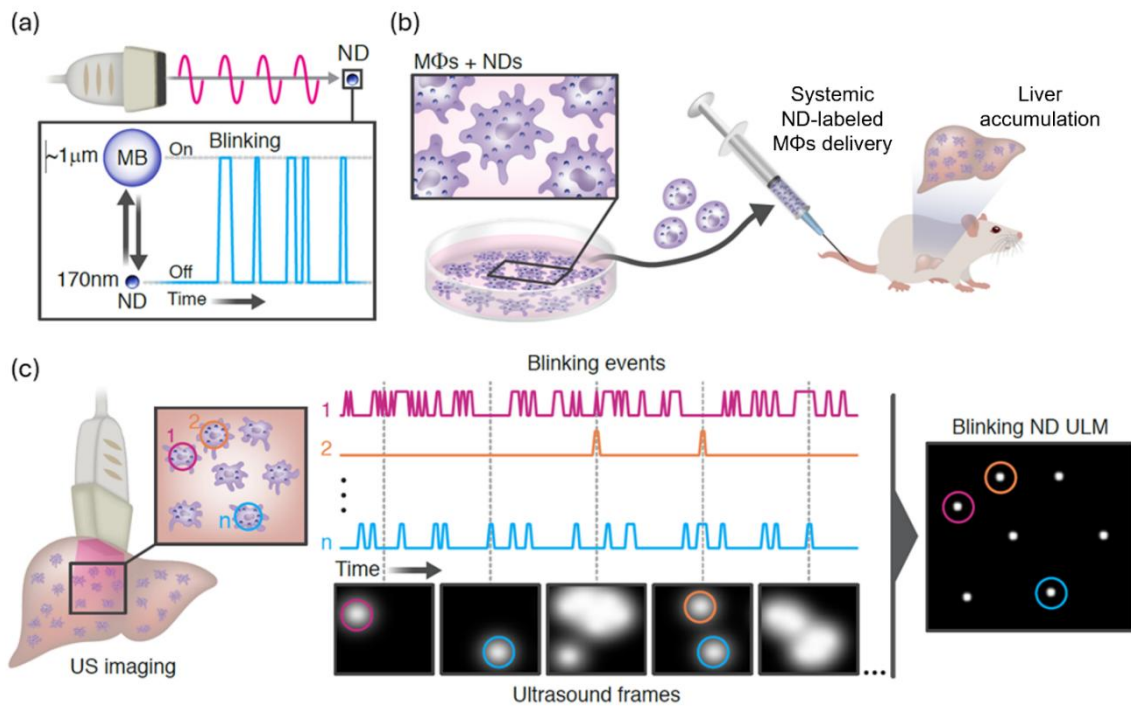


Figure 1: Schematic overview of super-resolution macrophage tracking using blinking NDs. (a) Blinking NDs mode of operation. Under ultrasound exposure, liquid NDs undergo reversible phase transitions into highly echogenic gaseous MBs, generating distinct “on” and “off” temporal signal. (b) Macrophages (MΦs) internalize NDs via

endocytosis, the resulting ND-labeled MΦs are systemically administered and subsequently accumulate in the liver. (c) Ultrasound imaging of the liver captures the stochastic blinking events of the ND-labeled MΦs. Fluctuations across sequential frames allow for the spatiotemporal separation of overlapping, diffraction-limited signals. The localizations are then summed to reconstruct a super-resolution ULM map of the macrophages.

2. Research objectives

This work aims to develop and validate an intracellular super-resolution ultrasound framework for tracking macrophages within biological tissues. By utilizing blinking NDs as contrast agents, this approach seeks to overcome standard acoustic diffraction limits and apply ULM to stationary cells, independent of vascular flow.

Specific aims:

1. Engineer reversible phase-changing NDs and implement a ULM post-processing framework. This involves synthesizing the NDs and developing a computational differential frame-by-frame subtraction algorithm to efficiently extract and separate overlapping signals based on their stochastic blinking events, enabling the localization of individual NDs.
2. Validate the intracellular imaging framework *in vitro*. This phase investigates the endocytic uptake efficiency of the engineered NDs by macrophages, assesses cellular viability following acoustic activation, and evaluates the framework's ability to mathematically resolve super-resolution images of macrophages.
3. Demonstrate super-resolution macrophage tracking and biodistribution *in vivo*. This objective applies the framework to track macrophages within a murine breast tumor model *in vivo* and subsequently maps their accumulation within the liver following systemic administration.

3. Theoretical background

3.1 Immune cell tracking and macrophage biology

Tracking the distribution of immune cells *in vivo* is of paramount clinical importance, as their accumulation serves as a powerful biomarker for disease progression and therapeutic efficacy.⁴⁴ Macrophages are specialized phagocytic cells and essential components of the innate immune system. They play a fundamental role in tissue homeostasis, immune surveillance, and the regulation of inflammatory responses.^{1,2} Originating from circulating monocytes that extravasate into target tissues, macrophages are highly plastic and can adapt their phenotype in response to local microenvironmental cues. Their infiltration into tissues serves as a key indicator of diverse pathologies, ranging from chronic inflammation to cancer progression.²⁻⁴ In pathological contexts such as the tumor microenvironment (TME), tumor-associated macrophages (TAMs) often constitute a dominant immune population, mediating processes such as angiogenesis, immunosuppression, and metastasis.^{45,46}

Beyond their diagnostic value as disease biomarkers, macrophages have emerged as primary targets and cellular vehicles for therapeutic interventions, including cancer immunotherapies and targeted drug delivery.⁴⁻⁶ A defining biological feature of macrophages is their robust endocytic activity. This intrinsic capacity to scavenge and internalize exogenous materials provides a natural mechanism for cellular labeling and drug delivery. Consequently, developing non-invasive modalities to track these cells *in vivo* offers vital insights for monitoring their biodistribution, assessing disease progression, and evaluating the efficacy of macrophage-targeted therapies.^{7,8}

3.2 Principles of medical ultrasound

Medical ultrasound is a highly accessible, cost-effective, and safe non-ionizing imaging modality that enables non-invasive whole-body imaging with substantial penetration depth. This technique employs longitudinal mechanical waves with frequencies above the threshold of human hearing (>20 kHz). Ultrasound waves are generated by a transducer containing piezoelectric elements, which convert electrical energy into mechanical vibrations and, conversely, mechanical vibrations into electrical signals. This dual functionality allows the same piezoelectric element to both transmit and receive ultrasound waves.¹¹

3.2.1 Acoustic impedance and pulse-echo imaging

Image formation in ultrasound is based on the pulse-echo principle. The transducer emits short acoustic pulses into the body. As these acoustic waves propagate through soft tissues, their behavior at interfaces is governed by the acoustic impedance (Z) of the tissue, which describes the resistance of a medium to sound propagation and is defined by:

$$Z = \rho \cdot c \quad (1)$$

where ρ represents the tissue density and c is the speed of sound. When the wavefront encounters an interface between tissues with differing densities, acoustic impedance mismatch occurs, causing partial reflections of the acoustic wave. These reflected waves return to the transducer as echoes. The depth of the reflecting structure is then determined based on the time between the transmission and reception (time-of-flight) of the waves. By mapping the spatial origin of these echoes, an image is reconstructed in which each pixel corresponds to a specific anatomical location, and its brightness (echogenicity) reflects the amplitude of the backscattered signal. Therefore, the intrinsic contrast of the resulting image is governed by the acoustic impedance of the media. Larger mismatches (e.g., between soft tissue and bone or gas) produce strong reflections that appear bright in the image (hyperechoic) and homogeneous media such as fluids appear dark (anechoic). The acoustic impedance differences between individual cells and their surrounding biological medium are inherently small, resulting in limited intrinsic contrast at the cellular scale. Consequently, visualization at the cell level typically requires the use of exogenous contrast agents.

Contrast agents are widely utilized to enhance intrinsic echogenicity in ultrasound imaging. Engineered micro and nanoparticles such as MBs, nanobubbles (NBs) and gas vesicles (GVs) consist of a gas core encapsulated by a stabilizing shell composed of lipids, proteins, or biocompatible polymers, whereas phase-changing NDs instead consist of a liquid core encapsulated by a similar stabilizing shell, which undergoes acoustically induced vaporization into a gas phase (Section 3.4). These contrast agents rely on the same acoustic concept: the presence of a gas phase (intrinsic to MBs, NBs, and GVs, or acoustically induced for NDs) introduces a profound impedance mismatch, resulting in robust backscattering and high-contrast images. Previous studies have

leveraged the natural endocytic capacity of immune cells to internalize these contrast agents, enabling highly sensitive in-vivo cell tracking with ultrasound.^{17–25} Despite these advances, cellular-scale visualization remains fundamentally constrained by the physical diffraction limit of the ultrasound beam.

3.2.2 The PSF

Due to the physical diffraction limit of the acoustic wave, targets smaller than this limit cannot be resolved according to their true geometric structure. Instead, the imaging system acts as a spatial low-pass filter, causing the target to appear as a spatially extended signal known as the PSF (Figure 2). The physical boundaries of the PSF are governed by the system's axial and lateral resolution limits, defined by Equations (2) and (3). Mathematically, the intensity profile of the ultrasound PSF is commonly approximated as a two-dimensional Gaussian distribution, whose extent along the axial and lateral directions defines the system's respective axial and lateral resolution limits, as derived below.

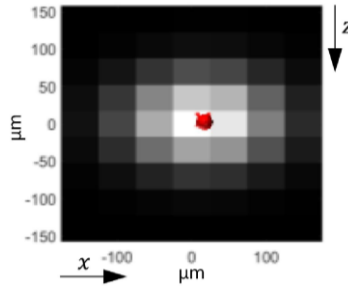


Figure 2: PSF of a MB in an ultrasound image. The PSF is much larger than the original size of the MB (the MB is illustrated as a red dot and magnified). This illustration is adapted from S. Dencks and G. Schmitz (2023).⁴⁷

3.2.3 Resolution and the diffraction limit

The spatial resolution of an ultrasound system, defined as the minimum distance required to distinguish two separate point targets, is therefore determined by the width of the PSF along each of its two orthogonal axes: axial and lateral, representing a fundamental constraint for cellular imaging. Axial resolution describes the ability to differentiate objects aligned along the direction of wave propagation. It is governed by the spatial pulse length and is given by:

$$R_{axial} = \frac{\lambda N}{2} \quad (2)$$

where λ represents the acoustic wavelength and N is the number of cycles within the transmitted pulse. Lateral resolution refers to the ability to distinguish objects perpendicular to the beam axis. It depends on the acoustic wavelength, imaging depth, and transducer aperture, and is defined as:

$$R_{lateral} = \frac{k\lambda z}{D} \quad (3)$$

where z represents the depth of the imaging target, D is the active aperture size of the transducer, and k is a constant determined by the aperture geometry of the transducer. For a rectangular aperture, as used in this work, the resulting diffraction pattern follows a sinc function, for which $k = 1.206$. Lateral resolution corresponds to the effective beam width and is often characterized by the full width at half maximum (FWHM) of the PSF's lateral profile. Both axial and lateral resolution depend on the acoustic wavelength and therefore improve with increasing transmitted frequency. However, because lateral resolution additionally depends on the transducer aperture and imaging depth, it inherently degrades as the distance from the transducer increases. For typical high-frequency ultrasound systems, this diffraction-limited resolution remains on the order of hundreds of microns, failing to resolve microvascular and cellular details. However, because the PSF follows predictable mathematical distribution, the true centroid of an isolated target can be calculated with sub-pixel accuracy.⁴⁷ This capability to localize targets far beyond the physical diffraction limit provides the fundamental motivation for super-resolution imaging.

3.3 ULM

3.3.1 Principles of localization in vascular imaging

ULM offers a powerful computational strategy to overcome the fundamental diffraction limit. Drawing inspiration from optical super-resolution techniques such as photoactivated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM), ULM relies on the localization of individual, spatially isolated point scatterers.³¹ MBs are typically used as contrast agents for this purpose, providing sufficient acoustic backscattering to enable the detection of single agents. The center of

mass from a single MB's PSF can be estimated with subwavelength precision based on mathematical models, typically achieving localizations down to a few micrometers. By accumulating the localized centroid coordinates of thousands of isolated agents over multiple acquisition frames, a super-resolved image is reconstructed, improving the spatial resolution by up to an order of magnitude.

