

TEL AVIV UNIVERSITY

THE IBY AND ALADAR FLEISCHMAN FACULTY OF ENGINEERING

School of Biomedical Engineering

**Ultrasound Localization Microscopy with
Micro - and Nanobubbles: Processing
Framework and Experimental Validation**

Thesis submitted toward the degree of
Master of Science in Biomedical Engineering
in Tel-Aviv University

by

Grigori Shapiro

March 2026

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This research work was carried out at Tel-Aviv University
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Abstract

Ultrasound Localization Microscopy (ULM) is a super-resolution imaging technique that relies on tracking individual contrast agents to visualize microvascular architectures with capillary-level resolution. While micron-sized microbubbles (MB) serve as the clinical standard for providing acoustic contrast, their size (1-10 μm) can limit extravasation and perfusion into the finest vascular beds. Sub-micron nanobubbles (NB) offer a potential solution for enhanced capillary penetration, yet they present significant challenges. Their reduced acoustic cross-section and weak backscattered signal require high transducer sensitivity and specialized processing to overcome low signal-to-noise ratios (SNR). To the best of our knowledge, this work provides the first demonstration of ULM using NBs as a contrast agent. Consequently, this thesis establishes a framework for NB-based ULM, focusing on sensitive signal processing and a comparative analysis with MBs to evaluate their respective performance in ULM super-resolution imaging. The study quantifies trade-offs regarding localization precision, tracking fidelity, and velocity mapping accuracy across varying flow regimes and geometries. To address the signal processing demands of NB imaging, a custom, end-to-end ULM processing pipeline was developed in MATLAB. A key contribution of this work is the engineering of a comprehensive Graphical User Interface (GUI) that enables real-time visualization and interactive parameter optimization at every stage of the pipeline. The framework incorporates SVD with adaptive singular value thresholding for clutter rejection, followed by sub-pixel localization using radial symmetry and Gaussian fitting. Tracking was performed using a comparative approach between nearest-neighbor (NN) and Kalman filter (KF) with a Smart Cost Matrix (SCM) to maintain trajectory continuity in high-density conditions. In vitro experiments conducted on custom vascular phantoms (100-500 μm channels) indicate that NB achieve hemodynamic mapping accuracy comparable to traditional MB, with both agents accurately retrieving theoretical velocity profiles. In addition, assessment of vascular partitioning at bifurcations using a localization-based Partitioning Ratio, showed that NB exhibited strong hemodynamic concordance with MB. The fractional distribution of NB across geometries and flow rates accurately reflected the structural resistance of the branching network, confirming their role as reliable, deterministic flow tracers. Furthermore, the predictive Kalman tracking (KT) significantly reduced trajectory fragmentation compared to greedy assignment, maintaining a tortuosity index near 1.0 even at the higher concentrations required for NB detection. The framework's practical utility was further validated through preliminary in

vivo renal vascular mapping in a rat model. These initial Temporal Mean Intensity Projections (TMIP) confirmed that NBs maintain sufficient systemic stability and echogenicity to resolve dense cortical microvascular networks, while the integrated SVD-filtering and two-stage motion correction successfully suppressed high-clutter tissue signals without blurring vascular boundaries. This research establishes a robust, physics-aware framework for ULM analysis, establishing NBs as viable and accurate contrast agents for super-resolution hemodynamic mapping. The results suggest that the integration of interactive optimization tools is essential for transitioning from standard MB to sub-micron agents, providing a versatile platform for future microvascular research and the characterization of new contrast agents.

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List of Abbreviations

FOV - Field of View
FPS - Frames Per Second
GUI - Graphical User Interface
HT - Hungarian Tracker
IQ - In phase/Quadrature (signal)
KT - Kalman Tracking
KF - Kalman Filter
MB - Microbubble
NB - Nanobubble
NN - Nearest Neighbor
PBS - Phosphate Buffered Saline
PSF - Point Spread Function
PW - Plane Wave
QC - Quality Control
RF - Radio Frequency
ROI - Region of Interest
SCM - Smart Cost Matrix
SNR - Signal-to-Noise Ratio
SSM - Spatial Similarity Matrix
SVD - Singular Value Decomposition
ULM - Ultrasound Localization Microscopy
US - Ultrasound
TMIP - Temporal Mean Intensity Projection

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1. Introduction

Ultrasound (US) imaging is a cornerstone of modern medical diagnostics, valued for its safety, low cost, and real-time capabilities. However, its spatial resolution is fundamentally constrained by the acoustic diffraction limit, which restricts the ability to resolve features smaller than approximately half the acoustic wavelength ($\lambda/2$). For conventional clinical transducers operating at 3-10 MHz, the acoustic diffraction limit is typically in the range of several hundred micrometers, effectively masking the intricate architecture of the microvascular bed [1]. This fundamental constraint precludes the high-fidelity visualization of the microvasculature; while the smallest capillaries ($< 10 \mu\text{m}$) remain entirely undetectable, the limitation extends to larger microvessels as well [1], [2]. Even vessels spanning tens to hundreds of micrometers, such as arterioles and venules, remain poorly resolved and often appear as blurred, indistinct structures that are difficult to differentiate from the surrounding tissue [1]. Consequently, ULM is essential not only for capillary-level imaging but also for providing the necessary resolution to distinguish and characterize the complex branching patterns and hemodynamic profiles of the intermediate microcirculatory network [1].

To overcome this barrier, ULM has emerged as a transformative super-resolution technique [2], [3]. Analogous to optical localization microscopy (e.g., PALM, STORM), ULM bypasses the diffraction limit by isolating and localizing the precise centers of individual, sparse contrast agents flowing within the vasculature [4]. By accumulating tens of thousands of these sub-pixel localizations over time, ULM can reconstruct vascular maps with a resolution an order of magnitude or more beyond the diffraction limit, revealing structures on the scale of tens of micrometers [2], [4].

1.1 The Engineering Challenge: Optimizing ULM for Sub-Micron Agents

While gas-filled MB are the established standard for ULM, their relatively large size (1-10 μm) limits their ability to perfuse the smallest capillary beds and prevents extravasation into the interstitial space - a key requirement for advanced tumor imaging and targeted therapy [5], [6], [7]. NB have emerged as a promising sub-micron alternative, offering the potential to reach deeper into the microvasculature [7], [8].

In this research, we propose the utilization of NB as a novel contrast agent for ULM to potentially overcome the physiological barriers encountered by larger MB [5], [9], [10]. However, the transition from MB to NB introduces significant technical hurdles that must

be addressed [5], [6]. Due to their smaller volume, NB possess a substantially lower acoustic cross-section, resulting in a lower SNR and a reduced signature [5], [10]. It is therefore hypothesized that standard ULM processing pipelines, which are typically tuned for the robust signals of MBs, may lack the sensitivity required to reliably detect or accurately track NBs. This study posits that achieving super-resolution with NBs requires a meticulous, multi-parameter optimization of the entire processing chain - from clutter filtering thresholds to tracking gate sizes [11], [12].

1.2 Technical Contribution: An Interactive Optimization Platform

A primary contribution of this thesis is the development of a custom, end-to-end ULM processing pipeline integrated within a specialized GUI. Unlike "black-box" processing scripts, this platform was designed specifically to address the sensitivity requirements of NB imaging.

A core advantage of this system is the consolidation of diverse signal processing techniques into a single interactive environment, enabling the researcher to systematically evaluate which combination of tools performs best for a specific dataset. For instance, the pipeline incorporates a wide array of clutter rejection strategies, including multiple SVD-based decomposition methods [13], [14], spatial convolution filters such as Gaussian smoothing, and temporal frequency-domain processing like Butterworth band-pass filters [15], [16].

Furthermore, the GUI provides a real-time, interactive feedback loop that allows for the immediate visualization of algorithmic adjustments at every stage of the pipeline. Within the adaptive clutter filtering module, users can interactively tune singular value thresholds to effectively isolate weak NB echoes from both stationary and moving tissue clutter [13], [17]. This is complemented by a detection tuning interface that enables real-time calibration of regional maxima and intensity thresholds, ensuring the identification of faint bubble candidates that might otherwise be lost [15], [18]. Moreover, the system facilitates tracking optimization through the comparative implementation of various algorithms, ranging from NN to complex KF, all with adjustable constraints on velocity and trajectory smoothness [19], [20]. Ultimately, this integrated framework serves as the primary engine for the systematic optimization of ULM data, ensuring that the highest possible localization density and tracking fidelity are achieved.

1.3 The Scientific Objective

1.3.1 Comparative Hydrodynamics

Beyond the software implementation, this work aims to quantify the functional differences between NBs and MBs in a controlled environment [21], [22]. First, the research focuses on hemodynamic fidelity by validating that sub-micron NB act as faithful flow tracers. This is achieved through a comparison of their cross-sectional velocity profiles against standard MB and theoretical parabolic flow models, ensuring they accurately report fluid dynamics without velocity bias [15], [23]. Moreover, the study investigates microvascular partitioning at bifurcations to test the hypothesis that NBs possess superior accessibility to constricted channels [24], [25]. This involves quantifying the fractional distribution of agents into secondary vessels independent of their absolute concentration. Finally, to enable a fair and rigorous comparison between contrast agents with vastly different native concentrations, robust, concentration-independent quantification strategies, such as the Partitioning Ratio and Tortuosity Index, are developed and applied [26], [27], [28].

1.3.2 In Vivo Renal Mapping

The rat kidney model serves as a robust *in vivo* platform to validate the comparative performance of NBs and MBs within a complex, hierarchical vascular network [25], [29]. For the purposes of this thesis, the kidney presents two critical research objectives. First, it evaluates multi-scale mapping capabilities by evaluating the ability of ULM to simultaneously resolve the high-velocity flow in the arcuate arteries (e.g., 10-40 mm/s) and the extremely slow, sparse flow in the peritubular capillaries (e.g., < 2 mm/s) [29], [30], identifying potential differences in the dynamic range captured by each agent. Furthermore, the study investigates size constraints and capillary accessibility to test the hypothesis that NBs offer superior penetration into distal micro-vessels [10], [25]. Given that glomerular capillaries have diameters ranging from 7-15 μm , traditional MBs may experience restricted flow or entrapment [31], [32]. This objective aims to determine if the sub-micron scale of NBs provides a more comprehensive and deeper map of renal perfusion compared to MBs [10], [33].

2. Theoretical Background

2.1 Principles of B-Mode Ultrasound and the Diffraction Limit

US imaging operates by converting electrical signals into acoustic pressure waves via a piezoelectric transducer. These waves propagate through tissue, where they are reflected, scattered, and absorbed [5], [34]. The returning "echo" waves are received by the transducer, converted back into electrical signals, and processed to form an image where brightness corresponds to the strength of the backscattered echo.

The spatial resolution of this system, or its ability to distinguish between two adjacent point sources, is governed by fundamental physics. Axial resolution, the ability to differentiate points parallel to the beam, is proportional to the wavelength of the US pulse. Lateral resolution, the ability to resolve objects perpendicular to the beam, is determined by the wavelength, imaging depth, and transducer aperture [5], [35], [36].

In addition to these parameters, the overall system performance is constrained by the acoustic diffraction limit, a physical barrier that restricts the lateral resolution to approximately half the wavelength of the transmitted pulse [35], [36]. Specifically, this limit dictates that any structure or point source smaller than this threshold cannot be resolved as an individual entity. Consequently, an object smaller than this limit, such as a single MB, does not appear as its true size. Instead, it appears as the Point Spread Function (PSF) of the system - a blurred spot whose size and shape are defined by the system's own resolution limits [35], [36]. For conventional medical US, this PSF is typically on the order of hundreds of micrometers, making the microvasculature invisible [5], [35].

2.2 Ultrasound Contrast Agents

The fundamental prerequisite for ULM is the presence of discrete, mobile scatterers within the bloodstream. While red blood cells scatter US, their high density and relatively weak acoustic impedance mismatch with plasma make them unsuitable for single-particle localization [5]. Therefore, ULM relies on gas-filled US Contrast Agents (UCAs) [35].

2.2.1 Structural Composition and Echogenicity

Both MB and NB share a similar structural architecture designed to maximize stability and echogenicity [5], [22]. They consist of:

High-Density Gas Core: Typically a perfluorocarbon (e.g., C_3F_8 , C_4F_{10}) or Sulfur Hexafluoride (SF_6) [5]. These gases are hydrophobic and have a high molecular weight, which significantly reduces diffusion across the shell and extends circulation time. Crucially, the gas core provides a drastic impedance mismatch with the surrounding tissue/fluid, acting as a potent acoustic scatterer [4], [10].

Stabilizing Shell: A biocompatible coating, commonly composed of phospholipids, proteins (e.g., albumin), or polymers [37]. The shell reduces surface tension to prevent collapse (Laplace pressure stabilization) and prevents coalescence [38], [39]. In this work, lipid-encapsulated bubbles were utilized, as their flexible shell allows for significant volumetric oscillation (compression and expansion) in response to the US field, enhancing the non-linear signal [5], [40].

2.2.2 MB: The Clinical Standard

MB with diameters typically in the range of 1-10 μm , are the current clinical standard for Contrast-Enhanced US and ULM [5], [38]. Their size is comparable to that of red blood cells, confining them strictly to the vascular lumen. They cannot pass through the endothelial junctions of healthy or tumor vessels, making them pure "blood pool" agents [10]. MBs are engineered to resonate within the standard diagnostic frequency range (2-10 MHz) [5], [35]. Near resonance, their scattering cross-section increases by orders of magnitude [5], generating a robust, high-SNR signal that is easily separable from tissue clutter. This makes MB-ULM relatively straightforward to implement with standard localization algorithms.

2.2.3 NB: The Sub-Micron Challenge

NB defined in this context as agents with diameters in the sub-micron range (typically 100-800 nm), represent the frontier of ULM research [5], [10]. However, their physical properties introduce severe detection challenges. The primary obstacle in NB-ULM is the drastic reduction in backscattered signal intensity [10]. In the acoustic regime where the bubble size (r) is much smaller than the acoustic wavelength (λ), the scattering cross-section (σ_s) is governed by the Rayleigh scattering approximation:

$$\sigma_s \propto \frac{r^6}{\lambda^4} \quad (1)$$

This r^6 dependency implies that a reduction in bubble diameter by a factor of 10 (e.g., from 3 μm to 300 nm) theoretically results in a signal reduction of 10^6 (60 dB), assuming all other factors remain constant [5], [33].

2.2.4 Relative Advantages of NB as Super-Resolution Contrast Agents

The transition from conventional MBs to NBs in ULM is primarily driven by the physical constraints of the microvasculature and the requirement for high localization density. First, the relative advantage of NBs stems from their sub-micron scale, which fundamentally expands the accessible vascular territory compared to the micron-range size of standard MBs [10], [33]. While MBs are physically restricted from entering the finest microcapillary networks due to their dimensions, the diminutive scale of NBs allows them to perfuse into these delicate vascular lattices [25], [33]. Moreover, this increased accessibility facilitates a significantly higher sampling density of the microvasculature, resulting in richer, more saturated ULM images that capture the full complexity of the circulatory system in regions where MB traversal events are typically sparse or physically prohibited [16].

Furthermore, NBs demonstrate enhanced in vivo stability and significantly longer circulation times compared to traditional clinical agents [22], [38], [41]. In addition, pharmacokinetic assessments in complex biological models, such as the rat kidney, have revealed that NBs exhibit a markedly slower washout rate, with a renal half-life that is substantially longer than that of standard microbubbles [22], [38], [42], [43], [44]. Finally, this prolonged retention facilitates extended data acquisition windows, enabling the accumulation of a larger number of localization events. This cumulative increase in detected trajectories directly refines the spatial resolution and overall quality of the super-resolved hemodynamic maps, allowing for a more precise characterization of microvascular flow patterns [16], [45].