The fundamental requirement for successful ULM is spatiotemporal sparsity. To correctly calculate the individual centers of mass, adjacent targets must be spatially separated by a distance greater than the FWHM of the system's PSF. In conventional implementations, this sparsity is provided by hemodynamics. As MBs flow through the vascular network in low concentrations, their continuous motion ensures both spatial separation and temporal signal variations (Figure 3).^{16,27–31,48}

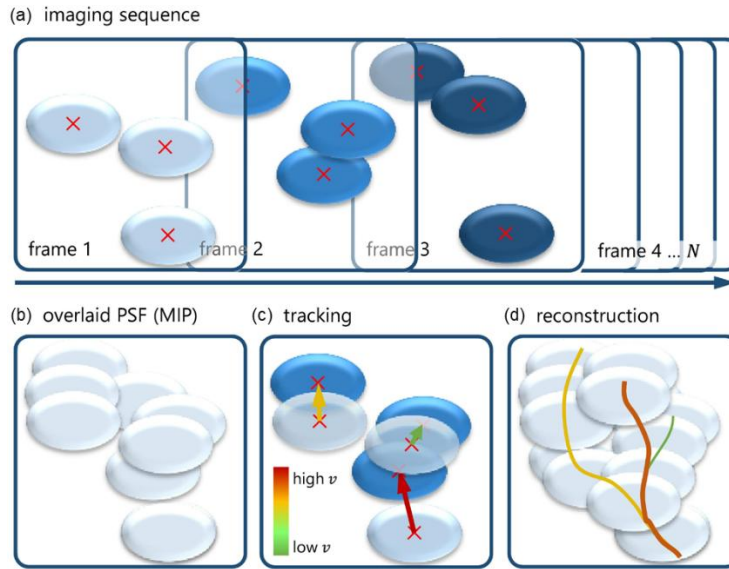


Figure 3: Basic concept of ULM in vascular imaging. (a) Schematic illustration of the localization of single MBs across frames 1 to 3 of an imaging sequence. (b) The overlaid PSF accumulation of frames 1 to 3. (c) Tracking MB positions from frame to frame (exemplified for frame 1 to 2) allows for the reconstruction of vessel courses and the determination of flow velocities and directions. (d) Reconstruction of the complete vascular tree from the full imaging sequence. This illustration is adapted from S. Dencks and G. Schmitz (2023).⁴⁷

3.3.2 Constraints in stationary cellular imaging

While targeted contrast agents internalized via endocytosis successfully enable deep-tissue tracking of macrophages *in vivo*, the predominantly stationary nature of these

cells introduces a fundamental physical constraint. When labeled macrophages accumulate in dense tissue clusters, the acoustic targets remain persistently active. Without the continuous motion provided by hemodynamics, neighboring PSFs overlap significantly. This persistent overlap prevents the target isolation required for sub-pixel localization, fundamentally limiting the applicability of ULM. Consequently, *in vivo* macrophage tracking remains restricted to macroscopic, diffraction-limited visualization, leaving cellular-scale super-resolution unexplored due to this inherent lack of spatiotemporal sparsity.

3.4 Phase-change NDs for ULM

3.4.1 Standard (irreversible) NDs

Phase-change NDs serve as an alternative class of contrast agents designed to bypass the physical limitations of MBs. Typically composed of a liquid PFC core encapsulated by a stabilizing lipid shell, NDs are formulated at nanoscale diameters ranging from 100 to 1000 nm. Upon exposure to an acoustic pulse exceeding a specific pressure threshold, the liquid core undergoes ADV, transitioning into a highly echogenic and stable gaseous MB (Figure 4).^{34–36,49}

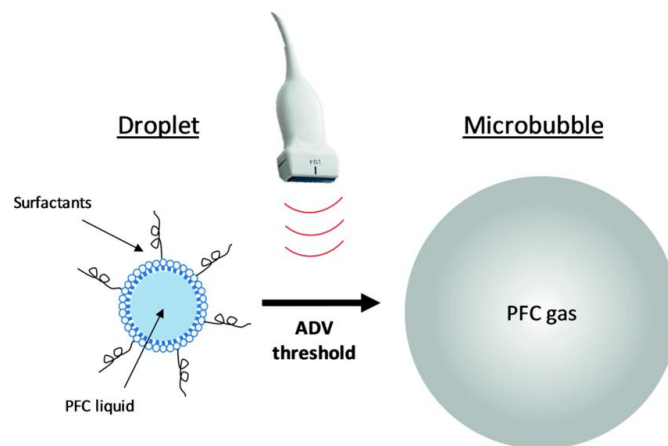


Figure 4: Schematic process of ADV. Vaporization of the PFC core as result of exposure to ultrasonic pulses leads to the formation of a gas bubble. This illustration is adapted from R. Melich et al. (2020)⁵⁰

The physical threshold required to initiate ADV depends fundamentally on the intrinsic thermodynamic properties of the core and the mechanical forces acting upon it. The PFCs range from high-boiling-point liquids like perfluorohexane (PFH, boiling point of 56°C) to low-boiling-point cores such as perfluorobutane (PFB, boiling point of

-2°C).²⁷ The interfacial tension of the encapsulating shell exerts an inward Laplace pressure that scales inversely with particle size. This internal compression effectively raises the vaporization temperature of the encapsulated PFC, enabling low-boiling-point cores like PFB to remain liquid at physiological temperatures (37°C). Because the vaporization temperature of a fluid depends directly on ambient pressure, the peak negative pressure (PNP) of an acoustic wave momentarily drops the local pressure within the core.⁵¹ This rapid decompression lowers the effective boiling point below the ambient temperature, overcoming the stability barrier to trigger vaporization and the subsequent formation of a highly echogenic MB (Figure 5).

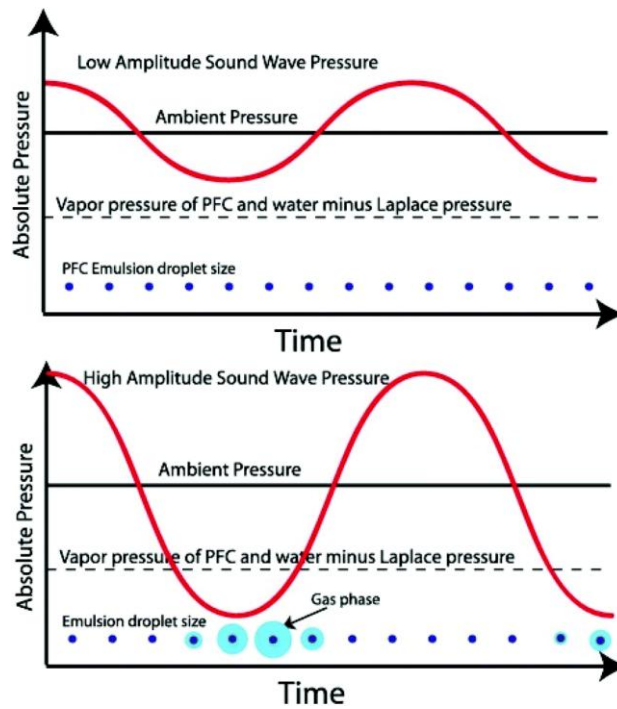


Figure 5: Schematic plot of an ultrasonic acoustic wave affecting a ND. The top graph shows an acoustic wave with low amplitude pressure above the ADV threshold. The bottom graph shows the vaporization of the liquid ND into a gas bubble as soon as the PNP of the wave drops below the ADV threshold (not to scale). This illustration is adapted from W. Pitt et al. (2014).⁵¹

Studies demonstrate that these irreversible phase-change NDs are used for ULM frameworks. They overcome persistent signal overlap by applying a sequential "vaporize-and-destroy" acoustic approach, achieving the spatial sparsity required for sub-pixel localization.^{27,37} An imaging sequence applies selective acoustic pressure to stochastically vaporize a sparse subpopulation of droplets, localize their respective

centroids, and immediately clear the active signals via high-intensity destructive pulses. While this interleaved cycle successfully isolates static targets across consecutive frames, it inherently drives the continuous consumption of the contrast agent, rapidly depleting the available unvaporized reservoir.^{27,37}

3.4.2 Blinking (reversible) NDs

To overcome the reliance on destructive acoustic pulses or continuous hemodynamics, reversible "blinking" NDs have emerged as a unique class of contrast agents.²⁷ Unlike standard NDs that undergo a permanent liquid-to-gas transition, blinking NDs undergo reversible, stochastic phase changes between liquid and gas states under continuous acoustic exposure.³⁸ Following initial vaporization, immediate heat dissipation into the surrounding medium combined with the subsequent positive pressure phase of the ultrasound wave rapidly raises the local vaporization threshold. This thermodynamic shift drives the condensation of the gas MB back into a liquid ND (Figure 6). This reversible phase-transition can be triggered purely acoustically using ultrasound, or optically via pulsed laser irradiation.^{39–42} Previous work demonstrates that ND shells incorporated with the surfactant Pluronic enhances ND phase-change probability.³⁸

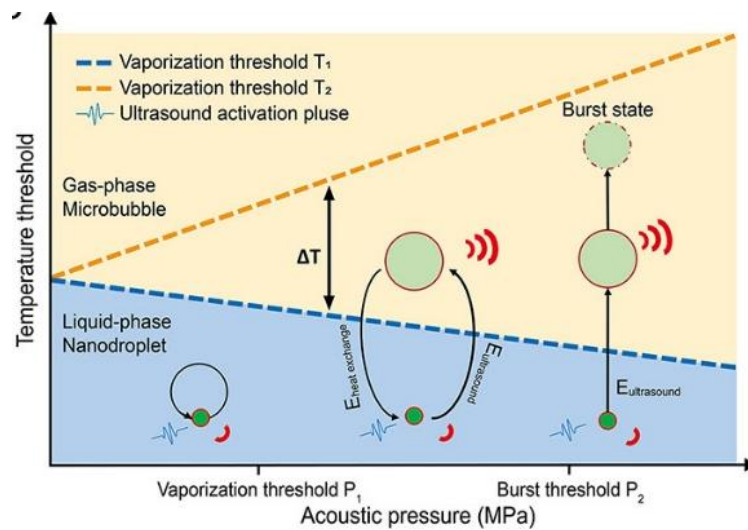


Figure 6: Diagram of phase transitions of blinking NDs under ultrasound stimulation. The negative pressure of the acoustic wave reduces the vaporization threshold (line T_1), triggering vaporization into a gas-phase MB. Following this transition, the wave's subsequent positive pressure raises the vaporization threshold (line T_2), driving liquefaction back to a liquid state. Acoustic pressures exceeding the burst

threshold (P_2) result in irreversible MB destruction. This illustration is adapted from F. Dong et al. (2021).³⁸

Transitions between anechoic liquid droplets and echogenic gas bubbles appear under ultrasound imaging as acoustic blinking sources. These stochastic signals provide a unique physical mechanism to achieve the temporal separation of overlapping PSFs required for ULM, independent of spatial motion and destructive pulses.²⁷ Recent ULM frameworks have utilized blinking NDs to map microvascular networks characterized by slow or zero blood flow.^{27,41}

To date, however, blinking ND-based ULM has been demonstrated primarily for freely circulating NDs following systemic administration within the vasculature. The feasibility of translating this stochastic framework into highly confined intracellular environments, specifically to resolve stationary agents internalized within dense cell populations, remains unestablished.

3.4.3 Stochastic logic for intracellular ULM

This work proposes a framework utilizing blinking NDs for the super-resolution ultrasound imaging of immune cells. Rather than relying on vascular circulation, this approach leverages the intrinsic endocytic activity of macrophages to naturally internalize the NDs prior to imaging. Once internalized, these NDs maintain their ability to undergo stochastic, ultrasound-induced phase-change transitions, effectively transforming the labeled macrophages into acoustic blinking sources.

This intracellular blinking provides a critical solution to the spatial resolution constraints of conventional ultrasound. Because macrophages frequently accumulate in dense, stationary clusters within tissues, standard contrast-enhanced imaging yields continuous, overlapping PSFs that merge into an indistinguishable composite signal. However, the distinct "on" and "off" temporal fluctuations generated by the internalized NDs provide the essential spatiotemporal sparsity required for ULM. By detecting and localizing these stochastic events across sequential frames, overlapping signals can be temporally separated and reconstructed. This enables the super-resolved tracking of individual macrophages within densely populated, stationary environments, establishing a new frontier for non-invasive intracellular imaging deep within living tissue.

4. Materials and methods

4.1 ND preparation and characterization

NDs were synthesized via a microfluidic fabrication process by mixing organic and aqueous solutions within distinct microchannels under controlled laminar flow. The organic solution comprised the lipid powders distearoylphosphatidylcholine (DSPC) (Sigma-Aldrich) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N [methoxy(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG2K) (Sigma-Aldrich). The lipid powders were dissolved in ethanol at a molar ratio of 90:10. For Pluronic-modified NDs, poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) (Pluronic L61) was added to the solution to achieve a molar ratio of 90:10:0.1 (DSPC:DSPE-PEG2K:Pluronic L61). In both cases, the final DSPC concentration was 1 mg/mL. The solutions were heated to 40°C and sonicated for five minutes. Subsequently, 1,1'-Diocadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) (Sigma-Aldrich) was dissolved in the organic solution, and the PFC perfluoro-n-hexane (PFH) (SynQuest Laboratories) was added to a final concentration of 2% (v/v) followed by vortexing to ensure homogeneity. The aqueous solution was made using water, propylene glycol (PG, Sigma Aldrich, Milwaukee, WI) and glycerol (Gly, Acros Organics) at a volume ratio of 7:2:1, as described previously.³⁸

The ND structure was formed using a microfluidic chip (0.2 mm channel width, Herringbone Mixer Glass Chip, Darwin Microfluidics, UK) and two syringe pumps (NE-300 InfusionONE Syringe Pump, Darwin Microfluidics, UK). The aqueous solution was pumped into the chip at inlet 1 at a velocity of 2 mL/min, while the organic solution was introduced simultaneously through inlet 2 at a velocity of 500 μ L/min (Figure 7a).

The ND suspension was dialyzed against phosphate-buffered saline (PBS) using a cellulose membrane (D9777, Sigma-Aldrich) to remove unencapsulated components and ethanol. The dialysis was performed in two consecutive cycles of 1.5 hours each, maintaining a 1:20 (v/v) sample-to-buffer ratio. The NDs were stored at 4°C.