2.2.5 Implications for ULM Processing

The distinct physical properties of MBs versus NBs establish the primary engineering and algorithmic challenges addressed in this thesis. While MBs yield robust, high-contrast acoustic signatures that are easily discernible from tissue clutter, NBs operate at the critical threshold of detection. Their sub-wavelength dimensions result in a significantly lower acoustic cross-section, often leaving their signals buried within the noise floor and residual clutter [5], [46].

These physical constraints require processing strategies that differ from standard ULM protocols. To address the low SNR inherent to NBs, this work utilizes a tailored processing pipeline designed to improve detection sensitivity [11]. A central component of this methodology is the interactive GUI, which facilitates the systematic calibration of

parameters such as clutter filter thresholds and tracking constraints [47]. This feedback mechanism is essential for isolating NB signals from background noise and ensuring the accuracy of the resulting hemodynamic maps [19]. By providing a platform for precise parameter adjustment, the framework enables reliable super-resolution imaging even under the strict detection limits of sub-micron contrast agents.

2.3 Ultrasound Localization Microscopy (ULM)

ULM overcomes the diffraction limit by treating imaging as a localization problem rather than a resolution problem [48]. The core principle is that the center of an isolated PSF can be localized with a precision (σ_{loc}) significantly higher than the width of the PSF itself, provided the SNR is sufficient [4], [48]:

$$\sigma_{loc} \propto \frac{FWHM}{\sqrt{SNR}} \quad (2)$$

where FWHM is the Full Width at Half Maximum of the system's PSF.

The core stages of the ULM processing pipeline are illustrated in the Figure 2.1: The main steps in ULM below. This visualization demonstrates the sequential transition from raw US data through clutter filtering and bubble detection to the final super-resolution trajectory tracking and mapping. The processing shown in this figure was performed on a raw dataset of an in vivo rat brain, sourced from the repository provided by Chavignon et al. [49].

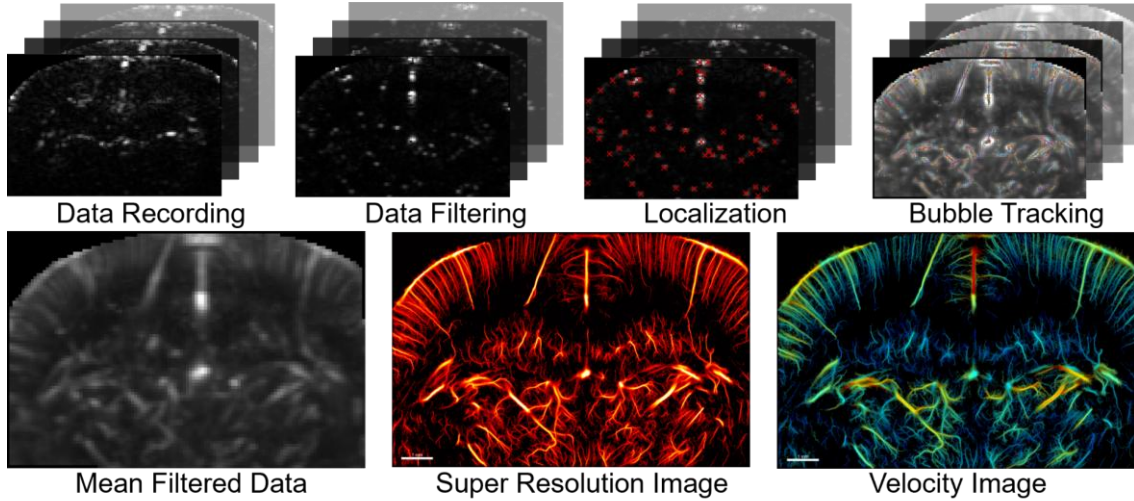


Figure 2.1: The main steps in ULM

The core methodology is as follows:

1. **Dilute Injection:** A low concentration of contrast agents (MBs or NBs) is injected, ensuring that in any given image frame, the agents are sparse enough that their individual PSFs are separable and do not overlap.

2. **Ultrafast Acquisition:** The area is imaged at very high FPS (hundreds to thousands of frames per second), typically using PW or coherently compounded PW imaging. This high temporal resolution minimizes the movement of a bubble between frames, which is essential for tracking.
3. **Localization:** In each frame, the center of each bubble's PSF is localized with sub-pixel accuracy, often using methods like radial symmetry, Gaussian fitting or weighted-average calculations.
4. **Tracking:** Localizations are linked across consecutive frames to form individual tracks. This step confirms that the localizations originate from a moving bubble rather than noise.
5. **Reconstruction:** Over thousands of frames, all the sub-pixel localizations are summed into a single image, creating a high-density map of all bubble positions. This cumulative map forms the final super-resolution image of the microvasculature. The calculated tracks are simultaneously used to generate velocity maps, providing detailed hemodynamic information.

While the previously described sequence outlines the general workflow of ULM, the transition from raw acoustic data to a high-fidelity super-resolution map is a multi-faceted process involving complex signal processing and physical considerations [45]. Achieving the theoretical limits of resolution depends heavily on the synergy between clutter filtering, localization precision, and tracking fidelity [16], [50]. Moreover, each stage in the processing pipeline presents unique technical challenges that directly impact the ultimate image quality and the quantitative accuracy of hemodynamic measurements [16]. Consequently, a deep understanding of the underlying mathematical frameworks is essential for adapting the system to complex biological models [29]. For these reasons, the following sub-sections provide an in-depth examination of the specific technical stages of the ULM process, exploring the physical models and algorithmic considerations that guide their implementation in this research [45].

2.3.1 Spatiotemporal Clutter Filtering

The fundamental challenge in ULM is the separation of high-intensity, low-frequency tissue signals (clutter) from the lower-intensity signals of dynamic contrast agents. To address this, the SVD effectively acts as a spatiotemporal filter that partitions the data into three subspaces based on energy and coherence: the low-rank clutter subspace, characterized by high-energy components with high spatial and temporal coherence; the mid-rank blood subspace, which represents flowing bubbles through moderate-energy

components with lower temporal correlation; and the high-rank noise subspace, consisting of low-energy, spatially and temporally uncorrelated electronic noise [14], [47].

To apply SVD, the recorded 3D IQ data block $I(z, x, t)$ of dimensions $N_z \times N_x \times N_t$ must be reshaped into a 2D Casorati matrix $X \in \mathbb{C}^{M \times N_t}$, where $M = N_z \times N_x$ represents the total number of pixels in a single frame [13], [14]. As illustrated in the spatiotemporal workflow (Figure 2.2 a-b), Each column of X contains the flattened spatial information of one frame, while each row represents the temporal evolution of a single pixel.

The decomposition is defined as:

$$X = U \Sigma V^* = \sum_{i=1}^{N_t} \sigma_i u_i v_i^* \quad (3)$$

where: u_i (Spatial Singular Vectors): These represent the principal spatial modes (e.g., the anatomical structure of the kidney or phantom). σ_i (Singular Values): These represent the energy or "weight" of each mode. In US, the first few singular values typically contain >95% of the total energy, corresponding to the tissue. v_i (Temporal Singular Vectors): These represent the temporal dynamics. High correlation between frames results in low-frequency temporal vectors [14], [47].

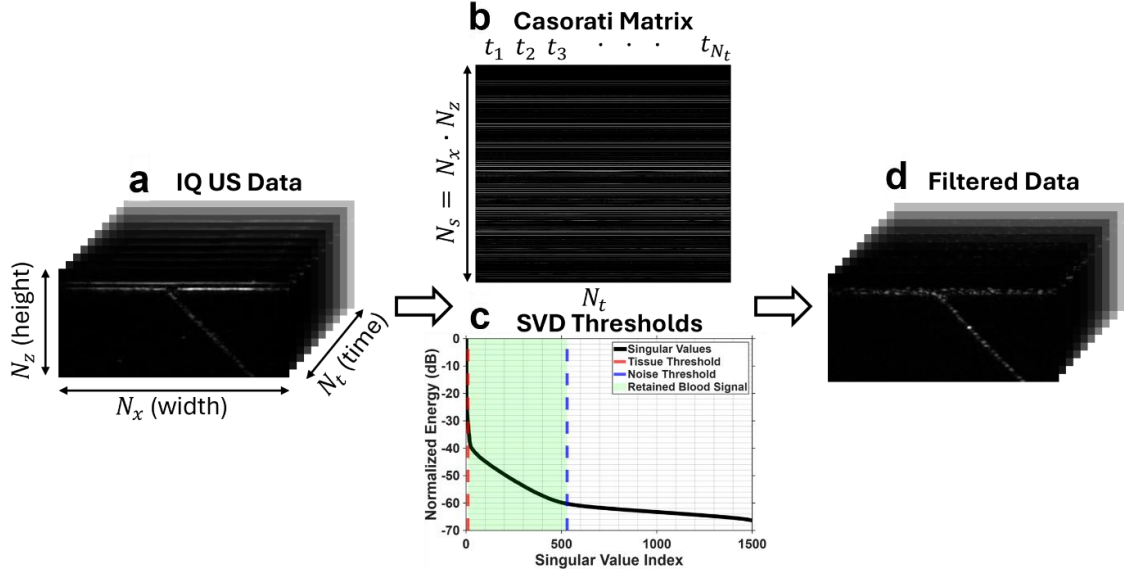


Figure 2.2: SVD-based clutter filtering workflow. (a) Raw IQ ultrasound data containing high-intensity tissue clutter. (b) The Casorati matrix, constructed from the spatiotemporal data. (c) Singular value (σ) distribution showing the selected cut-off thresholds used to isolate the bubble signal from tissue and noise. (d) Filtered frames demonstrating successful microbubble extraction.

Following the decomposition, the critical operational step involves the selection of the singular value range that isolates the contrast agent signal from the surrounding environment. First, the singular value distribution is analyzed to identify the cutoff

thresholds (Figure 2.2 c). Because tissue clutter is characterized by high spatial and temporal coherence, it is localized within the first few singular values ($i < \text{threshold}_{low}$). Conversely, electronic noise is spatially and temporally uncorrelated, occupying the highest indices ($i > \text{threshold}_{high}$). Moreover, the dynamic signals of nanobubbles and microbubbles are found within the intermediate singular values, which represent the "blood" subspace [17], [47].

Furthermore, the reconstruction of the filtered signal is achieved by defining a truncated singular value matrix Σ_{filt} , where σ_i is set to zero for all indices outside the selected blood subspace. The filtered Casorati matrix is then calculated using the inverse operation [14], [47]. This mathematical reconstruction effectively discards the high-energy tissue clutter and low-energy noise, retaining only the transient signals required for localization [15]. Finally, the filtered matrix is reshaped back into the original 3D dimensions to produce the clutter-filtered IQ sequence (Figure 2.2 d).

2.3.2 Sub-Pixel Localization

Super-resolution is achieved by isolating the signals of individual point-like emitters (MBs or NBs) and estimating their centroids with precision far exceeding the original image resolution [35], [50].

In ULM literature, two mathematical frameworks are predominantly used for sub-pixel centroid estimation, the first of which is 2D Gaussian fitting based on non-linear least squares optimization [51]. This method assumes that the intensity distribution of a bubble follows a 2D Gaussian bell curve, employing iterative optimization techniques (such as the Levenberg-Marquardt algorithm) to find the model parameters that best fit the observed pixel data [52], [53]. While this approach is widely considered the “Gold Standard” for localization precision, as it can theoretically approach the fundamental limits of estimation [45], [51], Its iterative nature makes it computationally expensive, which poses a significant bottleneck when processing the millions of frames typical of high-throughput ULM . Alternatively, gradient-based radial symmetry offers a non-iterative geometric approach based on the principle that for a radially symmetric PSF, the intensity gradient at any given pixel must point directly toward the true center [53], [54]. By calculating the optimal intersection point of all gradient lines within a local Region of Interest (ROI), this method provides an analytical, closed-form solution that is orders of magnitude faster than Gaussian fitting [51], [53], although it can be more sensitive to high noise levels or deviations from perfect circular symmetry in the PSF [15], [50].

2.3.3 Bubble Tracking and Velocity Mapping

The transition from a collection of discrete sub-pixel localizations to a continuous super-resolution map necessitates the reconstruction of individual bubble trajectories. This process, often framed as a specialized Multi-Target Tracking (MTT) problem, is fundamental to ULM because it bridges the gap between static structural imaging and dynamic functional mapping [45]. Beyond providing morphological detail, the primary importance of tracking lies in its ability to enable precise velocity estimation. By linking localizations across consecutive frames, the algorithm reconstructs the Lagrangian motion of each contrast agent, which serves as the basis for calculating flow vectors within the microvasculature [26], [55].

At the core of this process is the data association problem, which involves determining the correct correspondence between a localization in frame $t + 1$ and a specific bubble identified in frame t . This task is inherently challenging in ULM due to high bubble concentrations that lead to overlapping PSFs and stochastic “blinking” caused by clutter filtering artifacts [19], [45]. To address these challenges, recursive state estimation, specifically the KF, is employed to track the state of a bubble over time [19]. In this framework, the state of a bubble at time k is represented by a vector x_k , assuming a constant velocity model [19]:

$$x_k = [x, y, v_x, v_y]^T \quad (4)$$

In this state vector, the components x and y represent the spatial coordinates of the bubble center, while v_x and v_y denote the instantaneous velocity components in the respective directions [19].

The system dynamics are described by two fundamental equations that model the physical behavior and the observation process [55]. First, the process equation defines how the bubble's state evolves from one frame to the next:

$$x_k = Fx_{k-1} + w_{k-1} \quad (5)$$

where F represents the state transition matrix, which encodes the physical assumption of constant velocity by projecting the previous position and velocity to the current time step. The term w_{k-1} represents the process noise, accounting for random deviations from the idealized motion model, such as Brownian motion or complex local flow fluctuations [19], [55]. Second, the measurement equation relates the internal state to the observed localizations z_k identified by the detector:

$$z_k = Hx_k + v_k \quad (6)$$

In this equation, z_k is the measured position vector (x, y) , and H is the observation matrix that projects the four-dimensional state space into the two-dimensional measurement

space by isolating the position coordinates [19]. The term v_k represents the measurement noise, which is inherently linked to the localization precision of the imaging system and the SNR of the bubble [19].

The selection of optimal links between predicted states and new measurements is performed by constructing a cost matrix, $C_{i,j}$ [18]. Each element in this matrix represents the “penalty” or cost associated with connecting an active track i to a candidate localization j , based on physical and geometric constraints [55]. To ensure computational efficiency and prevent non-physical assignments, a gating mechanism is applied, whereby any potential link exceeding a predefined cost threshold is discarded [15], [45]. Consequently, the tracking problem is transformed into a global optimization task, typically solved using the Hungarian algorithm (or its Munkres implementation) [50]. The algorithm seeks to find a set of binary assignments, $a_{i,j}$, that minimizes the total global cost function [18]:

$$\text{Min} \sum_i \sum_j C_{ij} \cdot a_{ij} \quad (7)$$

The variable $a_{i,j}$ acts as a decision indicator, where a value of 1 signifies a valid link and 0 signifies no connection. This global minimization is subject to the constraint that each track can be linked to at most one localization, and each localization can be assigned to at most one track, thereby resolving spatial conflicts that a simple “greedy” approach might misinterpret [55].

Finally, following the reconstruction of these trajectories, the Lagrangian paths are differentiated to calculate local velocities. For a trajectory consisting of discrete positions p_t , the instantaneous velocity is estimated as:

$$v_t = \frac{|p_t - p_{t-1}|}{\Delta t} \quad (8)$$

where $|p_t - p_{t-1}|$ represents the Euclidean distance (displacement) traveled by the bubble between two frames, and Δt is the temporal sampling interval defined by the pulse repetition frequency [18], [26]. These calculated velocities are subsequently projected onto a high-resolution Eulerian grid, where the statistical average of all velocity vectors passing through each super-resolution pixel is computed [18], [26], [56]. This final stage transforms individual tracks into a comprehensive map of microvascular perfusion, allowing for the visualization of laminar flow profiles and the quantification of hemodynamics at the capillary level [21], [26].

2.4 Challenges and Stability in In-Vivo Renal ULM

Imaging the renal microvasculature in vivo [29] presents a unique convergence of acoustic and physiological hurdles that are typically absent in controlled phantom environments. First, the anatomical location of the kidney in a rat model (at a depth of 10-20 mm) requires imaging through multiple layers of skin, muscle, and perirenal fat, which introduces significant frequency-dependent attenuation. This loss of signal is particularly detrimental when utilizing NBs, as their smaller scattering cross-section compared to traditional MBs results in a lower initial SCR. Furthermore, the renal cortex exhibits high hemodynamic heterogeneity with dense, overlapping vascular structures, where resolving crossing vessels requires sophisticated data association to prevent track fragmentation [57], [58]. Consequently, maintaining signal integrity and spatial accuracy at these depths necessitates the advanced processing and motion rejection strategies developed in this work.

2.4.1 The Impact of Motion on Clutter Filtering

The primary challenge to the ULM framework arises from complex multi-modal motion. Unlike static phantoms, the kidney is subject to continuous 3D displacement, where respiratory cycles induce large-scale translations and cardiac activity causes periodic, non-rigid elastic deformations [27], [29]. From a mathematical perspective, clutter filtering algorithms like SVD rely on the high temporal correlation of stationary tissue to concentrate energy in a low-rank subspace. However, respiratory motion causes rank expansion, where tissue energy “leaks” into higher-order singular values that normally represent blood flow [14].

This spectral overlap makes it impossible to separate moving tissue from flowing contrast agents based on rank or frequency cutoffs alone. If not properly corrected, this motion manifests as localization jitter, directly blurring the microvascular map and degrading the final resolution. Performing SVD filtering on uncorrected data leads to either severe “flashing” artifacts or the complete suppression of microvascular signals, depending on the chosen threshold [59].

2.4.2 Multi-Stage Image Registration

To restore the spatiotemporal coherence required for effective ULM, a multi-stage registration pipeline is applied to the IQ data prior to clutter filtering. First, a rigid registration step is utilized to correct for global, first-order translations ($\Delta x, \Delta z$) and rotations (θ) caused by bulk movement or probe tremors [29]. Furthermore, non-rigid

(B-Spline) registration is implemented to compensate for local elastic deformations, such as the expansion and contraction of the kidney during the cardiac and minor respiratory phases [27], [30]. However, while these registration techniques are effective for low-amplitude drift and elastic deformation, the pipeline faces fundamental limits when confronted with high-velocity physiological events like deep inhalation.

2.4.3 Detection and Rejection of Breathing Regions

To address the limitations of registration during high-amplitude motion, the processing framework incorporates a proactive artifact rejection strategy. This begins with a temporal stability analysis using inter-frame correlation (ρ) to identify rapid tissue displacements. Rather than relying on static thresholds, the pipeline identifies and discards entire “breathing regions”-temporal windows encapsulating the full duration of a respiratory phase [29]. By removing these periods of instability before the clutter filtering stage, the system prevents motion-induced signal contamination of the blood subspace. Ultimately, this hierarchical approach ensures that the resulting super-resolution images are derived from high-fidelity, stationary signals, effectively neutralizing physiological interference while preserving the fine microvascular detail of the kidney [57].

3. Materials and Methods

The comparative analysis of NBs and MBs for super-resolution ULM demands a rigorous, multi-disciplinary approach. Unlike standard US imaging, ULM’s performance is intrinsically linked to the delicate interplay between contrast agent physics, hemodynamic conditions, and signal processing fidelity [16]. To address these complexities, the methodological framework developed for this research departs from standard, off-the-shelf processing tools in favor of a custom-engineered, end-to-end pipeline.

Establishing a reliable comparison between MBs and NBs requires deep optimization across several distinct domains. While NBs might offer the advantage of accessing smaller vascular beds, their reduced size results in significantly lower backscattered energy and lower SNR [10]. Furthermore, the fluid dynamics within these smaller vessels are characterized by extremely low flow velocities [29]. To prevent these subtle signals from being suppressed during clutter filtering, which is typically designed to remove stationary tissue, the acquisition and processing must be meticulously calibrated [17], [59].

The methodology is structured around four primary development and optimization phases designed to ensure the technical rigor of the super-resolution framework. First, controlled agent synthesis involves the precise optimization of lipid shell formulations to maintain consistent physical characteristics for both MB and NB [60]. Moreover, a custom high-speed data acquisition architecture was developed, utilizing specialized US sequence scripts that allow for the rapid recording of raw acoustic data across diverse acquisition modes [15]. In addition to these hardware-level optimizations, an adaptive pipeline strategy is implemented through a dedicated GUI that serves as an interactive environment for real-time calibration. This interface enables the meticulous tuning of filtering and tracking parameters to accommodate the specific acoustic signatures of each agent type [11]. Finally, a comprehensive evaluation framework is employed to contrast the functional performance of the agents through multi-parametric quantitative analysis. This includes the reconstruction of high-fidelity super-resolved velocity maps and a detailed investigation into agent partitioning across various channel geometries, supported by a statistical examination of trajectory characteristics such as track length distributions and tortuosity indices [26].

3.1 Contrast Agent Synthesis and Preparation

3.1.1 MB Synthesis (Modified Shell Formulation)

Lipid-shelled MBs were synthesized using a custom phospholipid formulation optimized for enhanced shell elasticity and acoustic stability [61], [62], [63]. The lipid shell was comprised of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2K) [40], [62].

To improve the viscoelastic properties of the bubble shell, a modified solvent ratio was employed compared to standard protocols. DSPC (5.9 mg) and DSPE-PEG2K (2.3 mg) were dissolved in a mixture of propylene glycol and glycerol at a 1:3 ratio (high-glycerol content) [60], [64]. Specifically, the lipids were dissolved in propylene glycol, while a separate phase of Phosphate-Buffered Saline (PBS) and glycerol (in a 3-fold molar excess relative to glycol) was prepared [62]. The phases were mixed and sonicated at 62°C to ensure homogeneity. This high-glycerol environment promotes a more flexible lipid arrangement, resulting in bubbles with a superior nonlinear acoustic signature [60], [64]. Aliquots of 1 ml were transferred to 3 ml headspace vials, sealed, and saturated with perfluorobutane (C_4F_{10}) gas [40], [61].

3.1.2 MB Activation and Purification Protocol

Prior to experimental use, MBs underwent a multi-step activation and purification process to ensure size uniformity and stability, significantly extending beyond standard mechanical agitation. First, the PBS used for dilution was degassed under vacuum for at least one hour prior to use to prevent the introduction of air nuclei. Furthermore, to ensure predictable shell behavior, the MB vials were removed from refrigerated storage and allowed to equilibrate to room temperature for 30 minutes before any mechanical intervention. Mechanical activation was performed by agitating the vials using a VialMix shaker for 45 seconds to generate the MB population. Immediately following agitation, the vials were allowed to cool and stabilize for 15 minutes. This step is crucial for the thermodynamic settling of the lipid shell. Finally, the generated bubbles were purified via centrifugation to isolate the desired size population and remove excess lipid debris [62].

3.1.3 NB Synthesis

NBs were formulated using a specialized lipid shell composition to minimize size while maintaining stability. The formulation included a mixture of 1,2-dibehenoyl-sn-glycero-3-phosphocholine (C22), 1,2-dipalmitoyl-sn-glycero-3-phosphate (DPPA), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE), and DSPE-PEG2K [25], [62]. Specifically, for a standard batch, 30.5 mg of C22, 5 mg of DPPA, 10 mg of DPPE, and 5 mg of DSPE-PEG2K were weighed and dissolved in a mixture of propylene glycol (0.5 ml), glycerol (0.5 ml), and PBS (4 ml) [60]. Due to the high phase transition temperature of the C22 lipids, the dissolution and sonication processes were conducted at an elevated temperature of 80°C [25], [62].

Following solution preparation, 1 ml aliquots were transferred to headspace vials, saturated with Octafluoropropane (C_3F_8) gas, and sealed. Activation was performed by mechanical shaking for 45 seconds [25]. To isolate the NB population from larger MBs, a buoyancy-based fractionation method was employed. The activated vials were centrifuged at 500 rpm for 5 minutes in an inverted position [60]. Following centrifugation, the NB-enriched population was extracted from the bottom of the inverted vial (adjacent to the septum), effectively filtering out the larger, more buoyant MBs [25], [62].

Post-synthesis characterization was performed using a particle counter system (Accusizer FXNano, Particle Sizing Systems, Entegris, MA) [25]. The MB exhibited a mean diameter of 1.3 μm , typical for lipid-shelled agents (Figure 3.1 a). In contrast, the NB displayed a distinct sub-micron profile with a mean diameter of 170 nm (Figure 3.1 b), confirming

the successful formulation of two independent contrast agent types, providing the distinct NB and MB populations required for the comparative analysis [25], [62].

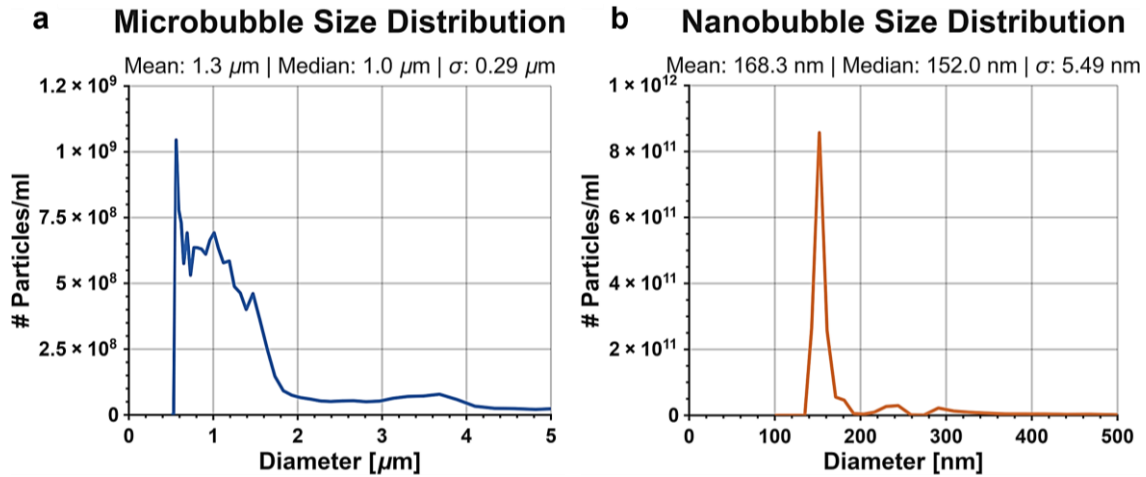


Figure 3.1: Size distribution of MB and NB. (a) Microbubbles. (b) Nanobubbles. measured by Accusizer FXNano, verifying the distinct size populations.

3.2 Optimization of MB Formulation for Enhanced Acoustic Response

To maximize the Contrast-to-Noise (CNR) ratio and improve bubble detectability in high-frequency ULM, the lipid-shelled MB formulation was optimized by adjusting the non-aqueous solvent ratios [60], [64]. Specifically, the ratio between propylene glycol (PG) and glycerol was varied to evaluate its impact on shell stability and acoustic oscillation [38].

3.2.1 PG:Glycerol Ratio Variations

The non-aqueous phase of the lipid shell formulation was modified by varying the ratio of propylene glycol to glycerol. Three specific ratios were tested: 1:1, 1:2, and 1:3 (PG:Glycerol). Increasing the glycerol content was hypothesized to enhance the mechanical stability of the lipid monolayer and increase the backscattered intensity [64]. Due to glycerol's high viscosity, an increase in its concentration typically reduces the rate of gas diffusion across the shell and modulates the damping constant, allowing for larger radial oscillations at higher transmit voltages without premature bubble destruction [64].

3.2.2 Experimental Design and Characterization

Physical characterization was performed using a Coulter counter to measure the mean diameter and size distribution (standard deviation) of the resulting MB populations [65], [66]. Following characterization, the acoustic performance was evaluated in a custom-made gelatin phantom. Acoustic characterization was performed using an L22-8v linear

array transducer operating at a transmit frequency of 10 MHz [15]. The imaging sequence utilized plane-wave B-mode acquisition with coherent compounding across three angles, at 200 FPS [15], [21]. To characterize the contrast agent response across a range of excitation levels, the transmit voltage was systematically varied from 5 V to 25 V in increments of 5 V.

3.2.3 CNR Analysis

The Contrast-to-Noise ratio was quantified by defining two identical Regions of Interest (ROIs): one centered within the phantom’s lumen during MB infusion and a second in the surrounding background tissue where no flow occurred [10]. The mean intensity was calculated across 20 frames per group, with a total of 10 groups per voltage setting to ensure statistical significance. The CNR was defined as:

$$CNR_{dB} = 20 \cdot \log_{10} \left(\frac{\mu_{vessel}}{\mu_{background}} \right) \quad (9)$$

Where μ_{vessel} represents the mean envelope amplitude of the backscattered signal within the channel ROI, and $\mu_{background}$ represents the mean envelope amplitude within a reference ROI of identical size, located in the non-perfused phantom tissue [10], [67].

Statistical analysis was performed using a one-way Analysis of Variance (ANOVA) at each discrete voltage level, to determine if significant differences existed between the three PG:Glycerol ratios (1:1, 1:2, and 1:3). Following the ANOVA, post-hoc pairwise comparisons were conducted using independent two-sample t-tests to identify specific differences between the formulations.

3.3 In Vitro Experimental Setup

To validate the super-resolution algorithms against a known “ground truth” geometry, custom tissue-mimicking phantoms were developed to provide a controlled environment that replicates the acoustic and mechanical properties of biological tissue. First, gelatin was selected as the primary matrix material because it can be engineered to match the speed of sound ($c \approx 1540$ m/s) and the attenuation coefficient (α) of renal parenchyma [24], [68]. Specifically, a 12% (w/v) gelatin solution was prepared by dissolving 18 g of gelatin powder in 150 ml of deionized water [15]. This concentration ensures that the acoustic impedance (Z) of the phantom minimizes reflection artifacts at the transducer interface, allowing the detected signals to originate primarily from the contrast agents within the channels. Furthermore, a critical design consideration was the implementation of a “wall-less” channel architecture. Unlike traditional microfluidics that utilize plastic or glass tubing, wall-less channels are cast directly into the gelatin matrix, thereby

eliminating the acoustic impedance mismatch inherent in tubing walls [24], [69]. This approach prevents strong reflections and reverberations that can saturate the transducer and mask the low-intensity signals of NBs [24], [69]. By allowing the contrast agent to flow in direct contact with the tissue-mimicking material, the setup effectively simulates the blood-endothelium interface, which is essential for accurately comparing the scattering cross-sections of NBs and MBs.

Following the material preparation, the phantom fabrication was executed using a high-precision molding process. The liquid gelatin mixture was poured into a custom-designed aluminum mold containing a negative relief of the target microvascular network, with channel diameters ranging from $100\ \mu\text{m}$ to $500\ \mu\text{m}$ (Figure 3.2 a, c) [15], [21]. To ensure structural integrity, the cast was subjected to thermal gelation at 4°C for two hours, followed by a bonding period of 24 hours to ensure a leak-proof seal between the phantom halves (Figure 3.2 b) [21].