The size distribution and concentration of the NDs were measured using a particle counter system (AccuSizer FX-Nano, Particle Sizing Systems, Entegris, MA, USA). To assess the effect of temperature on ND size, NDs were measured with dynamic light scattering (DLS, Zetasizer Ultra, Malvern Panalytical, UK) after incubation in 4°, 25°

and 37°C for up to eight hours. The morphology of the NDs was further examined using Transmission electron microscopy (TEM, JEM-1400Plus, JEOL, Tokyo, Japan) as previously described.⁵²

4.2 Ultrasound acquisition

Ultrasound imaging and ND activation were performed using a programmable ultrasound system (Vantage 256, Verasonics Inc., Redmond, WA, USA) equipped with L12-3v and L22-14vX linear array transducers (Verasonics). Ray line sequences of focused ultrasound pulses were implemented in MATLAB (R2025a, MathWorks, Natick, MA). In each experiment, the region of interest was aligned with the focal spot of the transducer. Center frequencies of 8 MHz (L12-3v) and 18 MHz (L22-14vX) were utilized for both the application of acoustic pressures to activate the blinking NDs and for acquiring B-mode ultrasound images. The minimal PNPs applied to induce ADV were 1.9 and 2.45 MPa, for 18 and 8 MHz respectively (Table 1). The normal operation PNPs used in this study ranged between 3-3.2 and 3.9-5.2 MPa, for 18 and 8 MHz respectively, with the exact values varying as a function of imaging depth and the corresponding F-number (Table 2). Data was recorded in blocks of 200 continuous frames at a frame rate of 20 Hz. The PNPs of both transducers were calibrated with a needle hydrophone (NH0200, Precision Acoustics, Dorchester, UK) in a degassed water tank.

Table 1: Ultrasound parameters and specifications for minimal ADV threshold

Transducer	Frequency [MHz]	Pulse length [cycle]	F-number	Focus depth [mm]	Active elements [#]	PNP [MPa]	MI
L12-3v	8	1	2	15	38	2.45	0.87
L22-14vX	18	1	1	10	100	1.9	0.43

Table 2: Ultrasound parameters and specifications for normal operation

Transducer	Frequency [MHz]	Pulse length [cycle]	F-number	Focus depth [mm]	Active elements [#]	PNP [MPa]	MI
L12-3v	8	1	2	10-20	25-50	3.9-5.2	1.4-1.7
L22-14vX	18	1	0.5-1	5-10	100-128	3-3.2	0.71-0.75

4.3 ULM image processing

Data analysis was conducted on the recorded 200-frame B-mode sequences. A differential frame-by-frame subtraction process was used to distinguish blinking ND signals from the surrounding static tissues. Data was normalized based on the global minimum and maximum intensities of each experiment. The maximum intensity projection (MIP) of the blinking events was calculated to provide a summarized image containing all NDs that appeared throughout the recording.

Blinking NDs were detected via spatial and temporal analysis. First, peak detection was conducted using the “Imregionalmax” function to detect local maxima on the MIP image. Second, each spatial peak was analyzed temporally, and the blinking events were detected with the “findpeaks” function. Two spatial peaks within a 10-pixel distance that shared a temporal similarity greater than 75% were considered duplicates; in such cases, the ND with the lower blinking intensity was removed.

Localization of NDs was then performed for every frame separately, within a 6x6 pixel window centered around each local maximum using an elliptical Gaussian fitting model. The confidence of each localization was calculated as the R^2 value. For each ND, the localization with the highest confidence was chosen, subject to a confidence threshold of 0.65. The final ULM images were reconstructed by summing all valid localization points detected in the sequence. All data processing was conducted in MATLAB.

Furthermore, to estimate the resolution enhancement, we analyzed dense clusters where individual signals were resolvable. The mean distance between the closest distinguishable peaks and standard deviation were calculated in ImageJ for both the MIP and ULM images. The ratio between the mean values (MIP/ULM) defined the fold-improvement in spatial resolution. All resolution enhancement calculations were analyzed in triplicates.

4.4 Ultrasound imaging of blinking NDs *in vitro*

To evaluate acoustic performance, both ND formulations were dispersed separately into liquid 1.5% (w/v) agarose solutions at 55°C to reach final concentrations of 8.8×10^8 NDs/mL. The solutions were then poured into custom molds and cooled to room temperature for gel formation, allowing the NDs to be immobilized within the phantom. The acoustic blinking ND activation and data acquisition were achieved using the L12-3v transducer. For all subsequent experiments, the Pluronic-modified formulation was utilized.

The effect of thermal incubation and the acoustic pressure was assessed by comparing NDs maintained at 4°C with those incubated at 37°C for two hours prior to gel embedding, with both groups yielding final concentrations of 8.8×10^8 NDs/mL. Acoustic blinking ND activation and data acquisition were performed using the L12-3v transducer at increasing PNPs ranging from 0-5.2 MPa.

4.5 Macrophage cell culture

RAW 264.7 murine macrophages were used as model cells for intracellular ultrasound imaging. The macrophages were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) penicillin-streptomycin, and 1% (v/v) L-glutamine. Cells were maintained at 37°C in a humidified incubator with 5% CO₂ until they reached approximately 80% confluency on the day of the experiment.

4.6 Preparation of ND-labeled MΦs and validation

Macrophages were grown in 6-well plates or T75 tissue culture flasks and maintained as described above. On the day of the experiment, the culture medium was replaced with a suspension of NDs in DMEM at a final surface concentration of 8×10^8 NDs/cm² of tissue plate's surface. For endocytic uptake of NDs, the cells were incubated with the ND suspension for either two or four hours for the validation experiment, and two hours for all subsequent procedures. Following incubation period, the ND suspension was removed, and the cells were rinsed to remove non-internalized NDs. For experiments using 6-well plates, the cells were rinsed five times with 1 mL DMEM, and for experiments using T75 flasks the rinse included five washes with 5 mL DMEM and two additional washes with 25 mL PBS.

To validate intracellular uptake, cells were imaged using an Echo Revolution microscope (ECHO, San Diego, CA, USA) equipped with a 60x oil immersion lens. To accommodate the short working distance of the immersion lens, ND-labeled macrophages were dissociated with a cell scraper and transferred to glass coverslips. The cells were incubated for an additional hour to ensure adherence prior to imaging. Intracellular NDs were identified via fluorescence imaging of the encapsulated DiI dye using the TRITC channel, overlaid with brightfield (BF) images. The number of NDs within each cell was quantified manually from the resulting fluorescence images.

4.7 ND-labeled MΦs' labeling rate and viability

Flow cytometry was performed using a Northern Lights spectral flow cytometer (Cytex Biosciences, Fremont, CA, USA). To assess the rate of endocytic ND uptake, ND-labeled macrophages were prepared in 6-well plates as previously described, using NDs labeled with the fluorescent dye DiI. The cells were then washed extensively to remove free NDs and maintained at 37°C for an additional 0, 2, 4, 6, or 24 hours prior to analysis. The fraction of ND-labeled macrophages was assessed by flow cytometry based on DiI fluorescence. Cells were additionally stained with Ghost Dye Red 710 (SKU 13-0871-T100, Cytex) to assess viability, with live cells quantified within the ND-labeled gated population. Unstained and single stain samples were used for spectral unmixing.⁵³ Spectral unmixing was done using SpectroFlo software.

For assessing viability in response to ultrasound activation, ND-labeled macrophages were prepared as described above in 6-well plates along with a control group of macrophages without NDs. The cells were then collected via dissociation with TrypLE Express (Gibco Corp, 12604-013, Grand Island, NY, USA) and resuspended in 500 µL of degassed DMEM. Ultrasound acquisition to induce ND blinking activation was applied to the cell suspensions within a rod-shaped cavity in a custom 1.5% (w/v) agarose phantom. The phantom was cast in a mold (65 mm × 25 mm × 20 mm) containing a centrally positioned aluminum rod (15 mm height, 6 mm diameter) to create a sample well. After removal from the mold, the phantom was aligned with the focal zone of the L12-3v transducer. The rod-shaped cavity was filled with the cell suspensions, which were gently pipetted to maintain cell distribution during the one-minute ultrasound acquisition at 5.2 MPa. Following exposure, the cell suspensions were re-seeded in 6-well plates and maintained at 37°C for 24 hours to monitor post-activation recovery. Finally, the cells were dissociated with TrypLE and quantified using a CellDrop automated cell counter (CellDrop, DeNovix Inc., Wilmington, DE, USA).

An additional assay evaluated viability specifically within the ND-labeled macrophage population immediately following ADV. Ultrasound activation was performed by loading the cell suspension into a rod-shaped cavity molded within a custom agarose phantom, where ADV was induced at 3 MPa (using the L22-14vX transducer) for one minute while the suspension was gently pipetted to maintain cell distribution.

Following exposure, cells were stained with Ghost Dye Red 710 and cell viability was quantified by flow cytometry, gated within the ND-labeled population.

4.8 ND-labeled MΦs' polarization assay

To assess the effect of ADV on macrophage polarization, ND-labeled macrophages were prepared as previously described and unlabeled macrophages served as control. Ultrasound activation was performed by loading the cell suspension into a rod-shaped cavity molded within a custom agarose phantom, where ADV was induced at 3 MPa (using the L22-14vX transducer) for one minute while the suspension was gently pipetted to maintain cell distribution. Following activation, cells were stained with Ghost Dye Red 710 to assess viability, followed by Fc receptor blocking (553141, BD Biosciences) to minimize non-specific antibody binding, and subsequent staining with Pacific Blue rat anti-mouse CD80 (104724, BioLegend) and APC rat anti-mouse CD206 (141708, BioLegend) to identify the M1 and M2 markers, respectively.⁵⁴ M1 and M2 polarized control macrophages were generated by treatment with LPS (10 ng/mL) and IFN- γ (20 ng/mL), or IL-4 (20 ng/mL) and IL-13 (20 ng/mL), respectively, for 24 hours prior to staining. The percentage of CD80⁺ and CD206⁺ cells was quantified by flow cytometry out of the viable cell population.

4.9 ND-labeled MΦs' migration assay

To assess whether ND labeling and ultrasound activation affect macrophage migratory capacity, we performed a migration assay using GFP-expressing macrophages. ND-labeled macrophages were prepared as previously described and unlabeled macrophages served as control. 4T1 murine mammary carcinoma cells (CRL-2539, ATCC) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium, supplemented with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin and maintained at 37°C within a 5% CO₂ humidified incubator. Ultrasound activation was performed by loading the cell suspension into a rod-shaped cavity molded within a custom agarose phantom, where ADV was induced at 3 MPa (using the L22-14vX transducer) for one minute while the suspension was gently pipetted to maintain cell distribution. Migration was evaluated using transwell inserts with 5 μ m pores (TCS004024, Jet Biofil), in 24-well plates, following a procedure similar to that previously described.^{17,21} 5 $\times$ 10⁵ macrophages were seeded in the upper insert, and 1.5 $\times$ 10⁵ 4T1 cells were seeded in the lower chamber, with wells containing medium

alone included as controls. Cells were allowed to attach overnight, after which the medium was replaced with serum-free DMEM in the upper chamber and serum-containing RPMI in the lower chamber, and the transwell inserts were assembled to allow migration toward the lower chamber. After three hours, migrated macrophages in the lower chamber were imaged by fluorescence microscopy, using the Echo Revolution microscope at 4x magnification.

4.10 Ultrasound imaging of ND-labeled MΦs *in vitro*

To confirm that the acoustic blinking signals originated from within the macrophages, ultrasound imaging was conducted on five agarose gels containing varying cell concentrations. Macrophages were seeded in five T75 flasks at varying initial densities to reach a range of confluences (40%-100%) by the day of the experiment. All flasks were treated with a constant ND surface concentration of 8×10^8 NDs/cm². The cells were dissociated with a cell scraper in 7 mL DMEM, quantified, centrifuged and resuspended in 1 mL of degassed PBS. The resulting ND-labeled macrophage suspensions (containing 2.5, 9, 17, 25 and 32×10^6 cells) were subsequently dispersed separately into 20 mL liquid 1.5% (w/v) agarose solutions at 55 °C, poured into custom molds and cooled to room temperature. This method allowed the cells to be immobilized within the phantoms. Acoustic activation and data acquisition were performed using the L12-3v transducer.

ADV for internalized NDs was measured for 8×10^6 ND-labeled macrophages embedded in agarose phantoms at a final cell concentration of 4×10^5 cells/mL. This was compared to the ADV of NDs in agarose phantoms at a concentration of 8.8×10^8 NDs/mL. The effect of extended incubation at 37°C was assessed for the NDs and ND-labeled macrophages after 2, 4, 6 and 24 hours of incubation prior to embedding in agarose phantoms. Acoustic blinking ND activation and data acquisition were performed using the L22-14vX transducer at increasing PNPs ranging from 0-3 MPa. Blinking activation was additionally assessed via B-mode data acquisition at 3 MPa using the same procedure, following ND labeling and compared to a control of macrophages without NDs.

Fluorescence microscopy was used to validate the distribution of ND-labeled macrophages within the agarose matrix. For this validation, 22.5×10^6 ND-labeled macrophages in 1 mL PBS were embedded in agarose gel as described above, from which thin slices were manually sectioned and placed onto glass slides. DAPI mounting

medium was applied to each slide, and the samples were imaged using the Echo Revolution microscope at 4x and 20x magnification.