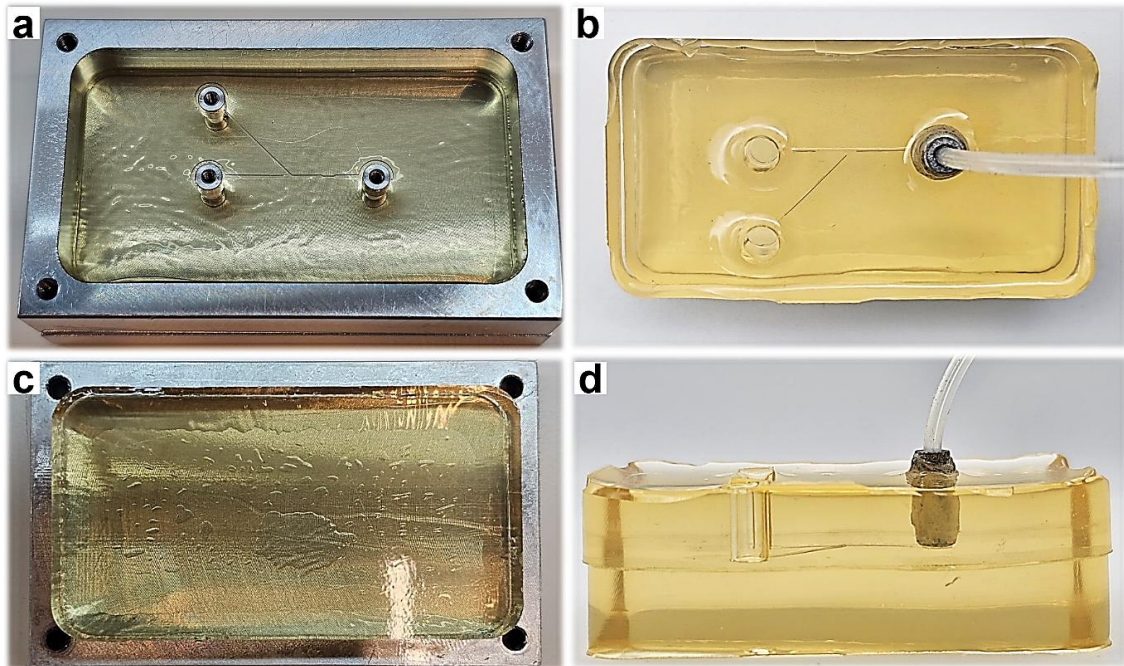


Figure 3.2: Design and assembly of the custom-made gelatin phantom. (a) The top mold component featuring the microvascular-mimicking channel structure and inlet/outlet connectors. (b) Superior view of the finalized phantom; the internal vascular channels are visible through the gelatin, with microfluidic tubing integrated for flow. (c) The bottom gelatin layer, designed with a smooth surface to interface with the top component and seal the microfluidic channels. (d) Lateral view of the complete dual-layer assembly, demonstrating the integrated structure ready for experimental use.

Finally, the experimental setup was completed by connecting the phantom to a programmable syringe pump (GenieTouch, Kent Scientific, Torrington) (Figure 3.3) [15], [21]. To mimic capillary blood flow conditions, diluted solutions of NBs or MBs were infused at constant flow rates ranging from 0.01 to 0.25 ml/min (Figure 3.2 d). This

controlled flow environment allowed for the systematic optimization of the SVD filtering and tracking parameters before transitioning to the complexities of the in vivo rat kidney model.

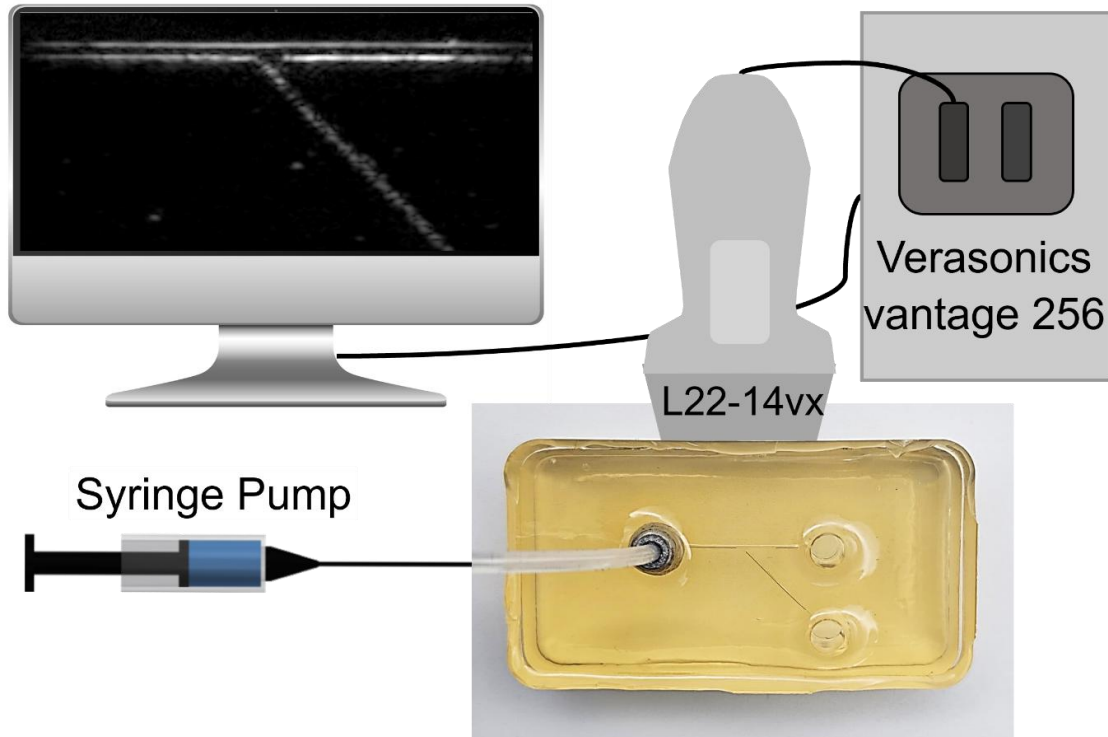


Figure 3.3: Experimental in vitro setup for ULM evaluation. The contrast agent (NBs/MBs) is administered via a syringe pump through microfluidic tubing into a custom-made gelatin phantom. Real-time B-mode imaging of the phantom’s internal channels is performed using a high-frequency linear array transducer connected to the Verasonics Vantage research system, with the live acquisition displayed on the primary monitor.

3.4 In Vivo Experimental Setup

In vivo validation was performed on a rat kidney model. All animal-related procedures were conducted in accordance with the guidelines provided by the Institutional Animal Research Ethical Committee.

Anesthesia was induced and maintained using Isoflurane vapor [29], [40], [59]. To prevent corneal desiccation during the prolonged anesthesia sessions, a protective ophthalmic ointment was applied to the animals' eyes [32], [70]. Prior to imaging, the abdominal region overlying the kidney was prepared by shaving the fur with electric clippers, followed by the application of a depilatory cream. This two-step process ensured an optimally smooth surface, minimizing air trapping and acoustic attenuation caused by hair [29], [40], [59], [71].

Physiological body temperature was maintained using a red heating lamp [50]. Imaging was performed transcutaneously on the kidney in its native anatomical position (in situ),

with no surgical exteriorization required [59], [72]. To guarantee mechanical stability and precise alignment, the US transducer was mounted on a dedicated heavy-duty adjustable arm attached to a high-precision 3-axis positioning stage (Quipu Probe Holder XYZ Stage, Pisa, Italy) [29], [58]. This setup allowed for fine micrometric adjustments to the imaging plane while eliminating motion artifacts associated with handheld scanning [58]. A catheter was inserted into the lateral tail vein for the intravenous administration of contrast agents [29], [59]. A stabilization period was allowed following each injection to ensure a steady-state concentration of bubbles in the blood pool prior to data acquisition [25], [58], [73].

3.5 Ultrasound Data Acquisition

All US data were acquired using the Verasonics Vantage 256 programmable research system (Verasonics Inc., Kirkland, WA, USA). This platform was selected for its open architecture, enabling the implementation of custom PW acquisition sequences and direct access to raw Radio Frequency (RF) data. For this research, the L22-14vX high-frequency linear array transducer was employed, specifically selected for its optimization in high-resolution imaging of small animals and superficial structures [12], [74], [75]. This transducer operates at a center frequency (f_c) of 18 MHz, corresponding to an acoustic wavelength of approximately $85.5 \mu\text{m}$ at a speed of sound of 1540 m/s. The array geometry consists of 128 elements with a 0.10 mm pitch and the elevation focusing is fixed at 8 mm, ensuring an optimal imaging zone within the first 10-15 mm of depth [75].

3.5.1 Acquisition Protocols and Data Management

A high-frame-rate PW B-mode imaging sequence was implemented for all NB and MB Acquisitions using the Verasonics Vantage system. To improve spatial resolution and SNR while maintaining high temporal fidelity, coherent PW compounding of three steered angles ($-5^\circ, 0^\circ, +5^\circ$) was utilized [11], [15], [76]. These steered acquisitions were beamformed and compounded in real-time into single complex IQ frames, ensuring high effective frame rates for accurate tracking of individual NB and MB. To maintain the system's maximum PRF and prevent frame loss, a two-stage data management strategy was employed. During the active sequence, compounded IQ frames were streamed directly into a pre-allocated circular buffer in the workstation's RAM, bypassing disk I/O bottlenecks. Once the acquisition sequence was completed, the accumulated data were transferred from volatile memory to the workstation's SSD in a single batch. This

approach ensured the temporal continuity of bubble trajectories required for robust ULM localization and tracking.

3.5.2 Temporal Resolution Optimization

The acquisition FPS was not fixed but was adaptively optimized for each experimental condition to maximize tracking fidelity. The primary optimization objective was to maintain an inter-frame bubble displacement of approximately 1-2 pixels [15]. This specific range is critical for ULM processing: displacements must be sufficiently large to be distinguishable from localization jitter (noise), yet small enough to ensure robust connectivity between [59]. The optimal FPS was calculated based on the induced flow rate, the vessel diameter, bubble concentration, and the transducer's PSF. For instance, a baseline FPS of 200 Hz was sufficient for typical capillary-mimicking flow rates between 0.01 and 0.03 ml/min. Furthermore, for higher flow velocities, such as the 0.25 ml/min rates observed in phantom experiments, the FPS was increased to 500 Hz or 1000 Hz [15], [76]. This adaptation was necessary to prevent motion aliasing and ensure that the target pixel displacement was maintained even under high-flow conditions [76].

3.5.3 FOV and Reconstruction Grid

The imaging FOV was adjusted specifically for each experiment to capture the relevant microvascular structures. As a representative configuration used in the foundational experiments (specifically the gelatin phantom studies), the axial acquisition window was defined between a depth of 14 mm and 20 mm, resulting in a 6 mm active imaging zone. For this geometry, the reconstruction grid yielded an image matrix of 122×260 pixels (Axial $\times$ Lateral). Here, the 260-pixel width corresponds to the transducer's aperture, while the 122-pixel height represents the 6 mm depth of interest.

This discretization corresponds to an effective spatial sampling (pixel size) of approximately $49 \mu\text{m}$ in both lateral and axial dimensions [12]. This sampling density ($\approx \lambda/2$) provides a sufficient basis for the subsequent ULM localization stage, where sub-pixel algorithms are applied to localize bubble centers with super-resolution precision [12].

3.6 The ULM Processing Pipeline

To address the challenge of resolving microvasculature across different scales, from major renal arteries to cortical capillaries, a custom and modular ULM processing framework was developed in MATLAB. The core processing logic is encapsulated within the `ULM_Processor` class, ensuring a standardized state-machine architecture that

manages data flow from raw IQ signals to final super-resolved reconstruction. The execution of this pipeline is orchestrated by high-level wrapper scripts (e.g., `run_ULM_Analysis_Kidney.m`), which handle data loading, batch management, and environment configuration.

3.6.1 Advanced Pre-Processing: Motion and Artifact Rejection

Processing in vivo data presents significant physiological challenges absent in controlled in vitro environments studies, specifically regarding respiratory motion and tissue deformation [30], [45]. To address these factors a dedicated pre-processing module was integrated into the ULM pipeline to ensure data integrity prior to the localization and tracking stages [45].

To mitigate the significant tissue displacement and decorrelation caused by physiological respiration, we developed an automated, adaptive frame-rejection pipeline. Unlike static thresholding methods, which are susceptible to noise and varying breathing amplitudes, this algorithm analyzes the temporal evolution of the global frame-to-frame cross-correlation coefficient (Figure 3.4 a, blue signal) [29]. The detection workflow operates in four stages to ensure robustness. The dominant respiratory frequency is first estimated via Fast Fourier Transform (FFT) of the correlation signal. This adaptive step automatically tunes the parameters for a band-pass Butterworth filter, isolating the respiratory signal component regardless of the specific physiological state of the animal. To enhance detectability, the filtered signal undergoes a transformation inspired by the Pan-Tompkins algorithm-involving differentiation, squaring, and Moving Window Integration (MWI). This converts oscillatory breathing patterns into a smooth, positive “motion energy” envelope (Figure 3.4 b, blue signal), where motion-dominant intervals are identified (Figure 3.4 b, grey regions). Furthermore a hierarchical detection strategy is employed. The primary pass identifies high-confidence - high-amplitude respiratory events, and a secondary “rescue” pass subsequently scans the quiescent intervals for subtle or irregular breathing patterns, that fall below standard thresholds but still induce sufficient decorrelation to disrupt super-resolution tracking. These detection passes are integrated to fully identify and refine the respiratory cycle minima (Figure 3.4 c, black markers). Based on these optimized minima, the energy-dominant intervals are dynamically identified within the MWI envelope (Figure 3.4 b, grey regions), which ultimately define the specific frames flagged for rejection (Figure 3.4 a, red markers). Lastly, the boundaries for frame removal are then dynamically defined based on statistical

deviations from the local baseline, ensuring the complete exclusion of motion artifacts while maximizing the retention of stable frames for ULM processing.