4.11 Comparison of ND activation using L12-3v and L22-14vX transducers

To assess the feasibility of using high-resolution ultrasound for *in vivo* imaging with ND-labeled macrophages, the blinking response of NDs activated by the L22-14vX transducer was compared to that induced by the L12-3v. For this comparison, NDs were embedded in two separate agarose gels as described previously, yielding final concentrations of 8.8×10^8 NDs/mL. The phantoms were placed at the respective focal points of each transducer, and acoustic activation and data acquisition were performed to evaluate the resulting signal yield.

4.12 Ultrasound imaging of ND-labeled MΦs in an *in vivo* tumor model

All animal procedures were performed according to the guidelines of the Institutional Animal Research Ethical Committee (approvals TAU-MD-IL-2603-120-3 and TAU-MD-IL-2605-129-5). Female BALB/c mice, aged between 8 to 12 weeks and weighing 20–25 g (sourced from Envigo, Jerusalem, Israel), were used as the breast cancer animal model. 4T1 murine mammary carcinoma cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium, supplemented with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin and maintained at 37°C within a 5% CO₂ humidified incubator. By the day of injection, they had achieved approximately 85% confluency. Cells were collected via dissociation with TrypLE and were subsequently suspended at a concentration of 9×10^5 cells in 25 μL PBS+/+. This cell suspension was then used for subcutaneous injection into the mammary fat pad, creating the primary tumor model. Tumors were allowed to grow until they reached a size of ~7 mm.

ND-labeled macrophages were prepared as described previously in T75 tissue culture flasks, dissociated with a cell scraper in 7 mL DMEM, quantified, centrifuged and resuspended. For ultrasound imaging of the ND-labeled macrophages *in vivo*, the mice were divided into two groups: the control group was injected with macrophages without NDs and the second with the ND-labeled macrophages. The tumors were placed in the focal point of the L22-14vX transducer, a needle was inserted intratumorally guided via ultrasound until clearly visualized as a high-intensity signal. A final load of 8×10^5

macrophages in 40 μ L was injected intratumorally. Subsequently, the acoustic activation and data acquisition were performed using the L22-14vX transducer.

4.13 Ultrasound imaging of ND-labeled M Φ s in the liver

To evaluate the biodistribution and liver accumulation of ND-labeled macrophages within an animal model, six BALB/c mice (8 to 12 weeks old, 20-25 g, Envigo, Jerusalem, Israel) were utilized.

ND-labeled macrophages were prepared as described previously in T75 tissue culture flasks, dissociated with a cell scraper in 7 mL DMEM, quantified and centrifuged. The cell pellet was resuspended to a final load of 1×10^7 cells in 150 μ L PBS for systemic administration, and a separate control group received no injection. Following a four-hour circulation period to allow for macrophage homing, the mice were sacrificed, and the livers and brains were harvested.

To detect the accumulated macrophages, ultrasound acquisition was applied to the excised organs within a custom 1.5% (w/v) agarose phantom. The phantom was cast in a mold (65 mm $\times$ 25 mm $\times$ 28 mm) containing a centrally positioned aluminum rod (24 mm height, 10 mm diameter) to create a sample well. After removal from the mold, the phantom was aligned with the focal zone of the transducer. The organs were positioned individually within the rod-shaped cavity, which was rinsed between samples. Acoustic activation and data acquisition were performed using the L22-14vX transducer.

5. Results

5.1 ND characterization and acoustic blinking activation

NDs were synthesized using a microfluidic Herringbone Mixer (Figure 7a) to precisely control particle size and concentration, yielding an average diameter of 170 ± 50 nm (Figure 7b). To evaluate the effect of shell composition on signal stability, two formulations were prepared, with and without the surfactant Pluronic (L61). TEM confirmed that both ND formulations shared a similar spherical morphology (Figure 7c, d). To evaluate their acoustic response, NDs were examined *in silico* within agarose gel phantoms. Upon exposure to ultrasonic waves, the NDs underwent stochastic blinking activation. Temporal analysis of this activity revealed unique patterns for each droplet (Figure 7g), with an average frequency of 2.09 ± 1.93 Hz for NDs synthesized with Pluronic (Figure 7e). These temporal signatures, combined with the spatial peaks

identified from the MIP of the subtracted frames (Figure 7f), enabled the accurate detection of distinct NDs.

However, a significant difference in signal stability was observed between the two formulations. While NDs lacking Pluronic failed to demonstrate sustained activity, the Pluronic-modified NDs exhibited robust, repetitive blinking, with individual droplets undergoing up to 60 blinking cycles during the 10-second recording period (Figure 7e).

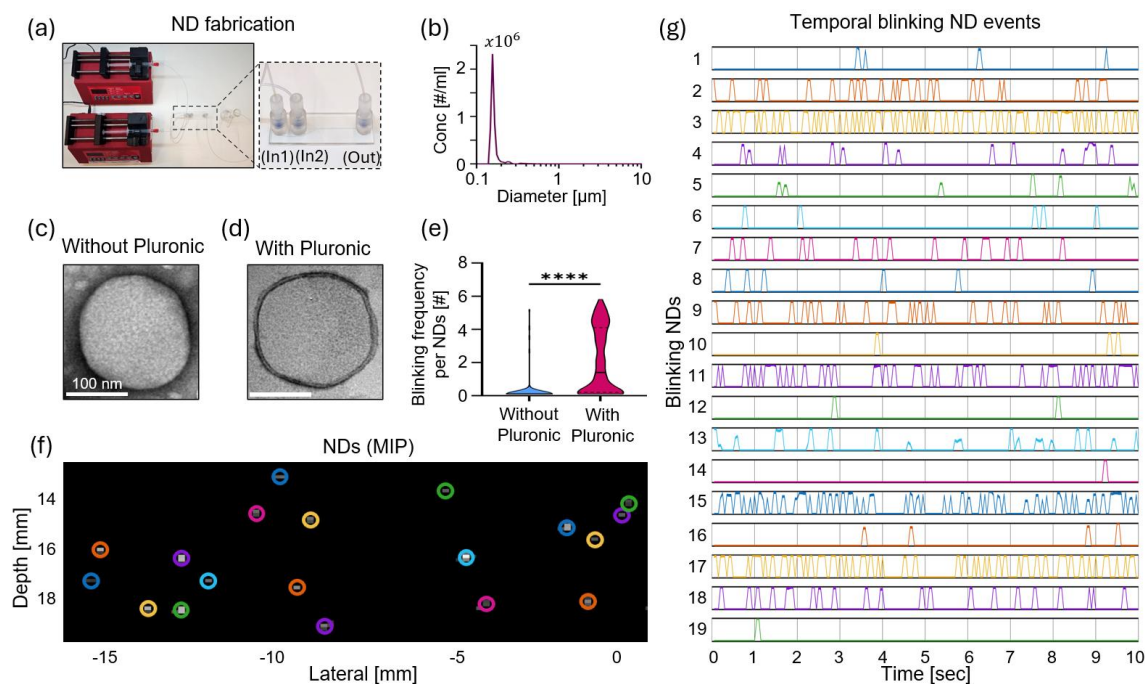


Figure 7: ND fabrication, size distribution and acoustic blinking characterization. (a) ND fabrication via microfluidic chip. (b) Typical size distribution of NDs with Pluronic (L61). (c, d) TEM of NDs. (c) Without Pluronic, and (d) with Pluronic. (e) Blinking frequencies for NDs formulated with and without Pluronic (L61). Unpaired t-test, $n = 8$. (f) MIP for NDs with Pluronic *in silico*, marked by colors. (g) Individual ND blinking patterns for corresponding NDs as marked in (f). NDs are marked from left to right (f) correspond to (g) from top to bottom. Adjusted p value was **** $p < 0.0001$. All data are plotted as the median $\pm$ quartiles.

The Pluronic-modified formulation was selected for all subsequent experiments. The size stability of this formulation over time was further assessed by DLS following incubation at 4°C, 25°C, and 37°C for up to eight hours, during which the average diameter remained within 177.1-186.5, 177.8-199.7, and 171.4-187 nm, respectively (Figure 8).

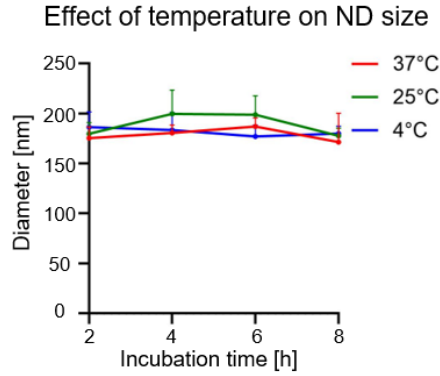


Figure 8: ND diameter as a function of incubation time. Measured after up to 8 hours at 4°C, 25°C and 37°C, $n = 3$. All data are plotted as the mean $\pm$ SD.

To clearly illustrate the complete processing pipeline, we visualized NDs embedded in an agarose phantom, where the NDs remain spatially confined. B-mode images were first acquired, followed by frame-by-frame subtraction to detect individual blinking events (Figure 9a, b). The resulting subtracted frames were then summed to generate the MIP (Figure 9c), and the temporal blinking profiles of individual NDs were subsequently extracted (Figure 9d). The analysis was performed at both 1.2 MPa and 3 MPa. No blinking was observed at 1.2 MPa, which is below the ADV threshold, whereas multiple blinking NDs were detected at 3 MPa, above the ADV threshold, resulting in clear blinking events in the subtracted images and the corresponding MIP.

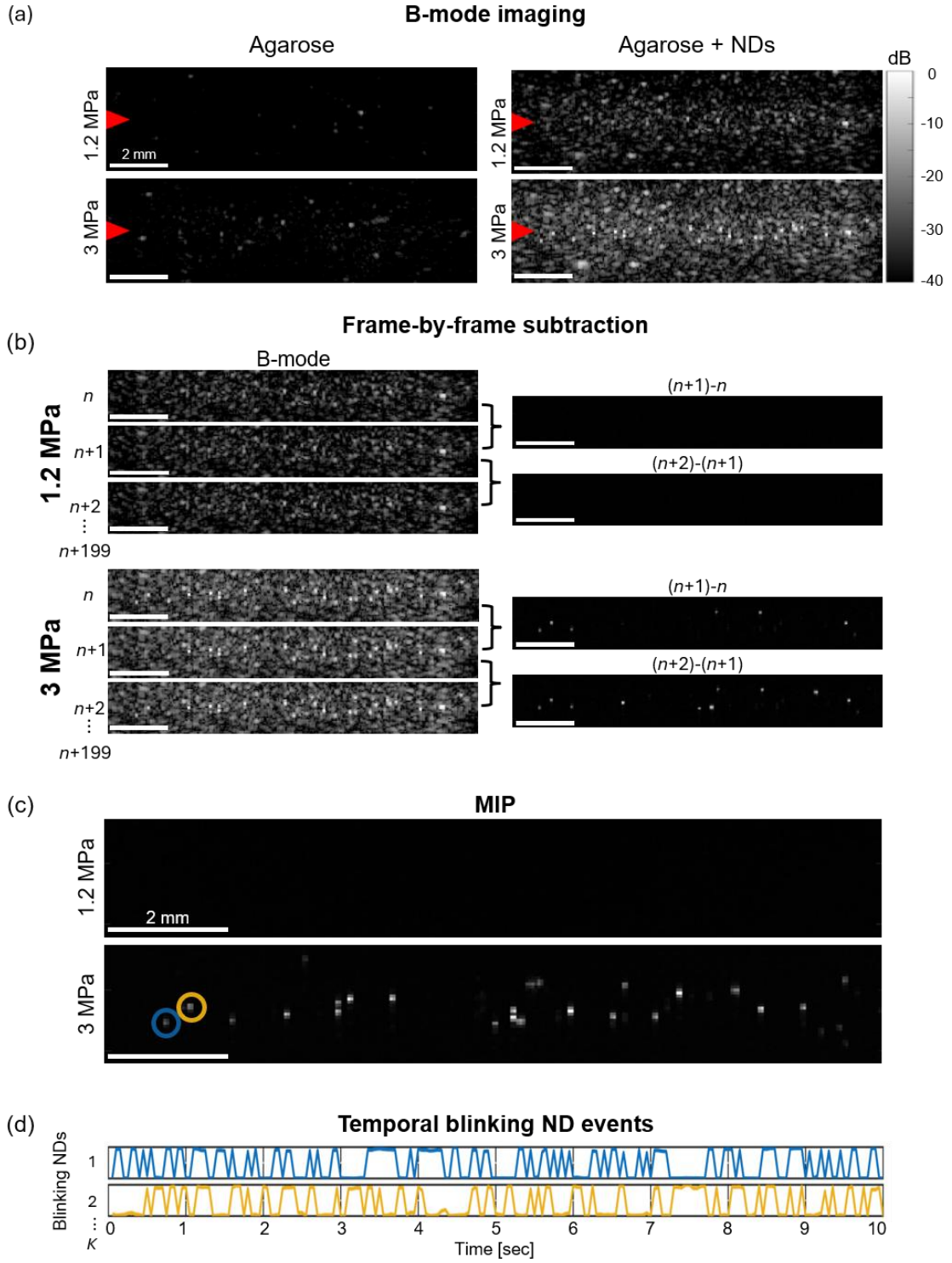


Figure 9: Blinking analysis for NDs in agarose phantom. (a) B-mode imaging at 1.2 (below ADV threshold) and 3 MPa (above ADV threshold). Red arrows show the focus depth at 10 mm. (b) Frame-by-frame subtraction shown on three following frames for agarose + NDs phantom at 1.2 and 3 MPa. B-mode images on the left (color bar in (a)) and the corresponding B-mode frame's subtraction on the right. Scale bar 2 mm. (c) MIP of all

subtracted frames. (d) Temporal blinking ND events of the two NDs detected and marked in (c), respectively.