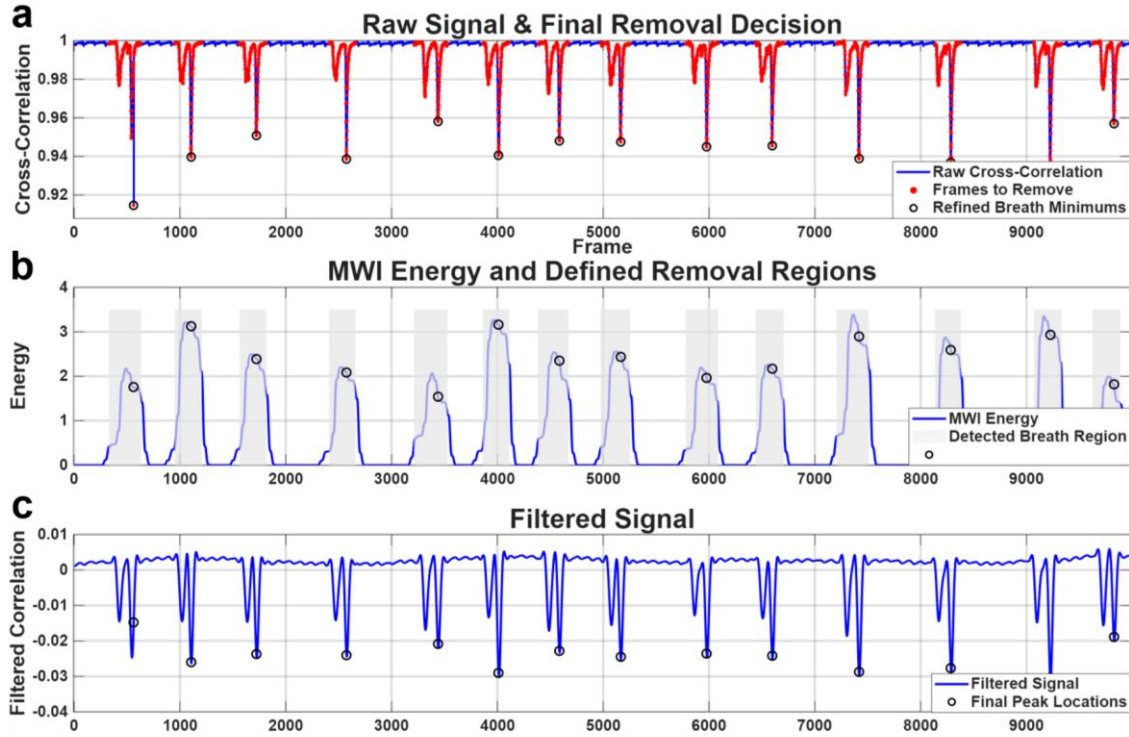


Figure 3.4: Automated respiratory phase detection and synchronization workflow. (a) Inter-frame cross-correlation (blue) and identified respiratory frames (red markers). (b) Moving Window Intensity (MWI) signal (blue) with energy-dominant respiratory phases highlighted (grey). (c) Filtered cross-correlation signal with black markers indicating local minima for cycle synchronization. The x-axis represents the frame index.

Once the respiratory motion is successfully eliminated, the focus shifts to correcting the more subtle, high-frequency tissue pulsatility and involuntary deformations driven by cardiac activity (Figure 3.5 b) [29], [77]. This hierarchical motion correction workflow is applied to the remaining “clean” frames to decouple physiological tissue motion from low-frequency positional drift [27], [77]. First, the process begins with intra-cycle rigid stabilization, where the continuous data stream is segmented into individual cardiac cycles based on the gaps identified during respiratory rejection [29], [77]. For each isolated cycle, a rigid transformation model, accounting for lateral translation (T_x), axial translation (T_y), and rotation (θ) (Figure 3.5 a), is calculated to stabilize the tissue against a local cycle-specific template [29], [77]. To ensure physically plausible motion trajectories and smooth transitions, the pipeline utilizes a non-linear least-squares optimizer with a “warm-start” mechanism, where the transformation parameters of the previous frame serve as the initial guess for the current frame [29]. Moreover, the algorithm incorporates inter-cycle drift Correction to compensate for gradual organ displacement or probe movement over the prolonged acquisition time. This is achieved

by automatically identifying a “Global Master Template” from the most stable cardiac cycle, subsequently aligning every other pre-stabilized cycle to this reference, ensuring long-term spatial consistency across the entire dataset [29]. Finally, to account for soft-tissue deformations such as the expansion and contraction of the renal parenchyma that rigid models cannot encapsulate, a non-rigid B-spline refinement stage is implemented. This phase utilizes a Free-Form Deformation model based on B-Splines which optimizes a dense grid of control points to warp the local tissue structures with sub-pixel accuracy, maximizing the correlation between the template and the moving frame [27]. Finally, the resulting transformation matrices are applied to re-grid the raw data, providing a stationary background that is critical for the subsequent SVD-based clutter filtering and for maintaining sub-pixel localization precision in the final super-resolution vascular maps [27].

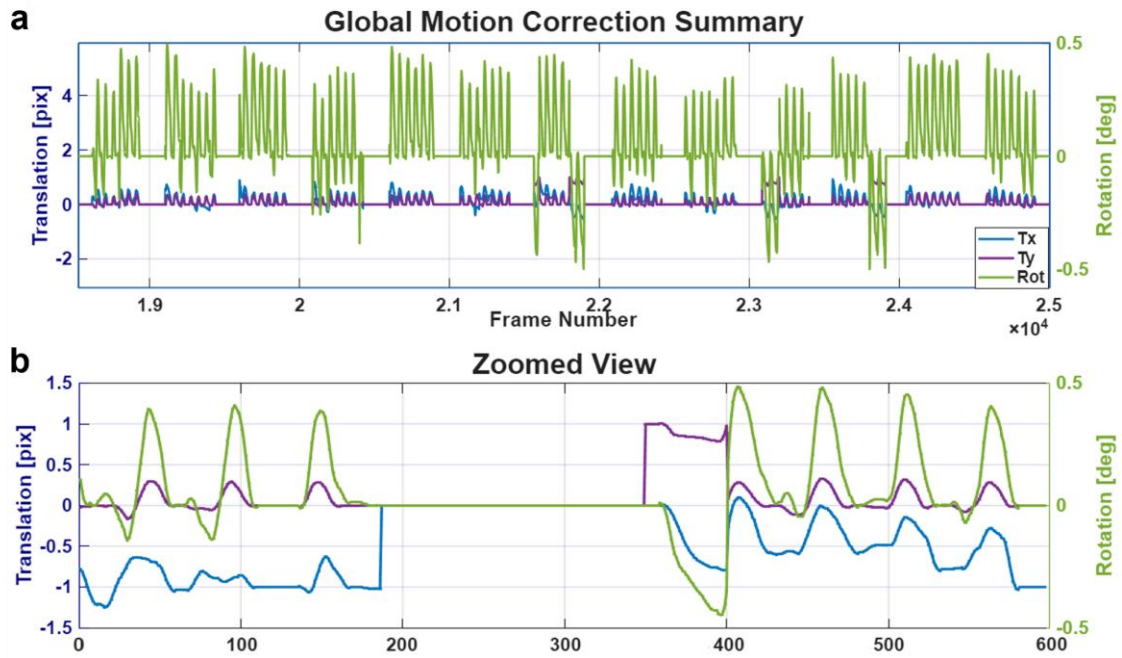


Figure 3.5: Rigid motion correction parameters for *in vivo* kidney imaging. (a) Global trajectories of lateral translation (blue), axial translation (purple), and rotation (green) estimated across the dataset. (b) A magnified view of the first 600 frames highlighting high-frequency fluctuations associated with cardiac pulsatility. The left vertical axis denotes translation in pixels, the right vertical axis denotes rotation in degrees, and the horizontal axis represents the frame index for all subplots.

3.6.2 Adaptive Clutter Filtering

Within the proposed ULM framework, the separation of MB echoes from stationary or slowly moving tissue clutter is treated as an iterative optimization process, utilizing a polymorphic filtering suite integrated into the ULM_Master_GUI.m. Rather than adhering to a single fixed algorithm, the pipeline allows for the selection of the most

effective clutter rejection strategy based on preliminary qualitative and quantitative assessments of each specific dataset. First, the SVD-SSM (Spatial Similarity Matrix) method, as proposed by Baranger et al. [13], is employed as a sophisticated diagnostic tool to identify the transition between clutter-dominated and bubble-dominated singular components. This approach calculates the Pearson correlation between the absolute values of the spatial singular vectors (U) to measure their spatial resemblance [13]. Grounded in the principle that tissue components exhibit high spatial coherence while MB signals appear as uncorrelated fluctuations [14], the resulting correlation matrix provides a visual and statistical cutoff that guides the final reconstruction.

Moreover, for scenarios requiring a more automated classification without manual thresholding, the pipeline incorporates a feature-based clustering approach inspired by the Density Canopy Clustering (DCC) SVD method developed by Han et al. [11]. This implementation evaluates each singular component within a three-dimensional feature space composed of normalized log-energy, power-weighted central temporal frequency, and spatial correlation to the mean spatial vector [11]. In this framework, the DCC algorithm is first employed to identify high-density peaks in the feature space, which serve as robust starting centroids. Furthermore, a K-means algorithm seeded by these canopy centers partitions these components into “Tissue”, “Blood”, and “Noise” clusters, effectively isolating the bubble signal by identifying the cluster that exhibits moderate energy and high temporal frequency signatures characteristic of moving contrast agents [11]. In addition to these SVD-based techniques, a temporal Butterworth bandpass filter is available as an auxiliary tool; however, its application is typically reserved for high-frame-rate datasets (e.g., 1000 Hz) where specific frequency isolation is required to minimize signal attenuation [14], [16].

Finally, the decision to utilize a specific filtering method, or a combination thereof, is determined during a preliminary testing phase within the custom GUI. This interactive environment allows for the immediate visualization of how different singular value cutoffs or clustering outcomes impact the signal-to-clutter ratio and the visibility of individual bubble trajectories. By leveraging the automated insights from the SSM correlation matrix to inform manual refinements in the standard SVD filter, the pipeline ensures that the high-SNR signal required for sub-pixel localization is maintained across diverse experimental conditions [50]. This integrated suite provides the necessary flexibility to accommodate the unique acoustic signatures of both NB and MB, ensuring that subsequent processing stages operate on the highest quality input data.

3.6.3 Localization and Super-Resolution

The precise identification of MB centroids is fundamental to the resolution of the final ULM maps. This process is executed via a hierarchical two-stage algorithm designed to maximize both detection sensitivity and localization fidelity under high-throughput conditions. First, candidate events are identified on the clutter-filtered envelope data using a regional maxima detection algorithm (`detectBubbles.m`). This step acts as a coarse spatial filter. It isolates potential MBs by identifying local intensity peaks that exceed a user-defined adaptive noise floor. This significantly reduces the data volume by discarding background pixels before the computationally intensive sub-pixel stage [15], [45]. Following coarse detection, the exact sub-pixel coordinates of each event must be determined. To ensure the pipeline's versatility across different experimental conditions, the custom software was architected with a modular localization engine. This design supports multiple algorithms, primarily Non-Linear Least Squares (NLLS) Gaussian Fitting and Gradient-Based Radial Symmetry [50], [51].

3.6.3.1 Gradient-Based Radial Symmetry

The Radial Symmetry algorithm, implemented in `localizeRadialSymmetry.m`, offers a robust, non-iterative alternative to standard fitting methods by exploiting the inherent spatial symmetry of the MB's acoustic PSF. It is based on the geometric postulate that for an ideal, radially symmetric intensity distribution (such as a bubble signature), the gradient vector at any given pixel within the ROI is oriented towards the true intensity centroid. Operationally, for each candidate event, the algorithm calculates the spatial intensity gradients $(\frac{\partial I}{\partial x}, \frac{\partial I}{\partial y})$ for every pixel in the defined ROI surrounding the candidate peak. The sub-pixel center $(\hat{x}, \hat{y})$ is determined as the unique point that minimizes the weighted perpendicular distances to the lines defined by these gradient vectors [54]. To improve precision in the presence of noise, a weighted least-squares approach is employed where the weights scale with the square of the gradient magnitude (∇I^2). This ensures that pixels on the “slopes” of the PSF, which carry the most significant directional information, contribute most heavily to the solution, while the influence of flat, noisy background regions and the zero-gradient peak center is suppressed [54].

3.6.3.2 Gaussian Fitting

In addition to radial symmetry, the pipeline incorporates a high-precision 2D Gaussian Fitting module, implemented in `fit2DGaussian_Fast.m`, which models each MB as a

bivariate Gaussian distribution [51]. This approach assumes that the acoustic signature of a single contrast agent can be mathematically described by the function:

$$I(x, y) = A \cdot \exp\left(-\frac{(x - x_0)^2 + (y - y_0)^2}{2\sigma^2}\right) + C \quad (10)$$

where A represents the peak amplitude, (x_0, y_0) the sub-pixel coordinates, σ the standard deviation of the PSF, and C the local background offset. The algorithm extracts a small ROI around each coarse detection and utilizes the Levenberg-Marquardt or Trust-Region-Reflective optimization algorithm via the `lsqcurvefit` function to minimize the residual error between the theoretical model and the empirical pixel data. Furthermore, to handle the high computational demand of processing millions of localizations, the module utilizes parallel processing (`parfor`) and vectorized initial guess estimations. A significant advantage of this iterative approach is the generation of a goodness-of-fit metric, specifically the R-squared (R^2) value, which provides a direct quantitative measure of how well the localized event matches the expected PSF of a single MB, allowing for the rejection of overlapping or distorted signals [11], [78].

3.6.3.3 Localization Quality Control

To ensure the purity of the resulting coordinates, a multi-layered Quality Control (QC) framework evaluates each candidate localized by either method. First, common rejection criteria are applied to discard localizations that fail basic physical constraints, such as the edge check, which removes candidates located too close to the image boundaries to allow for a complete ROI extraction [79]. Furthermore, a divergence check is performed to reject any sub-pixel solution that shifts more than a specified distance (typically 1–2 pixels) from the initial coarse peak, as such shifts often indicate a failure to converge or the influence of neighboring noise [52]. In addition to these general checks, algorithm-specific filters are applied; for example, Radial Symmetry localizations are rejected if the gradient field is insufficient or if the linear system's determinant indicates numerical instability. Conversely, for Gaussian Fitting, candidates are discarded if the optimization fails to reach a successful exit code or if the R^2 value falls below a strict threshold (e.g., $R^2 < 0.5$) [11], [78], [80]. Finally, this integrated QC layer ensures that only high-confidence centroids are passed to the tracking stage, maintaining the spatial integrity required for super-resolution imaging.

3.6.4 Tracking Algorithms

Linking isolated localizations into coherent trajectories is a computationally complex task, particularly in high-density environments where bubble PSFs may overlap or cross

paths [20]. To evaluate the trade-offs between precision and computational efficiency, the pipeline was architected with a polymorphic tracking engine supporting four distinct algorithmic approaches. First, the Nearest Neighbor (NN) approach, implemented in `trackNearestNeighbor.m`, serves as a high-speed greedy linker. This method iterates through active tracks and assigns each to the single closest localization in the subsequent frame within a predefined search radius [45]. While computationally efficient, the primary limitation of this approach is its local optimization strategy, which often leads to track fragmentation in dense regions when a locally optimal choice for one track precludes a more globally accurate assignment for another [45].

To address these limitations, the Global Hungarian Tracker (HT), implemented in `trackHungarian.m`, treats frame-to-frame linking as a formal linear assignment problem [48]. By utilizing the Munkres (Hungarian) solver, the algorithm finds the set of assignments that minimizes the total global cost for all tracks simultaneously, significantly improving robustness in complex flow scenarios [18], [45]. Furthermore, the cost calculation is enhanced by a “smart” cost matrix, implemented in `calculateCostMatrix.m`. This module moves beyond simple Euclidean distance by incorporating a weighted product of directional persistence and intensity similarity, ensuring that tracking decisions are grounded in the physical behavior of MB [45], [58]. Although the details of this weighted cost logic are explored in depth in the following chapter, its integration here allows the HT and subsequent trackers to penalize sharp turns and unrealistic fluctuations in bubble brightness [57], [81].

Moreover, to transition from reactive linking to predictive state estimation, the pipeline incorporates a standard KT in `trackKalman.m`. Unlike proximity-based linkers, the KT maintains a recursive state estimate for each bubble, including both position and velocity, using a constant-velocity motion model [19], [45]. This predictive capability allows the tracker to bridge temporal gaps caused by signal “blinking”, maintaining track continuity even when a bubble is not detected for several consecutive frames [58]. Finally, the most sophisticated method integrated into this work is the Hierarchical Kalman Tracker (HKT), implemented in `trackKalman_Advanced.m`. This algorithm was developed based on the methodology proposed by Taghavi et al. [20], specifically designed to resolve the challenges of tracking heterogeneous populations with varying velocity profiles [20].