5.2 MΦ internalization of NDs for cellular labeling

To internalize the NDs, we leveraged the natural endocytic activity of macrophages. NDs were introduced to the cell culture media, and intracellular uptake was evaluated following incubation periods of two and four hours (Figure 10a, b). Microscopy analysis demonstrated significant ND accumulation within the cells at both time points. An average of 5.37 ± 6.7 NDs per cell was observed after a two-hour incubation period, and 7.6 ± 6.47 after four hours. Notably, these values likely underestimate the true number of internalized NDs. Due to the diffraction limit of conventional fluorescence microscopy, clustered sub-diffraction NDs cannot be individually resolved, and weakly fluorescent droplets may fall below detection sensitivity. Furthermore, quantification was limited to a single optical plane. Therefore, these counts represent a conservative, relative measure of cellular labeling rather than an absolute quantification. As no significant difference in uptake efficiency was observed between the two durations, a two-hour incubation period was selected for all subsequent experiments. In addition, fluorescence imaging of the labeled cells embedded in an agarose gel phantom revealed a uniform spatial distribution (Figure 10c). These results verify the formation of ND-labeled macrophages, establishing the basis for investigating their acoustic blinking properties.

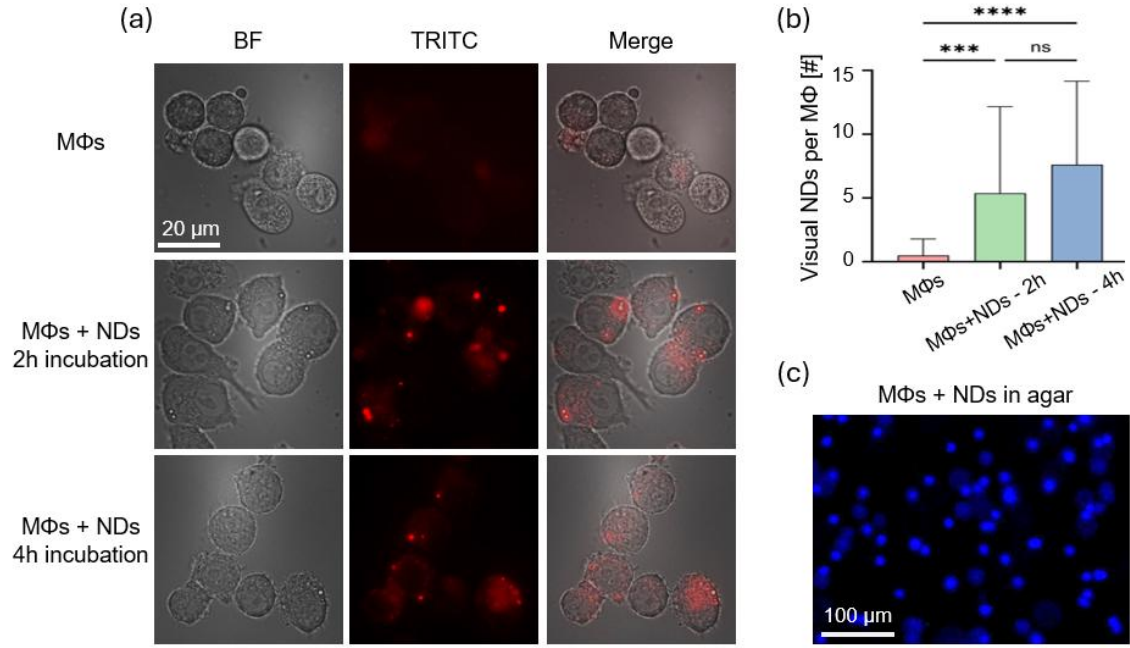


Figure 10: MΦs load blinking NDs, forming ND-labeled MΦs. (a) Fluorescence microscopy images comparing untreated MΦs with MΦs incubated with fluorescent red NDs (DiI) for two and four hours (60x magnification). (b) Number of visually detected NDs internalized in MΦs, per cell cross-section. One-way ANOVA with Tukey's multiple comparison test. (c) Fluorescence microscopy of ND-labeled MΦs in agarose gel, stained blue with DAPI (4x magnification). Adjusted p values were *** $p < 0.001$, and **** $p < 0.0001$. $n = 4$. All data are plotted as the mean $\pm$ SD.

5.3 Ultrasound induced intracellular ND blinking in MΦs

Prior to cellular imaging, we assessed the impact of the biological labeling conditions on ND acoustic performance. Comparisons between fresh NDs and those subjected to a two-hour incubation at 37°C revealed no significant degradation in activity, the number of detected blinking NDs was comparable between the two groups (Figure 11a). For both conditions, minimal blinking initiated at a PNP of 2.45 MPa, with the number of events increasing until reaching a maximum at 5.2 MPa, the upper pressure limit of the L12-3v transducer.

Because direct optical confirmation of intracellular ND vaporization was not feasible at the resolution and imaging plane required, we relied on indirect evidence to support intracellular ND activation and assessed the blinking signal across phantoms containing increasing concentrations of ND-labeled macrophages. Although a constant initial

concentration of NDs was applied to all groups, the number of detected blinking events increased proportionally with the number of labeled cells (Figure 11c). This trend, observed consistently across three different focal depths, suggests an intracellular origin of the blinking signal rather than non-specific background particles.

Next, the stochastic nature of these blinking signals was leveraged to achieve super-resolution ULM (Figure 11d). To demonstrate this, we analyzed two adjacent ND-labeled macrophages that appeared as a single, unresolvable cluster in the standard MIP image (Figure 11e). By analyzing their temporal fluctuations (Figure 11g), we successfully distinguished the neighboring sources. Because the two NDs exhibited distinct blinking patterns, with blinking frequency of 4.86 Hz and 2.14 Hz, respectively, they could be separated and localized in different frames, achieving sub-pixel resolution (Figure 11f). Applying this ULM algorithm across the entire field of view enhanced the spatial resolution by a factor of 2.4 over the standard MIP images, enabling the visualization of adjacent macrophages at a resolution of $182.7 \pm 10.8 \mu\text{m}$.

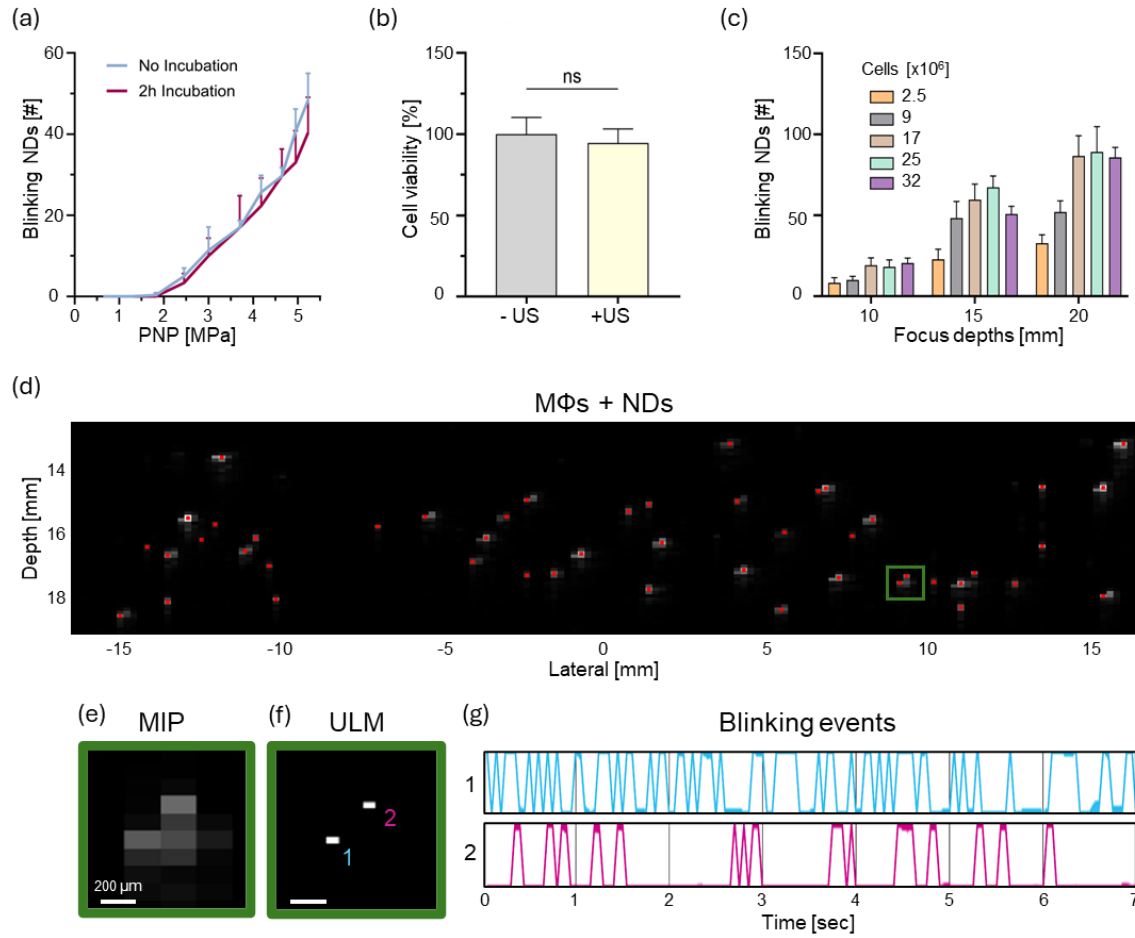


Figure 11: Acoustic activation of ND-labeled MΦs *in vitro*. (a) Acoustic pressure and incubation effects detection of NDs, $n = 3$. (b) Viability of ND-labeled MΦs after ultrasound activation. Unpaired t-test, $n = 6$, $p > 0.05$. (c) Detected ND-labeled MΦs for different cell amounts, $n = 6$. (d) MIP for ND-labeled MΦs *in vitro*, ULM localizations marked in red. (e, f) Separation of ND-labeled MΦs, MIP (e) and ULM image (f) of the area marked green in (d). (g) Corresponding blinking events for the ND-labeled MΦs in (f). All data are plotted as the mean $\pm$ SD.

To determine whether prolonged intracellular residence compromises ND structural integrity or acoustic performance, cellular uptake and acoustic activity of ND-labeled macrophages were examined for cells maintained under physiological conditions over extended periods. The fraction of ND-labeled macrophages was assessed using flow cytometry, based on the fluorescence of the ND's encapsulated DiI dye. The macrophages' uptake ratio with NDs was stable during the extended incubation periods. A percentage rate of 78.03%, 79.04%, 78.99%, 76.67% and 65.69% labeled macrophages was observed for the respective 0, 2, 4, 6 and 24 hours of incubation at

37°C (Figure 12a). In addition, flow cytometry viability assay quantified the live cells only within the ND-labeled macrophage population. These results confirmed high viability for the ND-labeled macrophages after extended incubation times, with 99.82%, 98.92%, 99.58%, 99.76% and 99.6% live cells for the respective 0, 2, 4, 6 and 24 hours of incubation (Figure 12b), demonstrating that prolonged intracellular retention of NDs does not adversely affect macrophage viability. Blinking kinetics were then assessed for ND-labeled macrophages and compared to NDs only. Both were incubated for 0, 2, 4, 6 and 24 hours, and imaged at 18 MHz with increasing PNPs (0, 1.2, 1.9, 2.5 and 3 MPa). ADV of ND-labeled macrophages was detected at 1.9 MPa with minimal blinking NDs, following an upward trend to a maximum average of 51, 52.67, 51.67, 57 and 31.67 blinking NDs after 0, 2, 4, 6 and 24 hours of incubation, respectively (Figure 12c). The ADV of the ND-labeled macrophages was compared to those of NDs only, showing similar behaviors for both free and intracellular NDs across the tested incubation periods (Figure 12d). Stable blinking ND kinetics was observed after up to six hours of incubation. A decrease in blinking NDs was observed after a 24-hour incubation period for both free and intracellular NDs, with no significant difference in blinking activity between the two populations at any of the tested acoustic pressures at this timepoint (two-way ANOVA with Sidak's multiple comparisons test; $p = 0.996, 0.999, \text{ and } 0.803$ at 1.9, 2.5, and 3 MPa, respectively) (Figure 12d, e). Blinking frequencies were also assessed for both free and intracellular NDs after different incubation periods and PNPs (Figure 12f). The average blinking frequency was in the range of 2.12-2.7 Hz, except for the free NDs examined at 1.9 MPa reaching an average frequency of 0.71 Hz (Figure 12f). This is due to less stable blinking activations observed for the free NDs at 1.9 MPa, suggesting a slightly lower ADV threshold for the intracellular NDs.

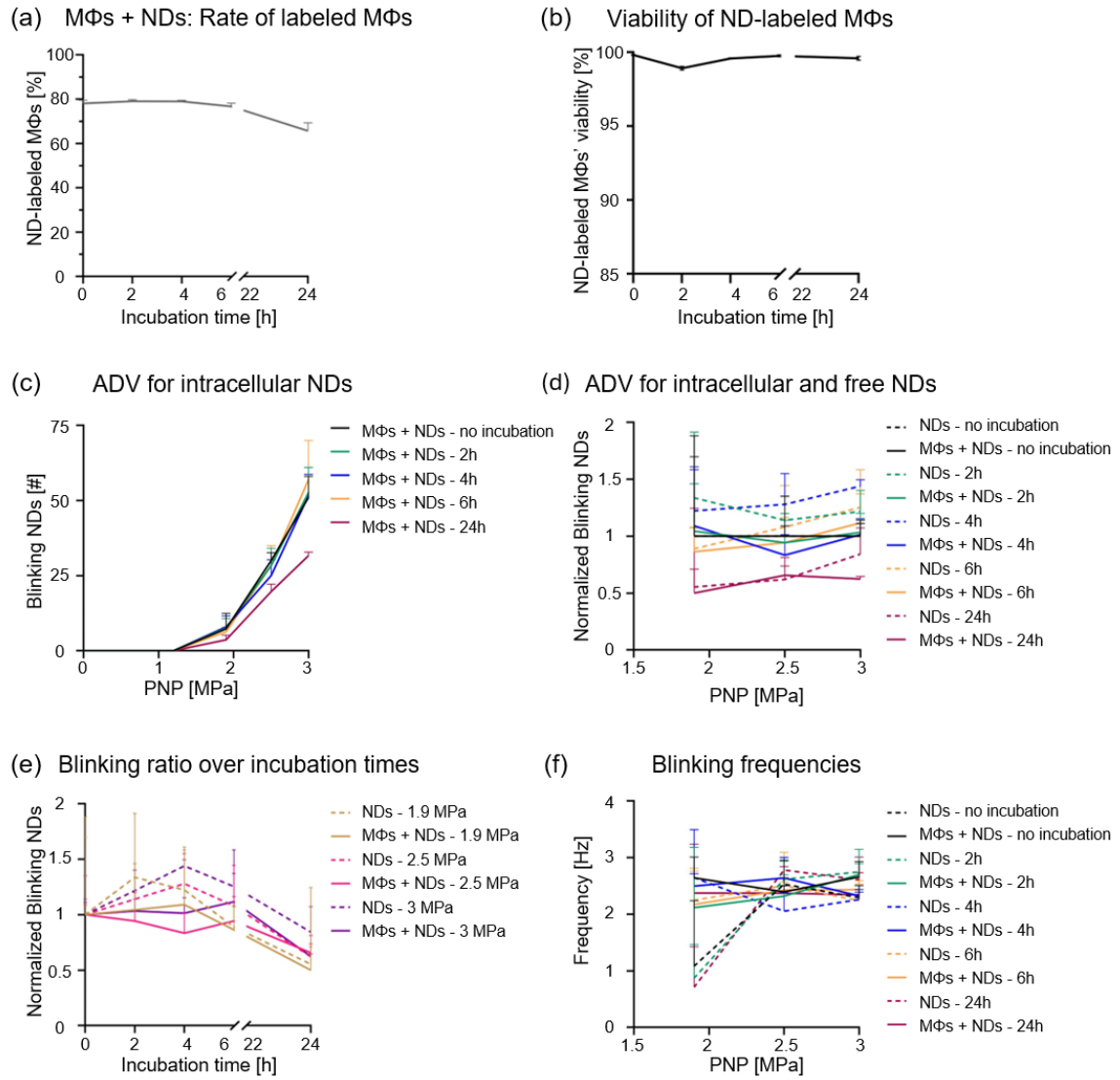


Figure 12: Effects of incubation time on ND-labeled MΦs. All results assess the effect of incubation at 37°C for 0, 2, 4, 6 and 24 hours (a) Percentage of MΦs containing NDs out of the total MΦ population, quantified by flow cytometry. (b) Effect of ND labeling on MΦs viability as a function of incubation time, quantified by flow cytometry. (c) ADV for intracellular NDs, at different incubation times measured at 1.2, 1.9, 2.5 and 3 MPa. (d) Comparison of ADV between free and MΦ-internalized NDs at 1.2, 1.9, 2.5, and 3 MPa to assess the effect of cellular internalization. (e) Blinking NDs for free and MΦ-internalized NDs over incubation times, comparing measurements at 1.2, 1.9, 2.5 and 3 MPa. (f) Blinking frequencies after different incubation times, measured at 1.2, 1.9, 2.5 and 3 MPa. All data are plotted as the mean $\pm$ SD, $n = 3$.

5.4 Functional integrity of ND-labeled MΦs following ultrasound activation

To evaluate the effect of ADV on ND-labeled macrophages, cell viability, polarization and migration were assessed following ultrasound exposure. Ultrasound activation was performed on the cell suspensions in a rod-shaped cavity molded within an agarose phantom, where ADV was induced at 3 MPa for one minute while the cell suspension was gently mixed. Cell viability was quantified following 24-hour post-activation recovery, confirming that ND-labeled macrophages maintain high cell viability of $94.43\% \pm 8.83\%$ (Figure 11b). An additional viability assay was performed to evaluate the live cells within the ND-labeled macrophage population. For this, cells were stained with a viability dye immediately after ultrasound activation, and live cells were quantified by flow cytometry, gated specifically within the ND-labeled population. The percentage of live cells was $99.88 \pm 0.03\%$, with no significant difference between macrophages exposed to ultrasound and non-sonicated controls (Figure 13).

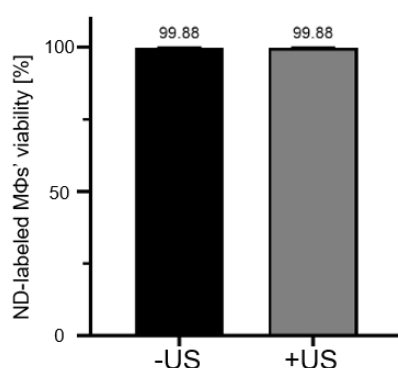


Figure 13: Flow cytometry analysis of ND-labeled MΦ viability, with and without ultrasound. Unpaired t-test, $n = 3$. All data are plotted as the mean $\pm$ SD.

To determine whether intracellular ND activation alters macrophage polarization state, macrophage phenotype was assessed with flow cytometry based on surface marker expression (CD80 for M1 and CD206 for M2) and compared to M1 and M2 polarized positive controls. The average CD80 expression was 85.96%, 84.78%, 85.51%, and 84.5% for the four experimental groups ($-$ NDs $-$ US, $-$ NDs $+$ US, $+$ NDs $-$ US, and $+$ NDs $+$ US, respectively), compared to 99.19% for the M1 control, with SD below 1%

across all groups (Figure 14a). The average CD206 expression was 15.34%, 14.55%, 18.36%, and 16.23% for the same four groups, respectively, compared to 24.73% for the M2 control, with SD below 5% across all groups (Figure 14b). In both markers, expression across the four experimental groups remained substantially lower than the corresponding M1 and M2 controls, and comparable to one another regardless of ND labeling or ultrasound activation.

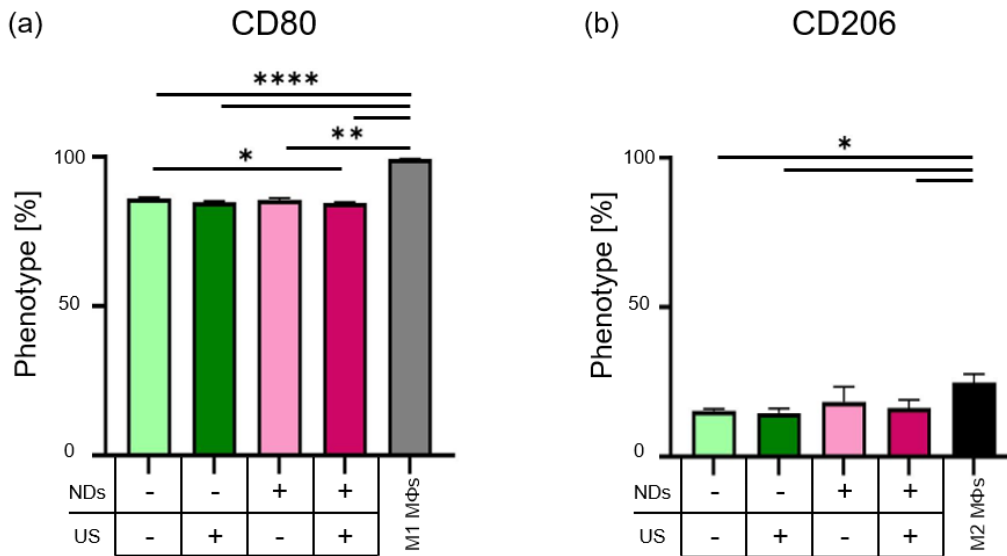


Figure 14: MΦ polarization following ND labeling and ultrasound activation, assessed by flow cytometry. (a) CD80 expression (M1 marker) and (b) CD206 expression (M2 marker) in MΦs across four experimental groups ($\pm$ NDs, $\pm$ ultrasound activation), compared to polarized M1 and M2 MΦ controls, respectively. Adjusted p values were $*p < 0.05$, $**p < 0.01$, and $****p < 0.0001$, $n = 3$. All data are plotted as the mean $\pm$ SD.

Since effective *in vivo* tracking relies on ND-labeled macrophages retaining their migratory and chemotactic capacity, migration was evaluated using a transwell migration assay with macrophages seeded in the upper chamber and 4T1 cells in the lower chamber, and wells containing medium alone included as controls. After three hours, the migrated macrophages in the lower chamber were imaged by fluorescence microscopy. The average number of migrated cells was 9.83 ± 3.37 , 13 ± 1.79 , 13.17 ± 5.56 , and 12.83 ± 2.93 for the four experimental groups ($-$ NDs $-$ US, $-$ NDs $+$ US, $+$ NDs $-$ US, and $+$ NDs $+$ US, respectively), with no significant differences detected between groups (Figure 15).

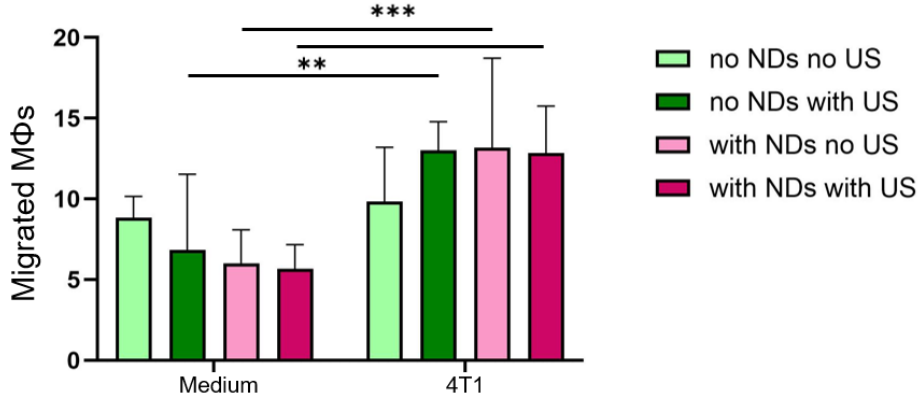


Figure 15: MΦ migration following ND labeling and ultrasound activation. Number of migrated MΦs is quantified by fluorescence microscopy (4x magnification), across the four experimental groups ($\pm$ NDs, $\pm$ ultrasound activation) toward wells containing medium alone (negative control) or seeded with 4T1 cells. Adjusted p values were $**p < 0.01$, and $***p < 0.001$, $n = 3$ wells, two fields imaged per well. All data are plotted as the mean $\pm$ SD.

5.5 *In vivo* ULM of breast cancer tumors using ND-labeled MΦs

We next sought to extend this approach to *in vivo* imaging in a murine model of breast cancer. Given the superficial nature of breast tumors, we aimed to exploit higher ultrasound frequencies to improve spatial resolution. To this end, we transitioned to the L22-14vX transducer (center frequency 18 MHz). As a first step, we verified that stochastic blinking of NDs could be efficiently detected at this higher frequency. An average of 54 ± 8.3 blinking NDs were detected using the L22-14vX, representing a 1.97-fold increase compared to the ones detected with the L12-3v (Figure 16a). Notably, this robust activation was achieved at a PNP of 3.2 MPa, corresponding to a mechanical index (MI) of 0.75.

Following this validation, we evaluated the feasibility of tracking immune cells in a localized tumor model, prior to investigating systemic distribution. ND-labeled macrophages were administered into the tumor, followed by ultrasound imaging. Imaging of a control group injected with unlabeled macrophages was also performed for comparison. We detected an average of 35.75 ± 17.06 blinking events from the ND-labeled macrophages *in vivo*, compared to 0.75 ± 0.96 in the unlabeled control group ($p < 0.01$, Figure 16b). Furthermore, we observed blinking frequencies ranging from 0 to 6 Hz (Figure 16c), confirming the stochastic nature of the phase-change events

necessary to achieve the temporal separation required for ULM. In the MIP images (Figure 16d), the dense accumulation of cells appears as a blurred continuous signal. By localizing the individual blinking events (Figure 16e), the reconstruction of the stochastic signals provided a super-resolved image with a spatial resolution of $30 \pm 5.6 \mu\text{m}$ *in vivo* (Figure 16f), representing a 4.5-fold enhancement compared to the diffraction-limited MIP images. These results validate that the ND-labeled macrophages can be robustly activated and resolved through biological tissue, successfully translating this tracking framework to an *in vivo* setting.