The HKT operates through a multi-pass “subtractive” or “peeling” logic, where tracking is decomposed into sequential velocity-dependent levels. First, the algorithm is initialized with parameters optimized for slow flow regimes (e.g., 0-8 mm/s). Once valid tracks are

formed and finalized at this level, their associated localizations are subtracted from the global pool, thereby preventing them from being falsely linked to faster-moving bubbles in subsequent passes [20]. Furthermore, the filter's noise parameters are adaptively scaled at each level: the process noise is adjusted proportional to the maximum expected velocity, while measurement uncertainty is refined across iterations to favor high-confidence localizations [20]. This hierarchical separation is critical for the accurate reconstruction of the rat kidney microvasculature, where slow-flowing peritubular capillaries and fast-flowing interlobular arteries must be resolved simultaneously without cross-linking errors [20].

Following the tracking phase, the pipeline offers an optional validation layer within the `applyQualityControl.m` module to provide a final stage of artifact rejection. While the SCM logic previously described handles most physical constraints during the linking process, these additional filters serve as a secondary refinement tool for finalized trajectories. Specifically, functions such as `applyVDCConstraint.m` and `applyDirectionConstraint.m` can be electively employed to verify Velocity Dispersion and directional consistency [19], [58]. Moreover, because the SCM already penalizes non-physiological jumps and turns during track formation, this post-tracking validation is not strictly mandatory and is typically reserved for datasets with exceptionally high noise levels or complex crossing scenarios where further trajectory pruning is required before super-resolution rendering [58].

3.6.5 Advanced Cost Matrix Logic

To resolve ambiguities in complex flow regions (e.g., bifurcations), a SCM was implemented (`calculateCostMatrix.m`). Unlike standard trackers that rely solely on Euclidean distance, this module computes a weighted cost C_{total} for linking a track i to a new detection j [58]. The cost function incorporates “soft penalties” that increase linearly with deviation from physical expectations, rather than just applying a binary cutoff. This total cost is mathematically defined as:

$$C_{total}(i, j) = C_{dist} \cdot (1 + W_{slope} \cdot P_{angle}) \cdot (1 + W_{int} \cdot P_{intensity}) \quad (11)$$

First, C_{dist} represents the base spatial distance cost, which is calculated differently depending on the active algorithm. In the Hungarian framework, C_{dist} is the normalized Euclidean distance from the track’s last known position, whereas in the KF, it is the distance from the track’s predicted state estimate [58], [81]. This distinction is critical because the Kalman approach implicitly penalizes deviations from the established

velocity vector, ensuring that the link remains consistent with the bubble's momentum [19], [58].

Moreover, to resolve ambiguities in regions of complex flow such as bifurcations, the cost function incorporates a directional penalty, P_{angle} . This term accounts for the deviation angle θ_{ij} relative to a user-defined safety gate, θ_{gate} , and is defined as

$$P_{angle} = \max(0, \theta_{ij} - \theta_{gate}) \quad (12)$$

If the angle exceeds a hard gating limit, the cost is set to infinity ($C_{total} = \infty$), effectively preventing physically impossible trajectories [12], [19]. However, for angles within the allowed range but beyond the safety gate, the cost scales linearly according to the weighting factor W_{slope} . Furthermore, the SCM maintains brightness consistency through the intensity penalty $P_{intensity}$, defined as:

$$P_{intensity} = \frac{|I_{current} - \overline{I_{track}}|}{\overline{I_{track}} + \epsilon} \quad (13)$$

where I_{track} is the stable mean brightness of the existing track and ϵ is a small constant used for numerical stability. This term discourages the tracker from jumping between bright MB signals and dim noise artifacts by penalizing significant fluctuations in amplitude, with the weight controlled by W_{int} [58], [81]. Finally, by integrating these spatial, directional, and intensity features into a single multiplicative cost, the pipeline ensures that tracking assignments are grounded in physical expectations, providing the stability necessary for accurate super-resolution reconstruction of the renal microvasculature [58], [81].

3.6.6 Super-Resolution Reconstruction and Rendering

The final stage of the ULM pipeline involves the transformation of discrete MB trajectories into continuous, high-resolution maps of the vascular architecture and hemodynamics. This reconstruction is achieved through a quantitative trajectory accumulation method, where MB transits are projected onto a high-resolution grid that is typically up-sampled by a factor of 10 relative to the native B-mode pixel size. To ensure topological continuity and mitigate high-frequency localization jitter prior to grid projection, a two-stage trajectory refinement process is applied. First, raw tracks are regularized using a Savitzky-Golay filter, which was selected for its unique ability to suppress high-frequency tracking noise while preserving the underlying structural shape and peaks of the vessel path [82]. Moreover, these smoothed tracks are densely interpolated using cubic splines to ensure a connected path across the sub-pixel grid,

effectively preventing aliasing artifacts that often arise from the discrete nature of the temporal sampling [45], [50].

Furthermore, a significant architectural contribution of this pipeline is the decoupling of the tracking analysis from the final visualization engine. This modular approach allows for rapid, iterative rendering of super-resolution maps using different post-processing thresholds, such as minimum track length or velocity gates, without the computational overhead of re-processing the entire dataset. In the rendering stage, the engine generates four distinct visualization modalities to provide a comprehensive assessment of the microvasculature. In addition to standard histogram-based maps, the system produces a vessel density map that utilizes power-law dynamic range compression ($\gamma = 0.3$). This transformation is critical for visualizing both high-flux main channels and delicate, low-density capillary structures simultaneously within a single frame [18], [83].

To correlate anatomical structure with functional hemodynamics, the pipeline further implements a dual-mode fusion overlay using an HSV (Hue-Saturation-Value) color space [45]. In this visualization, the flow velocity is encoded into the Hue component (Blue to Red), while the Value (Brightness) is modulated by the local vessel density [20]. This allows for the immediate identification of hemodynamic patterns, such as the distinction between slow-flow peritubular capillaries and high-speed interlobular arteries in the rat kidney model. Finally, the quantification of flow speed is presented through both raw and filtered velocity maps. The raw map provides a mathematically exact representation by calculating the arithmetic mean of the velocities of all tracks traversing each specific pixel [26], [78], whereas the filtered map utilizes a slight Gaussian smoothing ($\sigma = 0.6$) to bridge spatial gaps and present a visually coherent hemodynamic gradient [15], [80]. This multi-layered rendering approach ensures that the final super-resolution images serve as both a qualitative visualization tool and a quantitative diagnostic record of the microvascular environment.

3.7 GUI for Adaptive ULM Optimization

To facilitate a comparative analysis between NBs and MBs across varying experimental conditions, a robust, end-to-end computational pipeline was required. A significant challenge in ULM processing is the high variability of signal characteristics; factors such as the acoustic FPS (e.g., 200 Hz vs. 1000 Hz), the target medium (in vitro phantoms vs. in vivo kidney tissue), and the flow velocity of the agents significantly alter the spectral footprint of the MB signal [50], [76]. While standard ULM research often relies on 'blind' batch scripts where static parameters are applied globally based on theoretical

assumptions, such a trial-and-error approach is prone to suboptimal reconstruction and artifacts [11].

To overcome these limitations, the ULM Master GUI was developed - a custom MATLAB-based platform designed to transition the workflow from arbitrary parameter estimation to systematic, empirical optimization [80]. Serving as a real-time calibration instrument, this interface provides granular control over the entire parameter space, allowing for the adaptive optimization of the pipeline for each specific dataset prior to batch execution [81]. By providing an interactive feedback loop, the GUI ensures that filtering thresholds, localization precision, and tracking constraints are meticulously tuned to resolve hierarchical vascular structures with maximum fidelity [15], [45], [81].

3.7.1 Metadata-Driven Initialization and Physical Calibration

The optimization workflow begins with a context-aware initialization protocol designed to ensure physical fidelity across diverse experimental setups. To bridge the gap between raw digital data and physical microvascular dimensions, the pipeline automatically parses an experiment-specific metadata file (info.txt) associated with each dataset. This file provides the “ground truth” parameters of the acquisition, including the transmit frequency, speed of sound, and the FOV dimensions.

By extracting the FPS and the axial/lateral FOV, the system computes the spatial calibration factors (δ_x, δ_z) required for super-resolution reconstruction:

$$\delta_x = \frac{FOV_x}{N_x}, \quad \delta_z = \frac{FOV_z}{N_z} \quad (14)$$

where N_x and N_z represent the matrix dimensions of the loaded US frames. This automated calibration step is fundamental to the pipeline’s reliability, as it ensures that all subsequent derivative metrics - most notably the particle velocity (mm/s) and the super-resolution upsampling factors - are physically grounded and invariant to changes in imaging depth or probe settings between the in vitro and in vivo models. The interface functions as an interactive “sandbox” environment, providing immediate visual and quantitative feedback.

3.7.2 Adaptive Clutter Filtering and SVD Optimization

The platform serves as a comparative environment for evaluating clutter filtering strategies. The interface provides real-time control over singular value (SV) thresholds, allowing for the immediate assessment of the filtered result stack. This interactive tuning is essential for identifying the optimal SV range that maximizes tissue clutter rejection while preserving the integrity of the slow-moving NB signals. Such precision is

particularly critical in high-frame-rate acquisitions, where the spectral overlap between microvascular flow and residual tissue motion is most pronounced.

3.7.3 Interactive Detection and Sensitivity Calibration

Following clutter suppression, the system enables granular calibration of the bubble detection stage. Users can adaptively tune the Normalized Intensity Threshold and the maximum permissible bubble count per frame. To ensure high specificity, the GUI provides a real-time visualization layer where detected candidates are superimposed as overlays (red markers) directly on the filtered B-mode image. This allows the user to visually distinguish between true MB signals and noise floor artifacts, ensuring high specificity before computational resources are committed to sub-pixel localization.

3.7.4 Sub-Pixel Localization Logic and Quality Assessment

The GUI incorporates multiple sub-pixel localization algorithms, including Gradient-Based Radial Symmetry and 2D Gaussian Fitting, allowing the user to select the method most suited to the dataset's SNR. Crucially, the interface provides quantitative feedback by displaying the convergence count per frame relative to the initial candidate count from the previous stage. This allows the user to monitor the candidate-to-localization drop-off rate in real-time. By observing how many initial detections are filtered out during the fitting process, the user can assess the strictness of the chosen quality constraints (such as goodness-of-fit thresholds), ensuring that only high-fidelity, symmetric MB signals are retained.

3.7.5 Trajectory Linkage and Tracking Calibration

The tracking module facilitates the construction of trajectories through an adjustable logic-based framework. The user can calibrate the Advanced Cost Matrix weights, gap-closing capabilities, and frame-to-frame linking distances. The GUI visualizes the construction of trajectories superimposed on a TMIP of the filtered data. This background map highlights the vascular morphology, enabling the user to verify that tracks correctly follow the vessel geometry, particularly in regions of high curvature or complex branching.

3.7.6 Super-Resolution Rendering and Reconstruction Control

The final stage of the pipeline decouples the tracking analysis from image generation, enabling the interactive refinement of the super-resolution maps. The user can adjust regularization parameters, such as the trajectory smoothing kernel size and the upsampling factor, in real-time. Furthermore, the system allows for the instantaneous

filtering of the final map by 'Minimum Track Length'. By visually inspecting the trade-off between vessel connectivity and spatial resolution, the user can determine the optimal parameters for reconstructing high-fidelity ULM images.

3.8 Data Analysis and Quantitative Metrics

To rigorously quantify the performance differences between NBs and MBs, as well as to evaluate the fidelity of the developed tracking algorithms, the final super-resolution datasets and trajectory tables were analyzed using a suite of dedicated metrics [26].

3.8.1 Trajectory Geometric Fidelity (Tortuosity)

To assess the physical realism of the reconstructed paths and identify tracking errors (such as spatial jitter or erratic linkages), the Tortuosity Index (τ) was calculated for each trajectory. It is defined as the ratio of the actual path length to the straight-line Euclidean distance between the start and end points [26], [56]:

$$\tau = \frac{\sum_{i=1}^{N-1} |p_{i+1} - p_i|}{|p_N - p_1|} \quad (15)$$

where p_i is the position of the bubble at frame i .

The analysis focused on the distribution of τ across all tracks:

$\tau \approx 1$: Indicates smooth, straight trajectories (Ideal laminar flow).

$\tau \gg 1$: Indicates “jittery” paths or zigzagging artifacts typical of localization noise or erroneous cross-linking [26].

Histograms of τ were generated to quantify the “noise tail” and evaluate The ability of advanced tracking algorithms to smooth high-frequency fluctuations.

3.8.2 Tracking Continuity and Selectivity

The robustness of the tracking algorithms was evaluated using two complementary metrics designed to assess the fidelity of the reconstructed trajectories. First, the relationship between the total track count and trajectory fragmentation was analyzed, based on the principle that a higher raw number of tracks does not inherently imply superior performance [84]. Moreover, an inflated count often indicates fragmentation, a phenomenon where a single continuous vessel is broken into multiple short segments due to localization jitter or signal dropout [45], [85]. Consequently, the total track yield was scrutinized in conjunction with track length distributions to ensure that high bubble density did not compromise the structural integrity of the microvascular map. Furthermore, the distribution of trajectory durations, measured in frames, served as a primary metric for temporal continuity [26]. A significant shift towards longer tracks

indicates successful gap-closing and stable locking onto the bubble center throughout its transit [29]. In contrast, a dominance of short tracks suggests frequent tracking failures or inappropriate filter thresholds, necessitating further iterative optimization within the custom GUI to achieve the desired super-resolution [45].

3.8.3 Spatially Resolved Velocity Profiling

Beyond global velocity averages, cross-sectional velocity profiles were extracted to validate the hemodynamic accuracy of the contrast agents. First, specific ROIs were strategically defined across the phantom geometry, including the pre-bifurcation, post-bifurcation, and branching segments [21]. Moreover, these experimental velocity profiles were compared against the theoretical parabolic profile, which is expected for laminar conditions in micro-channels [15], [21], [23]. Furthermore, this comparative analysis was performed to assess whether the contrast agents accurately trace the hydrodynamic gradient from the vessel center, where velocity is maximal, to the walls, where it reaches zero [15]. Finally, this assessment served as a critical validation of the flow-tracing fidelity of the agents, ensuring that the tracking algorithms within the custom pipeline accurately reflect the underlying fluid dynamics.

3.8.4 Quantification of Microvascular Partitioning and Perfusion Efficiency (In Vitro)

To evaluate the functional perfusion capabilities of NB compared to MB, a comparative metric was required that is robust against variations in absolute bubble concentration [57]. Since the native concentration of NBs is approximately two orders of magnitude higher than that of MBs, standard temporal metrics such as “Time-to-Saturation” or raw track counts are inherently biased by the initial bolus dose rather than the agent's actual hemodynamic efficiency [21], [25].

To overcome this limitation, a Vascular Partitioning Analysis strategy was developed. This method evaluates the fractional distribution of contrast agents at a bifurcation point, effectively normalizing the data against the total incoming flux to isolate the hemodynamic behavior [86]. For each phantom geometry, three ROIs were defined on the super-resolved density map. Pre-bifurcation in the main channel upstream of the split, post-bifurcation in the continuing low-resistance channel, and the bifurcation branch in the secondary (collateral) channel [21].

The total number of accumulated localizations (N) was aggregated for each ROI over the entire acquisition duration. The Partitioning Ratio (P_{branch}) was calculated as the

percentage of bubbles entering the branch relative to the total number of bubbles that navigated the bifurcation:

$$P_{branch} = \frac{N_{branch}}{N_{branch} + N_{post_main}} \times 100 \quad (16)$$

This metric serves as the primary tool for comparing the two agents. If NBs possess a superior ability to perfuse small capillaries due to their size, this would be reflected in a higher P_{branch} compared to MBs in the same geometry [25]. Conversely, equal ratios indicate that both agents strictly follow the bulk fluid flow distribution governed by the hydraulic resistance network [15].