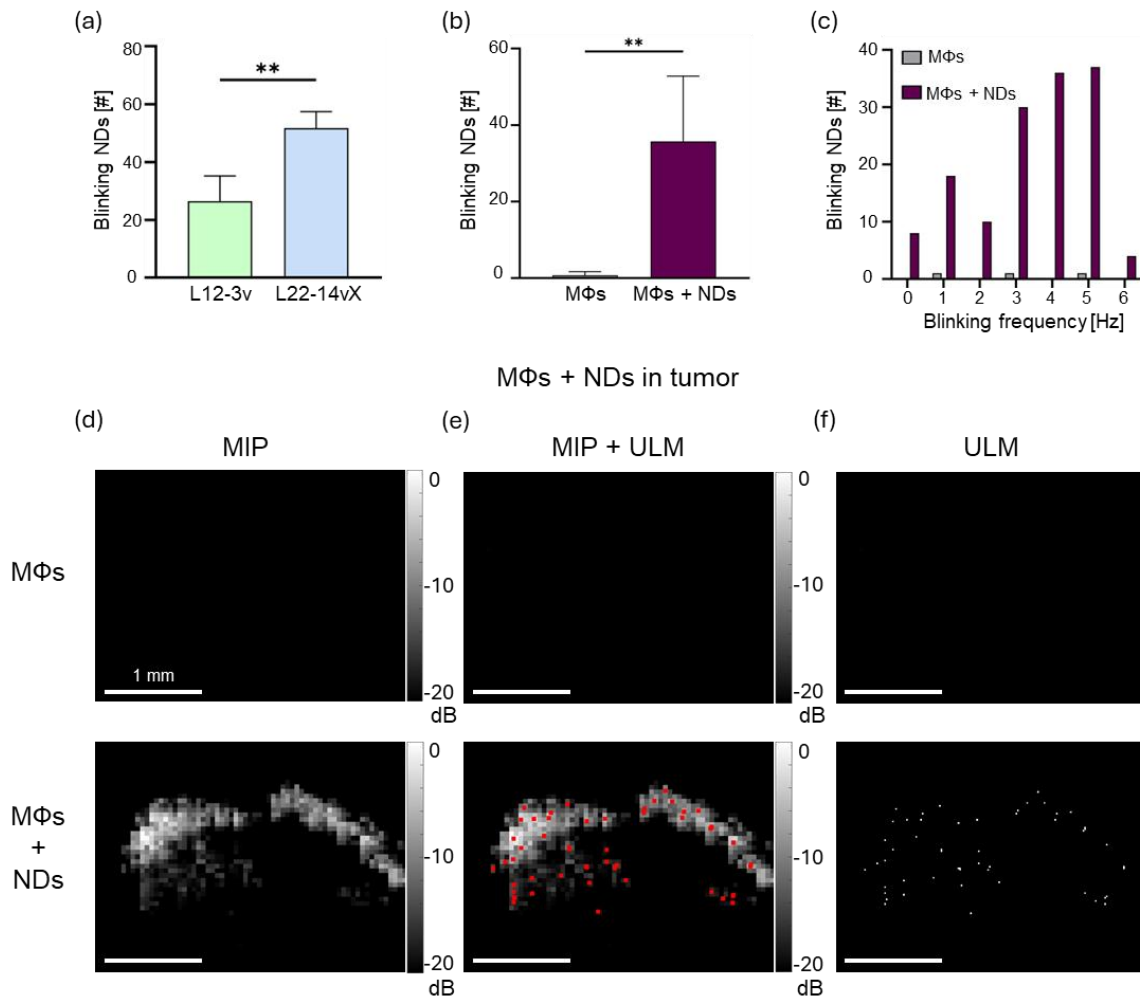


Figure 16: ULM of ND-labeled MΦs in a breast cancer mouse model. (a) Number of blinking NDs detected with the transducers L12-3v (5.1 MPa) and L22-14vX (3.2 MPa). Unpaired t-test, $n = 7$. (b) Detected ND-labeled MΦs *in vivo*. Unpaired t-test, $n = 4$ tumors. (c) Blinking frequencies for ND-labeled MΦs *in vivo*, $n = 4$ tumors. (d, e, f) MIP and ULM images of MΦs in the tumor (top) and ND-labeled MΦs (bottom); (d) MIP. (e) MIP with

ULM localizations labeled in red. (f) ULM. Adjusted p values were $^{**}p < 0.01$. All data are plotted as the mean $\pm$ SD.

5.6 ULM imaging of ND-labeled MΦs in the liver

Following *in vivo* tumor imaging, we next sought to validate systemic administration and assess the biodistribution of ND-labeled macrophages in a highly perfused organ. To this end, we evaluated their accumulation in the liver. ND-labeled macrophages were administered intravenously and allowed to circulate for four hours to facilitate biodistribution and active liver homing *in vivo*, consistent with previously reported homing timepoints for systemically administered immune cells.⁵⁵ Due to its proximity to the lungs and heart, the liver was imaged *ex vivo* to minimize respiratory and cardiac motion artifacts, ensuring that detected blinking signals originate from NDs rather than tissue motion. The brain was also assessed as a negative control organ, given that macrophages are not expected to cross the intact blood-brain barrier (BBB).

Ultrasound imaging of the liver revealed robust blinking activity from the internalized NDs. These temporal events remained stable, exhibiting up to 60 blinking cycles during the 10-second recording period (Figure 17e), a performance comparable to the *in vitro* observations (Figure 7e). This suggests that the ND-labeled macrophages maintain their acoustic activity throughout the injection, circulation, and tissue accumulation processes. Leveraging these signals, ULM resolved diffraction-limited MIP features into super-resolved maps (Figure 17a-c), achieving a spatial resolution of 26.3 ± 3.2 μm . This represents a 6.1-fold improvement over MIP. Blinking activity was minimal in the brain (Figure 17d).

The ULM images revealed that macrophages accumulate within the liver in distinct clusters of approximately 1-2 mm, with three such clusters observed within a single image plane (Figure 17a-c).

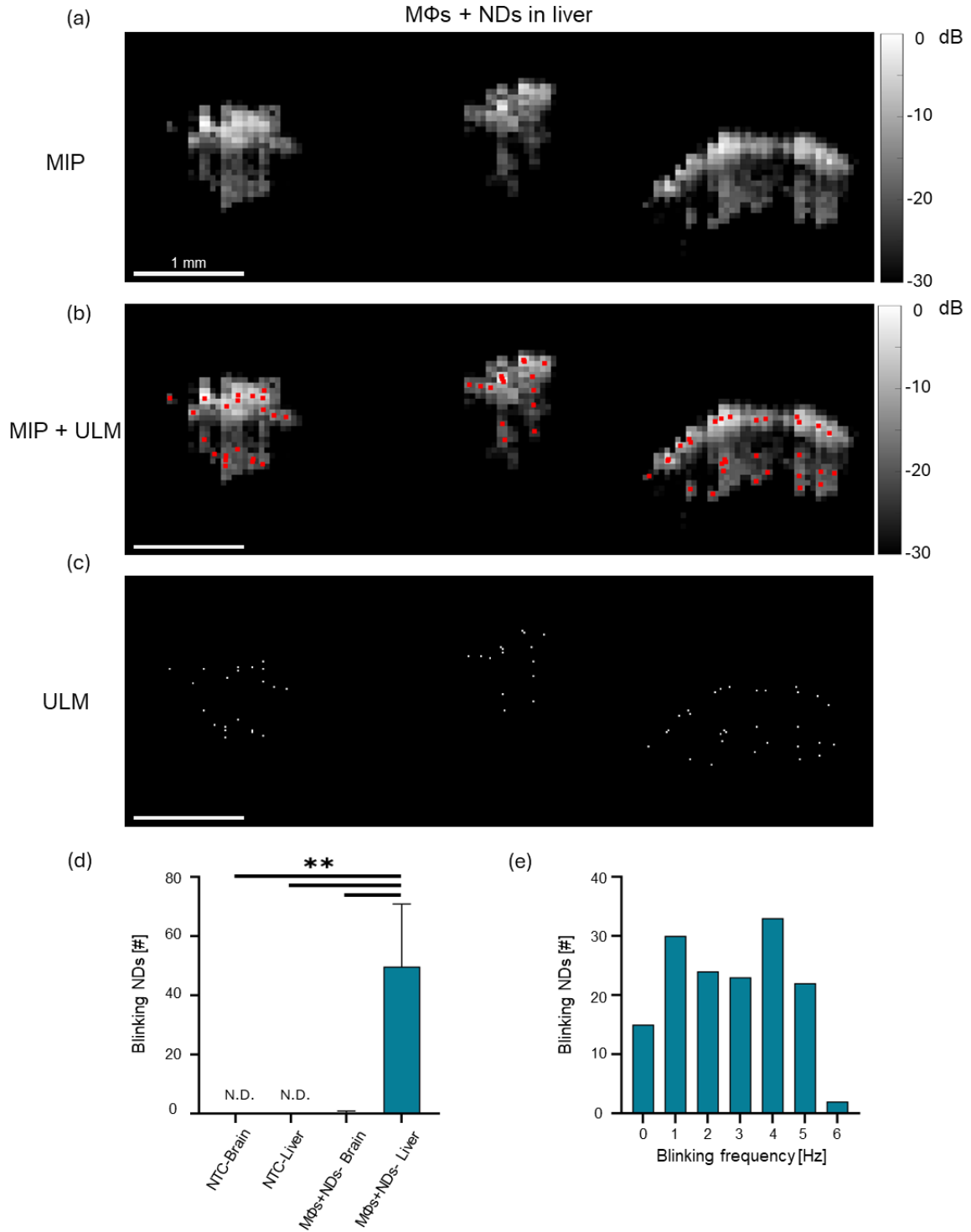


Figure 17: ULM of ND-labeled MΦs in the liver. (a) MIP. (b) MIP with ULM localizations labeled in red. (c) ULM. (d) Detected ND-labeled MΦs in the liver. One-way ANOVA with Tukey's multiple comparison test. (e) Blinking frequencies for ND-labeled MΦs in the liver. Adjusted p values were $**p < 0.01$, $n = 3$. All data are plotted as the mean $\pm$ SD.

6. Discussion

The ability to non-invasively track immune cell dynamics deep within living tissue holds immense potential for monitoring disease progression and evaluating the efficacy of immunotherapies. However, conventional ultrasound-based cell-tracking methods are fundamentally limited by the acoustic diffraction limit, rendering dense cellular populations unresolvable. In this work, we developed and validated an intracellular super-resolution imaging framework that overcomes this barrier by utilizing stochastic, phase-changing NDs. By leveraging the natural endocytic activity of macrophages to internalize these NDs, we successfully transformed stationary immune cells into stochastically blinking acoustic scatterers, enabling their super-resolved localization *in vivo*.

The success of this framework fundamentally relies on the precise engineering of the NDs to support continuous, reversible phase-transitions. Crucially, a significant difference in signal stability was observed between the tested formulations (Figure 7e). The absence of repetition in the non-Pluronic group is consistent with previous studies demonstrating that Pluronic incorporation enhances ND blinking probability.^{38,56} This indicates that the Pluronic triblock copolymer is mechanically necessary to sustain repeatable vaporization cycles. Furthermore, the encapsulation of PFH, a PFC with a naturally high boiling point of 56°C, ensured thermal stability at physiological temperatures. The inward Laplace pressure exerted on these nanoscale NDs effectively increases the internal pressure, raising the boiling point even further. This prevented spontaneous vaporization, ensuring the droplets remained in a stable liquid state until the PNP of the acoustic wave momentarily lowered the local pressure to trigger the phase transition. The ND activation threshold (2.45 and 1.9 MPa for 8 and 18 MHz respectively) is lower than previously reported for PFH NDs.³⁸ One possible explanation for this effect is that Pluronic reduces the ND's interfacial tension; since Laplace pressure scales directly with interfacial tension, this reduction is expected to lower the ND's effective Laplace pressure, thereby lowering the thermodynamic barrier to vaporization.⁵⁷ Prior work showed that the PNP threshold decreases as higher ratios of Pluronic are incorporated, consistent with the relatively high Pluronic:lipid ratio used in our formulation.³⁸

Translating this acoustic technology into a biological context required validating the efficiency of cellular uptake and the subsequent viability of the labeled cells. Following

a two-hour incubation period, quantitative fluorescence analysis confirmed robust internalization, yielding an average of 5.4 NDs per cellular cross-section (Figure 10a, b). Because this quantification represents only a two-dimensional focal plane, the volumetric nature of macrophages dictates that the true intracellular payload is significantly higher. Moreover, conventional fluorescence microscopy is itself diffraction-limited, such that closely clustered NDs cannot be individually resolved, and weakly fluorescent droplets may fall below detection sensitivity. The reported count therefore reflects the number of NDs visually resolved rather than the true intracellular number. Importantly, the two-hour incubation at 37°C used for macrophage labeling did not affect the acoustic activity of the NDs, demonstrating that the labeling conditions do not compromise their acoustic sensitivity (Figure 11a).

Establishing that these blinking events truly originate from within the labeled macrophages, rather than from residual free NDs or imaging artifacts, is essential for validating the intracellular specificity of this approach. Direct confirmation of intracellular ND vaporization would require simultaneous optical and ultrasound imaging of the same cell; however, the substantial disparity in spatial resolution and imaging plane between the two modalities makes such one-to-one correlation highly challenging. Although combined optical microscopy-ultrasound systems have been used to visualize the response of individual bubbles to ultrasound excitation,¹⁵ our approach requires not only ultrasound excitation but also ultrasound image formation and localization of blinking events, spatially registered with the microscopic field of view. We therefore relied on indirect evidence to support intracellular ND activation. Increasing the cellular concentration across multiple phantom models while maintaining a constant initial ND concentration yielded a proportional increase in blinking events (Figure 11c), confirming that the signals arise specifically from within the macrophages rather than from uninternalized background particles.

Our temporal stability studies show that ND acoustic activation remains stable for at least six hours under physiological conditions, based on characterization across independent incubation timepoints (Figure 12c–f), defining an optimal imaging window for ND-labeled macrophages. Beyond six hours, the detected blinking signal declines for both free and intracellular NDs, with no significant difference in decline between the two populations (Figure 12d, e), indicating that this decline is not driven by cellular-induced degradation but more plausibly reflects an intrinsic degradation of the NDs themselves. In addition, at later time points than those examined in this work,

further signal loss might occur due to cellular degradation or cellular proliferation, given the reported ~11-hour doubling time of RAW 264.7 macrophages.⁵⁸ Nonetheless, applications requiring longer tracking periods remain feasible, albeit with reduced ADV activity and consequently lower imaging sensitivity. Notably, this six-hour imaging window already represents a substantial extension over previously reported tracking durations for engineered immune cells. Labeling of CAR-T cells with NBs (nanobubbles) has previously been shown to be effective for *in vivo* tracking, though limited to a 75-minute imaging window due to NB stability constraints.⁵⁹ Our approach may therefore broaden the applicability of ultrasound-based tracking to CAR-T, CAR-M and other endocytic immune cell therapies.

Importantly, these robust intracellular blinking events did not induce cytotoxicity, with cells maintaining high viability following ultrasound exposure (Figures 11b and 13). Moreover, neither ND internalization nor ultrasound-induced ADV drove a shift in macrophage polarization state or altered macrophage migratory capacity toward 4T1 cells. Together, these functional assays indicate that the labeling and imaging procedure leaves macrophage viability, phenotype, and migratory function intact, supporting the suitability of this approach for *in vivo* tracking without compromising the biological behavior of the immune cells being monitored.

While previous ULM frameworks have utilized blinking contrast agents to map stationary microvasculature, this work successfully translates this flow-independent mechanism directly to intracellular cell tracking. As demonstrated in our *in vitro* models, even when adjacent labeled macrophages merged into a single diffraction-limited cluster, their independent, uncoordinated phase transitions provided the spatiotemporal sparsity needed to mathematically separate their overlapping PSFs (Figure 11e-g). The ULM framework enhanced the spatial resolution by a factor of 2.4, localizing adjacent macrophages at a resolution of $182.7 \pm 10.8 \mu\text{m}$. Importantly, this improvement reflects the specific spatial distribution of cells within the phantom, where macrophages were relatively sparsely packed. As such, the measured factor should not be interpreted as an intrinsic limit of the method. In more densely populated environments, such as *in vivo* tissues where macrophages are expected to be in closer proximity, the increased spatial overlap combined with stochastic blinking is expected to further enhance the achievable resolution. These results highlight that the stochastic intracellular blinking provides the temporal sparsity required to resolve dense,

overlapping targets, establishing a foundation for super-resolution imaging of cellular populations *in vivo*.

Building upon these fundamental validations, we examined the tracking of macrophages *in vivo* within a breast tumor model. TAMs are critical mediators of the tumor microenvironment, often orchestrating angiogenesis and metastasis.^{45,46} The ability to pinpoint their exact accumulation sites offers substantial diagnostic value. Furthermore, beyond assessing endogenous immune responses, this high-resolution tracking provides a critical non-invasive tool for monitoring the biodistribution and targeted delivery of macrophage-mediated immunotherapies. Because high-frequency ultrasound yields superior spatial resolution at the cost of penetration depth, we capitalized on the superficial nature of the murine breast tumors to safely transition to an 18 MHz transducer. This shift maximized the baseline spatial resolution prior to algorithmic processing. By subsequently applying our ULM framework to the stochastic blinking events, the macrophages within the tumor were successfully tracked and localized with a resolution of $30 \pm 5.6 \mu\text{m}$ *in vivo* (Figure 16f), representing a 4.5-fold enhancement compared to diffraction-limited imaging. Such resolution could potentially enable discrimination between engineered immune cells residing in the peritumoral stroma and those that have successfully infiltrated the tumor parenchyma, depending on the tumor architecture and stromal composition. This distinction has direct relevance to adoptive cell therapies such as CAR-T and CAR-M, where confirming successful tumor infiltration rather than peripheral accumulation is critical for assessing therapeutic efficacy. Furthermore, since achievable spatial resolution scales with the imaging wavelength, the use of higher-frequency transducers could further improve localization precision in applications requiring finer spatial resolution. Following local tumor tracking, we next evaluated the systemic biodistribution of intravenously administered ND-labeled macrophages. Because our framework utilizes differential frame analysis to detect blinking events and isolate them from the surrounding tissue, a static imaging environment is crucial for accurate localization. While superficial tumors could be adequately stabilized *in vivo*, assessing systemic biodistribution within deep internal organs required an *ex vivo* approach to circumvent the respiratory and cardiac motion artifacts inherent to their anatomical locations. Importantly, while the ultrasound acquisition was performed *ex vivo*, the biological circulation and subsequent organ accumulation occurred entirely *in vivo*. In the liver, the ULM reconstructions achieved a spatial resolution of $26.3 \pm 3.2 \mu\text{m}$ (Figure 17c), a

6.1-fold improvement over MIP, while the brain exhibited minimal background signal. This minimal signal in the brain serves as a critical biological validation; because circulating macrophages and nanoscale NDs are not expected to cross the intact BBB, this finding supports the spatial specificity of our tracking and indicates that the detected signals do not represent systemic artifacts. Interestingly, the localized macrophage accumulations in the liver exceeded the dimensions of individual murine lobules (~0.5 mm). This distribution pattern suggests the macrophages clustered across supralobular vascular domains spanning multiple adjacent functional units. These domains comprise sinusoidal networks where resident macrophages naturally accumulate to filter the blood.^{60,61} This anatomical correspondence suggests that our method maps the cellular-scale distribution of macrophages within the hepatic architecture, marking a significant step toward super-resolution intracellular immune cell tracking via ultrasound imaging.

A critical advantage of this specific framework is its excellent operational safety profile. By utilizing reversible phase-changing NDs, significant blinking activation was successfully achieved at a MI of only 0.75 within the intracellular environment of the tracked macrophages, falling within the FDA diagnostic safety limit ($MI < 1.9$). Ultimately, achieving robust temporal sparsity within this low-MI regime confirms that continuous, super-resolution tracking of immune cells can be performed safely, without risking mechanical cytotoxicity or tissue damage.

While this study establishes a robust framework for *in vivo* tracking of macrophages, several limitations remain. Deep-tissue imaging in dynamic organs is susceptible to motion artifacts; therefore, systemic biodistribution was assessed *ex vivo* to ensure reliable localization. Future studies will incorporate motion compensation algorithms to enable fully *in vivo* implementation of the method. In addition, the performance of the framework at higher cellular densities remains to be systematically explored. The observed 6.1-fold resolution improvement reflects the specific *in vivo* cell distribution and may vary in denser populations, although such conditions are not directly controllable *in vivo*. Furthermore, the stochastic blinking behavior of NDs is strongly influenced by ultrasound excitation parameters, imaging frame rate, and extended intracellular residence time, and further work is needed to optimize control over this behavior. In addition, variability in intracellular ND uptake may introduce heterogeneity in signal intensity across cells. Finally, current particle characterization methods cannot reliably distinguish acoustically active, PFH-loaded NDs from inactive

particles within the synthesized suspension, such that the reported ND concentration likely overestimates the fraction of functional agents. This is a common challenge across nanoscale ultrasound contrast agents, where distinguishing functional particles from inactive ones remains technically difficult using conventional sizing methods.⁶² Future work will focus on motion correction strategies, improved control of blinking dynamics, and integration with volumetric ultrasound imaging to enable fully *in vivo* super-resolution mapping of immune cell dynamics.

7. Conclusions

This study establishes an intracellular super-resolution ultrasound imaging framework that overcomes the diffraction limit to enable non-invasive tracking of macrophages in deep tissue. By leveraging blinking NDs as intracellular contrast agents, we demonstrate ULM without reliance on vascular flow, addressing a central limitation of conventional approaches. The stochastic blinking behavior of NDs was preserved following cellular internalization and across imaging configurations using transducers operating at distinct center frequencies, confirming the robustness and translatability of the platform. We validated this framework across imaging scales, from *in vitro* systems to *in vivo* tumor models, where dense macrophage accumulations were resolved with enhanced spatial resolution. Following systemic administration, ND-labeled macrophages were successfully tracked after homing to the liver, achieving a 6.1-fold improvement in spatial resolution and reaching $26.3 \pm 3.2 \mu\text{m}$. Together, these results demonstrate that intracellular blinking provides the temporal sparsity required for super-resolution imaging in dense cellular environments, establishing a new paradigm for ultrasound-based cellular imaging. This capability advances ultrasound from vascular imaging toward functional, cell-resolved imaging, with potential applications in non-invasive diagnostics and the monitoring of cell-based therapies.

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תקציר

אולטרסאונד היא אחת משיטות הדימות הרפואי הנפוצות והנגישות ביותר בשימוש קליני יומיומי, אך זיהוי ומעקב של תאים באמצעות אולטרסאונד מהווה אתגר משמעותי הנובע משני קשיים פיזיקליים: ראשית, מגבלת העקיפה מכתובה רזולוציה מרחבית שאינה מספיקה כדי להבחין בין תאים בודדים; ושנית, הניגודיות של תאים בדימות אולטרסאונד נמוכה מאוד, כך שלא ניתן להבחין בין תאים ספציפיים לבין הרקמה. תאי מאקרופאגים הינם תאים מרכזיים במערכת החיסון, המסוגלים לבלוע ולעכל פתוגנים, תאים פגומים וחלקיקים זרים בתהליך הנקרא אנדוציטוזה, ובכך ממלאים תפקיד חיוני בשמירה וויסות של תהליכים בגוף. מעקב אחר תאי מערכת החיסון בגוף הוא בעל חשיבות קלינית רבה, שכן הצטברותם ברקמות משמשת כסמן ביולוגי להתקדמות מחלות ולהערכת יעילותם של טיפולים מבוססי-תאים.

במחקר זה, פותחה שיטת דימות תוך תאית בסופר רזולוציה, המבוססת על חומר ניגוד מסוג ננו-טיפות "מהבהבות" בשילוב עם ULM (Ultrasound localization microscopy). ULM היא שיטת עיבוד תמונות אולטרסאונד המבוססת על איכון של מיקומי חומרי ניגוד מבודדים מרחבית על פני פריימים רבים. הצטברות מיקומיהם השונים על פני הפריימים מאפשרת לשחזר תמונה בסופר רזולוציה. במסגרת המחקר, פיתחנו ננו-טיפות בגודל של כ-170 ננומטר העשויות מליבה נוזלית של Perfluorocarbon ומעטפת ליפידית. תוך שימוש בפעילות האנדוציטוזה הטבעית של מאקרופאגים, הננו-טיפות נבלעות על ידי התאים ובכך מתקבלים מאקרופאגים מסומנים בהן. כאשר הננו-טיפות נחשפות לגלי הקול של מקור האולטרסאונד הן עוברות שינוי אקראי והפך ממצב נוזל לגז, ובחזרה. בועות גז מהוות חומר ניגוד חזק באולטרסאונד, בניגוד לנוזל, כך שהמעבר האקראי בין מצבי הצבירה יוצר אותות אקוסטיים שנראים כהבהובים של החלקיקים בתמונת האולטרסאונד. ההבהוב האקראי של הננו-טיפות מאפשר להפריד מרחבית וזמנית ביניהם ועל ידי כך לבנות תמונות סופר רזולוציה של הננו טיפות בתוך תאי המאקרופג.

השיטה אומתה בתרבויות תאים ובמודל גידול סרטני בעכברים, בהם הדגמנו את סימון התאים על ידי אותות מהבהבים, תוך שמירה על פונקציונליות וחיות של כ-94% מהתאים. כמו כן הדגמנו את שחזור מיקומי התאים בסופר רזולוציה בסביבת תאים צפופה. בעקבות הזרקה מערכתית של התאים, מאקרופאגים מסומנים אותרו לאחר הגעתם לכבד, והתמונה שוחזרה ב-ULM ברזולוציה של 26.3 ± 3.2 מיקרומטר, שיפור של פי 6.1 בהשוואה לרזולוצייה של המתמר. עבודה זו מציגה גישה חדשה לדימות תוך-תאית מבוססת אולטרסאונד בסופר רזולוציה, המאפשרת הדמיה לא פולשנית של התארגנות תאי מערכת החיסון בגוף חי. הגישה מרחיבה את יישומי האולטרסאונד לדימות תאית פונקציונלית, ומהווה תשתית לתחומי האימונולוגיה, האבחון, וטיפולים מבוססי תאים.

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