3.9 In Vivo Validation Dataset (Rat Brain)

To validate the robustness of the custom ULM processing pipeline on complex in vivo microvasculature prior to analyzing the experimental kidney data, an external benchmark dataset was utilized. The dataset, provided by Chavignon et al. [49], consists of high-frame-rate contrast-enhanced US recordings of a rat brain.

The data was acquired using a Verasonics Vantage 256 system equipped with a high-frequency linear array (128 elements, 0.1 mm pitch), operating at a center frequency of 15.625 MHz. To ensure high temporal resolution, a coherent PW compounding sequence with five angles was employed (-6° to $+6^\circ$), yielding a post-compounding FPS of 1000 Hz.

The dataset was utilized in its beamformed IQ format, which facilitated the direct assessment of the developed SVD-based clutter filtering, sub-pixel localization, and tracking algorithms. The successful reconstruction of the intricate cerebral angio-architecture confirmed the pipeline’s capacity to resolve hierarchical vascular networks, ranging from major cortical arterioles to fine capillary beds. This validation step established the necessary baseline for the subsequent comparative analysis of NB and MB in the complex in vivo kidney model.

4. Results

4.1 Influence of Glycerol Concentration on Acoustic CNR

Building upon the optimization protocols established in the methodology (Section 3.2), this section evaluates the physical and acoustic outcomes of the modified PG:Glycerol ratio formulations. The objective was to confirm that the increased glycerol concentration translates into improved signal and a more uniform bubble population, which are prerequisites for achieving a consistent PSF and reliable localization in the subsequent in-vivo kidney experiments.

4.1.1 Size Distribution and Uniformity

Physical characterization via Coulter counter, indicated that increasing the glycerol ratio shifted the population toward a more uniform distribution. The 1:3 ratio demonstrated a higher concentration of bubbles within the 1-2 μm range, which is ideal for achieving a consistent PSF in ULM (Figure 4.1).

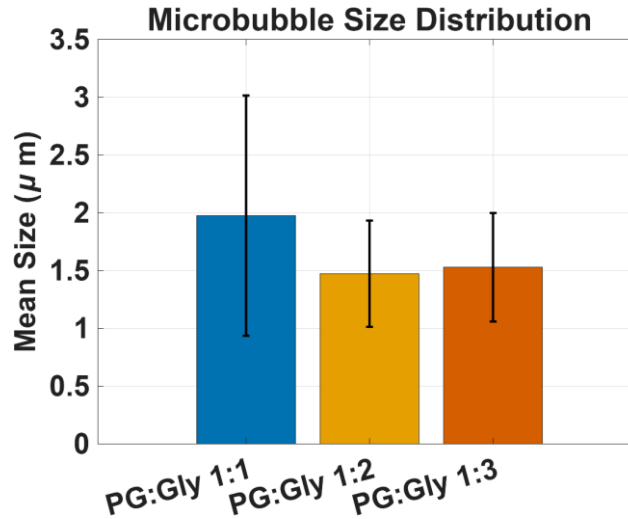


Figure 4.1: Size distribution of MB synthesized with varying Propylene Glycol to Glycerol ratios (1:1 blue, 1:2, yellow and 1:3 orange). Measurements were obtained via Coulter counter characterization.

4.1.2 CNR Performance and Statistical Significance

The acoustic results demonstrate that the 1:3 PG:Glycerol ratio consistently provided the highest CNR across the entire voltage range (5V to 25V) (Figure 4.2).

At the lowest excitation level, the 1:3 ratio showed a significant improvement over the 1:1 ($p < 0.001$) and 1:2 ($p < 0.001$) formulations, suggesting a lower threshold for acoustic activation. The enhancement was most pronounced at the highest transmit voltage. The 1:3 recipe achieved a highly significant CNR increase compared to the 1:1 recipe ($p < 0.001$), indicating that the increased glycerol concentration successfully stabilized the bubbles against the higher mechanical index (MI) of the 25V pulses (Figure 4.2).

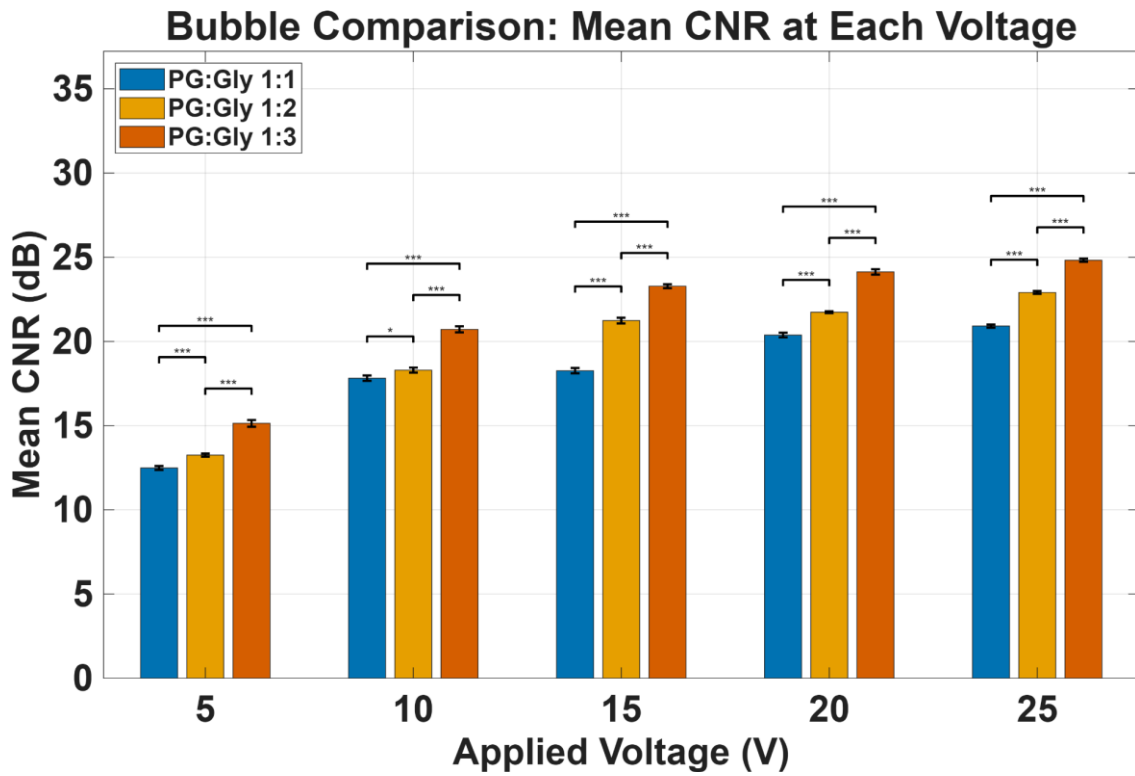


Figure 4.2: Quantitative comparison of acoustic CNR across transmit voltages ranging from 5V to 25V for different PG:Glycerol formulations. Significant differences are indicated by: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. n=10.

The statistical analysis confirms that the addition of glycerol is a dominant factor in enhancing the contrast signal. As shown in the pairwise comparisons, the 1:3 ratio outperformed the 1:2 ratio significantly at every voltage step above 5V ($p < 0.001$) (Figure 4.2). This optimized 1:3 formulation was therefore selected for all subsequent in vivo validation steps to ensure maximum sensitivity during the trans-capillary flow of the rat kidney.

4.2 “ULM Master GUI”: An Interactive Optimization Environment

4.2.1 The Visual Feedback Loop

The core engineering contribution of this platform is the reduction of the optimization iteration time. In a standard workflow, assessing the impact of a tracking parameter (e.g., *Max Gap Closing*) requires re-running the entire dataset. The ULM Master GUI implements a real-time feedback loop. User adjustments to sliders or dropdown menus trigger an immediate re-calculation on the cached dataset, visualizing the results instantly. This capability allows for sensitive analysis by rapidly identifying the “breaking point” of parameters (e.g., finding the exact SVD rank where tissue is suppressed but capillaries remain). Furthermore, it allows a interactive gating by Visually matching the detection threshold to the noise floor of the specific acquisition.

The GUI is organized into a logical left-to-right workflow, mirroring the sequential phases of super-resolution processing pipeline, ensuring an intuitive user experience. The interface is architecturally divided into three primary functional zones (Figure 4.3). First, the control module, situated in the right panel, serves as a vertical sidebar housing the parameter controls for each processing stage. Within this module, the upper tabs facilitate workflow navigation, allowing the user to isolate specific algorithmic steps (Figure 4.3 a). Moreover, a dynamic panel located beneath these tabs displays the active controls relevant to the selected phase (Figure 4.3 b); for instance, in the “Track” tab, this area exposes tracking engine selections, such as KT or Hungarian algorithms, alongside various physical constraint toggles.

In addition to the control infrastructure, the Visualization Canvas occupies the center of the interface, providing high-resolution, real-time feedback (Figure 4.3 c). This central window is critical for the validation of the ULM results, as it allows the user to overlay detection centroids and tracking trajectories directly onto the structural B-mode image to ensure that the calculated paths align accurately with the underlying physical anatomy. Furthermore, temporal navigation is managed via the Playback Control Bar located at the bottom of the canvas (Figure 4.3 d). This module, which includes a frame slider and index input, enables the user to scrub through the entire image sequence to verify temporal consistency and identify transient artifacts across the dataset.

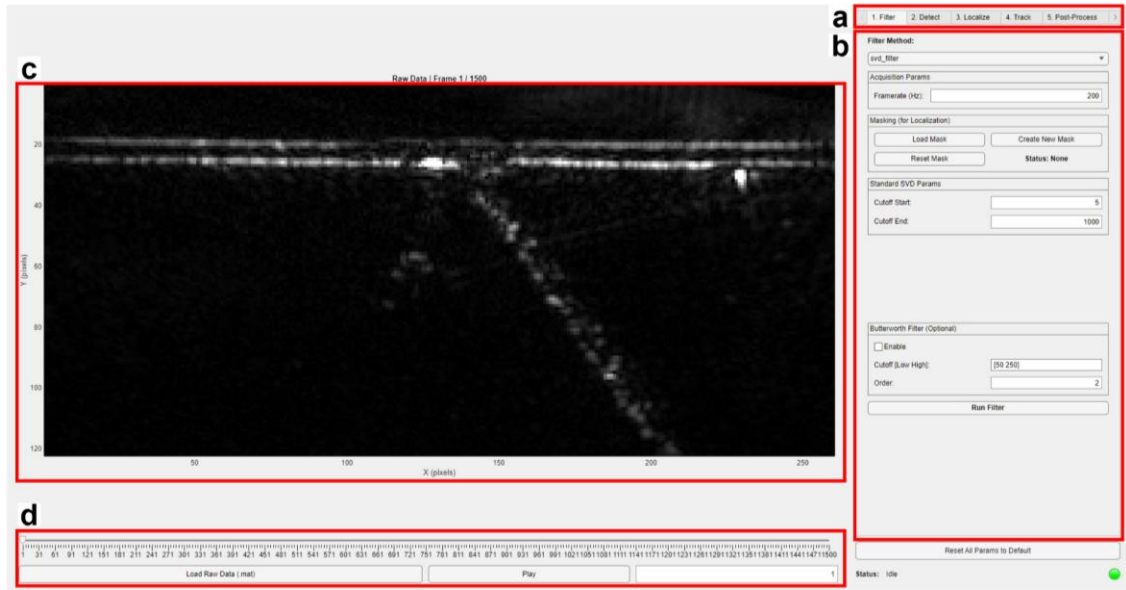


Figure 4.3: ULM Master GUI interface. The graphical interface is designed to guide the user through the optimization pipeline. Key functional zones are marked: **(a)** Workflow Tabs, allowing navigation between processing stages (Filter, Detect, Localize, Track). **(b)** Parameter Control Panel, housing the specific sliders and dropdowns for the active stage. **(c)** Main Visualization Canvas, providing real-time overlay of results on the B-mode data. **(d)** Playback & Navigation Bar, enabling frame-by-frame inspection and video looping.

Finally, by centralizing these interactive zones, the platform empowers the user to perform a rigorous sensitivity analysis, systematically varying individual parameters, such as the SVD Rank, while holding others constant to visually assess their impact on the final vessel reconstruction fidelity.

4.2.2 Signal Isolation and Clutter Rejection

The first critical stage in the super-resolution pipeline is the isolation of the dynamic contrast agent signal from the stationary or slowly moving tissue background. Within the custom-developed GUI, this process is facilitated through a multi-layered approach that combines spatial pre-processing, adaptive decomposition, and multi-dimensional filtering. First, the platform provides robust pre-processing capabilities, including a dedicated spatial cropping tool that allows the user to isolate a specific ROI within the raw dataset. This localized analysis is further enhanced by an integrated masking module, which enables the user to either manually define a binary mask or load a pre-existing one to focus the processing engine exclusively on relevant anatomical structures.

Furthermore, the core of the clutter rejection strategy resides in the SVD filtering engine. While the interface supports manual rank selection for meticulous control, it also incorporates advanced, automated thresholding techniques, such as the SSM and DCC-SVD, to improve objectivity and repeatability. The GUI allows users to interactively tune

these SVD filter parameters and immediately assess the impact on signal separation. This optimization process is demonstrated on a 300 μm channel phantom with a bifurcation (MBs, flow rate: 0.03 ml/min). In the raw B-mode image (Figure 4.4 a), the MB are significantly obscured by strong echogenic reflections from the gelatin phantom material and the channel walls. By varying the singular value cutoff range through the GUI, the effectiveness of the filter is revealed. A threshold range of [5, 1500] leaves significant background noise visible (Figure 4.4 b), whereas a range of [5, 1000] reduces noise but still allows interference with bubble isolation (Figure 4.4 c). Finally, a rank selection of [5, 500] provides optimal clutter rejection, clearly isolating the bubbles from the background (Figure 4.4 d).

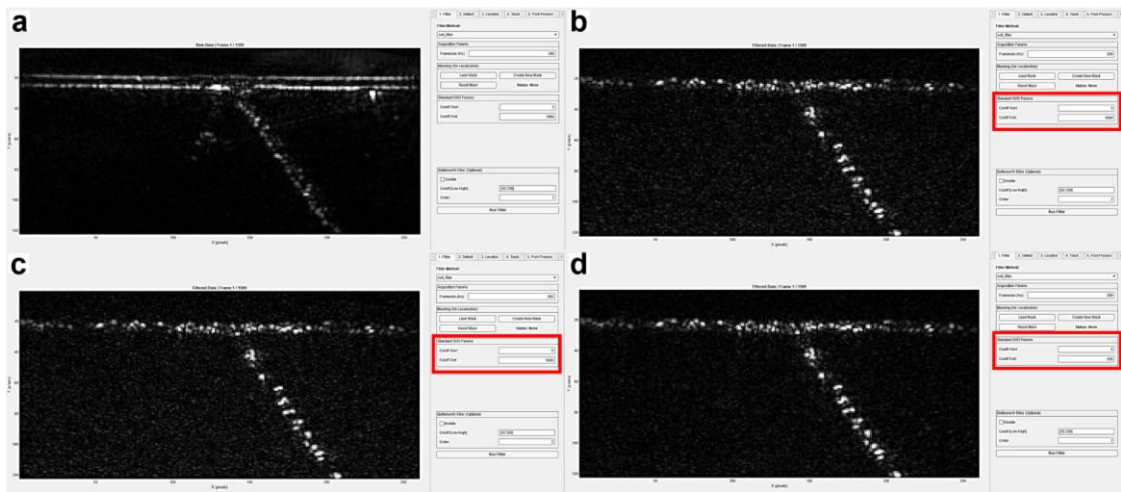


Figure 4.4: SVD clutter filtering optimization in a 300 μm bifurcation phantom (MBs, 0.03 ml/min). (a) Raw B-mode image. (b–d) Impact of singular value rank selection on clutter rejection: (b) Rank [5, 1500]. (c) Rank [5, 1000]. (d) Rank [5, 500], representing optimal filtering performance.

Moreover, the filtering panel includes secondary spatial and temporal processing layers to further refine the isolated signal. Users can apply spatial noise reduction through a configurable Gaussian filter, where the smoothing intensity is controlled by adjusting the sigma parameter directly within the interface. In addition, a temporal band-pass filter is situated at the base of the control panel, allowing for the isolation of specific frequency ranges across the frame dimension. By centralizing these diverse filtering modalities into a single interactive environment, the platform enables a highly iterative optimization process, ensuring that the dynamic scattering of NBs is preserved with maximum fidelity while suppressing high-amplitude clutter.

4.2.3 Spatial Constraints and Detection Sensitivity

Following the clutter rejection stage, the pipeline must distinguish true MB signals from residual noise and artifacts. While the initial structural refinement is performed during

the filtering phase, the detection module provides an interface to manage these spatial constraints. The manual ROI management allows the user to visualize and maintain the mask established during the initial filtering stage (Figure 4.5 a). By restricting the analysis to specific anatomical structures, such as a phantom channel or the kidney cortex, the system effectively reduces the computational load for the subsequent tracking engine. Furthermore, the platform features an advanced, optional histogram-based auto-masking module that provides an alternative to manual delineation. This tool generates a “Flow Probability Mask” by calculating the temporal average of the SVD-filtered image sequence, where regions of consistent bubble flow appear as high-intensity pixels. To refine the isolation of these flow regions, the user can apply image enhancement algorithms such as Contrast Limited Adaptive Histogram Equalization (CLAHE), Top-Hat filtering for vesselness enhancement, or sharpening filters. A binary mask is then generated by selecting a specific intensity threshold from the resulting histogram (Figure 4.5 b). This automated approach provides an objective method for isolating perfused flow regions and suppressing detections in non-perfused background areas.

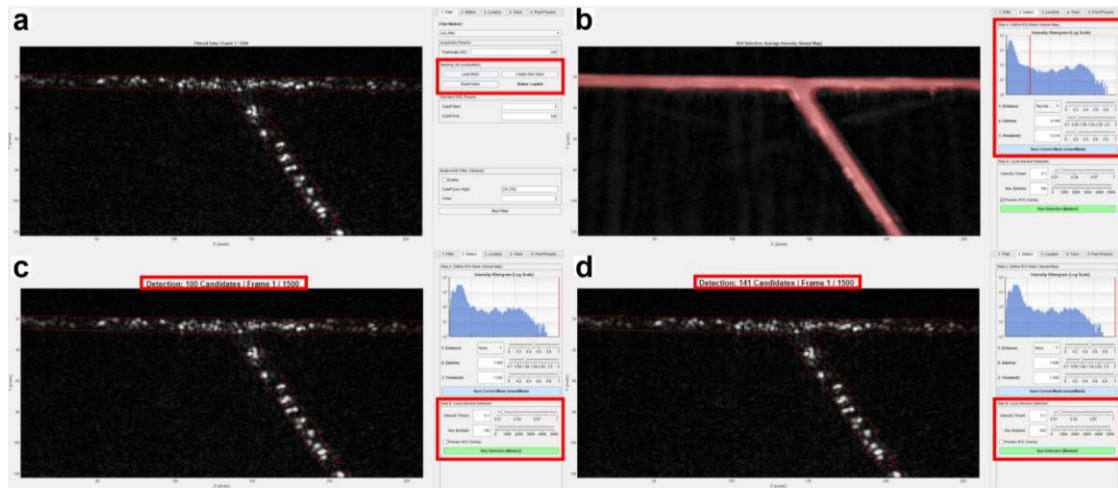


Figure 4.5: Spatial masking and detection sensitivity optimization. (a) Manual ROI established during the initial filtering stage. (b) Optional auto-masking using temporal averages and enhancement tools (CLAHE/Top-Hat). (c) Saturated detection results where the 100-candidate limit truncates valid signals. (d) Optimized detection with a 200-candidate limit, identifying 141 bubbles without saturation.

The sensitivity of the detection algorithm is further optimized by managing the interplay between the normalized intensity threshold and the maximum candidate limit per frame. The primary objective is to define an intensity threshold that effectively excludes low-level spurious noise while ensuring that the candidate limit is high enough to avoid artificially truncating valid signals. This challenge is evident when the algorithm is configured with a conservative limit of 100 candidates. In this scenario, the detector identifies exactly 100 points, signifying a state of algorithm saturation where valid bubble

events are likely omitted because the actual bubble flux exceeds the predefined ceiling (Figure 4.5 c).

To resolve this saturation and ensure detection fidelity, the candidate ceiling is increased to 200 while maintaining the original intensity constraints. Under these relaxed parameters, the pipeline successfully localizes 141 distinct bubbles within the same frame. This configuration confirms that the previous limit was insufficient to capture the high-flux events, whereas the updated limit allows for complete data recovery while still providing an upper bound to prevent the inclusion of excessive noise (Figure 4.5 d). By providing real-time feedback on the detection count relative to these set limits, the GUI enables the user to achieve optimal localization density.

4.2.4 Sub-Pixel Localization and Fidelity Check

Once candidate peaks are identified at the integer pixel level, the pipeline transitions to sub-pixel localization to achieve the high spatial resolution characteristic of ULM. The ULM Master GUI facilitates this by offering two selectable localization engines: a gradient-based Radial Symmetry algorithm and a 2D Gaussian fitting model. While the previous detection stage relies primarily on intensity, it cannot differentiate between valid bubble signals and high-amplitude noise or artifacts. Consequently, the interface implements a localization QC layer that serves as a secondary noise-rejection filter, ensuring that only candidates conforming to the expected PSF geometry are retained for tracking.

This fidelity filtering process is based on several geometric and statistical constraints that evaluate the physical validity of each candidate. Within the Radial Symmetry engine, the system calculates image gradients within a small ROI and solves for a center of symmetry. Candidates are automatically rejected if they exhibit low gradient consistency, unstable determinant values in the analytical solution, or excessive divergence between the sub-pixel center and the original integer peak. Similarly, the Gaussian fitting engine utilizes an R-squared metric to assess the “Goodness of Fit” relative to a theoretical PSF model. If a candidate fails these criteria or requires a coordinate shift larger than a predefined pixel threshold, it is discarded as a spurious detection.

The practical impact of this fidelity check is demonstrated by processing the 141 high-flux candidates identified in the previous detection stage. The visualization shows the localization engine identifying sub-pixel coordinates for each candidate across the representative frame (Figure 4.6). However, the subsequent application of the QC layer results in the retention of only 107 valid MBs, which are visualized as small red dots

superimposed on the structural B-mode image (Figure 4.6). This reduction from 141 to 107 points indicates that 34 candidates were filtered out due to PSF distortion, low fit-reliability, or divergence. By stripping away these low-fidelity signals, the pipeline ensures that the subsequent tracking algorithm operates only on high-confidence data points, thereby preventing the formation of erratic or non-physical trajectories.

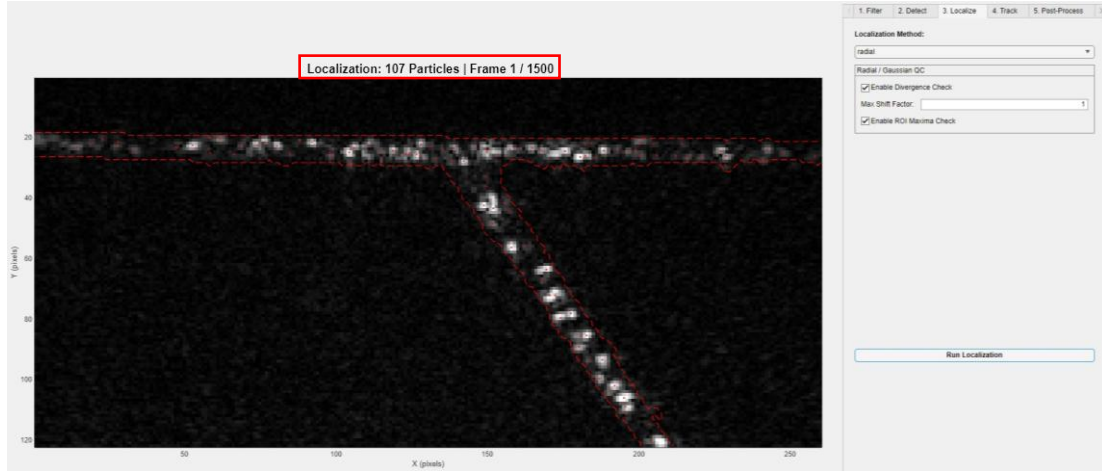


Figure 4.6: Sub-Pixel localization and fidelity filtering. The visualization demonstrates the result of the sub-pixel localization stage on the representative frame from Figure 4.5.

4.2.5 Physics-Based Tracking and Algorithm Comparison

The final and most critical stage of the ULM pipeline is temporal tracking, where individual localizations are linked into continuous trajectories to estimate blood flow velocity. The ULM Master GUI was explicitly designed to benchmark different tracking algorithms, addressing the trade-off between computational speed and tracking fidelity. The choice of algorithm dramatically impacts the biological fidelity of the reconstruction (Figure 4.7). A baseline NN approach yielded a high number of tracks ($N = 5,615$), but exhibited significant noise near channel walls and failed to capture a consistent flow direction, resulting in a disordered map (Figure 4.7 b-e). The Hungarian algorithm ($N = 5,460$) improved global directionality but produced “jittery” trajectories that failed to resolve the smooth curvature required at the bifurcation point (Figure 4.7 f-i). In contrast, the KF with a comprehensive cost matrix ($N = 5,173$) successfully modeled the fluid dynamics, reconstructing smooth, continuous curves at the branching point (Figure 4.7 j-m). Finally, applying a strict minimum length filter ($MinLen = 10$), distilled the dataset to 1,706 high-fidelity trajectories (Figure 4.7 n-q). While this reduced the total count, it eliminated fragmented, ambiguous segments, sharpening the final image and ensuring that only robust, physically validated data contributed to the super-resolution map.

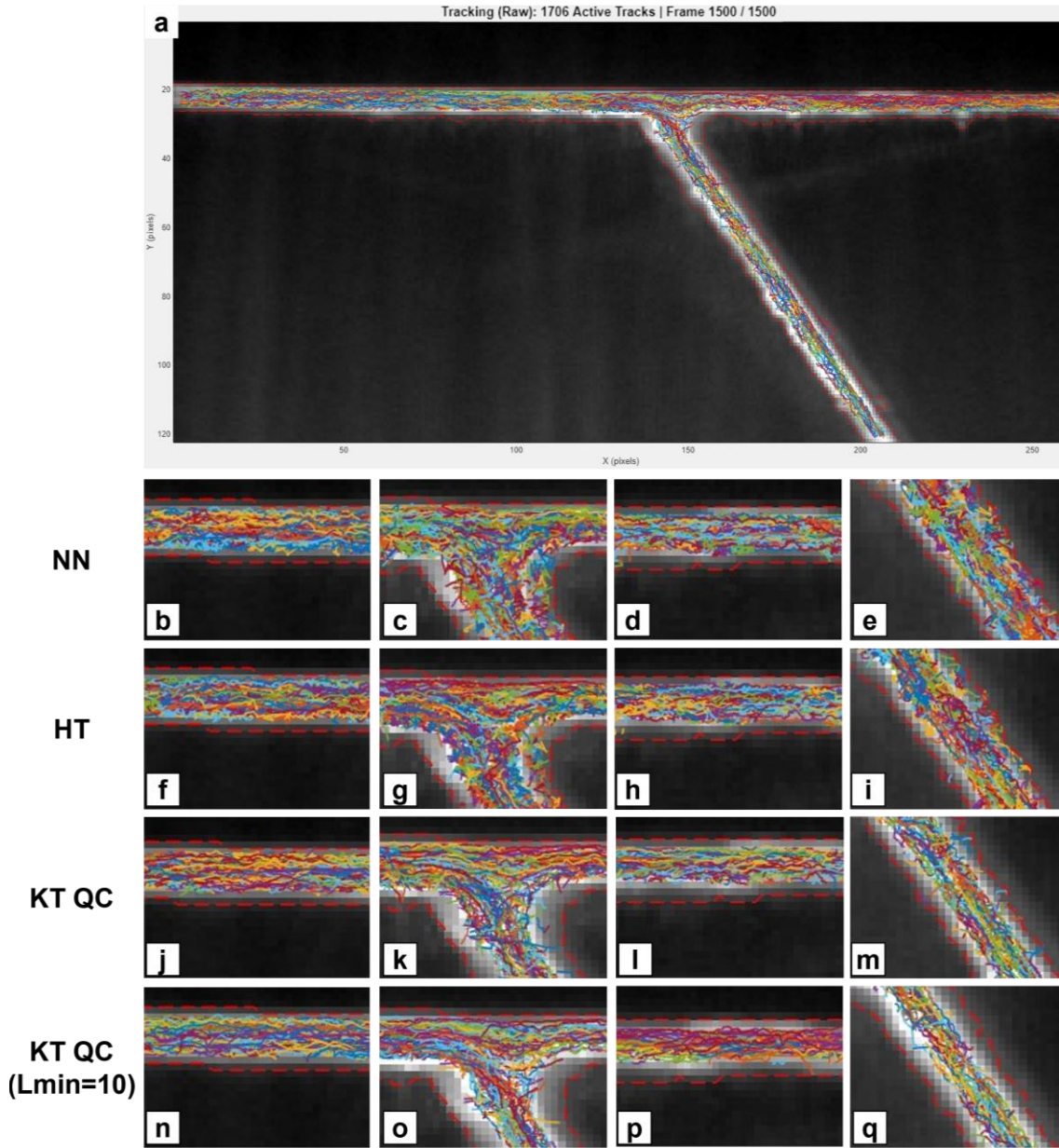


Figure 4.7: Evolution of tracking fidelity and quantitative selection. (a) Visual display of the received tracks inside the phantom. (b-q) micro-vascular reconstruction detail (zoomed view). Magnified inspection of the bifurcation region reconstructed by the four tracking protocols. (b-e) NN: Baseline tracking (N=5615 tracks). (f-i) Hungarian Algorithm: Standard frame-to-frame assignment (N=5460 tracks). (j-m) Kalman Filter + SCM + QC: Motion-model tracking with kinematic constraints (N=5173 tracks). (n-q) High fidelity filtering: Final result after applying minimum length constraint (MinLen=10), retaining only robust trajectories (N=1706 tracks).

4.2.6 Track Post-Processing

Following the tracking stage, the resulting trajectories consist of discrete localization points defined on the coarse imaging grid. To achieve the target super-resolution, typically reaching a scale of $\lambda/10$, these discrete tracks must be converted into continuous flow lines that align with a high-definition sub-pixel grid. The ULM Master GUI

facilitates this transition through the “PROCESS” module, which allows the user to interactively calibrate the trajectory smoothing and interpolation parameters. Within this module, the primary tool for optimization is the “Smoothing Factor”, which controls the intensity of the spline-based interpolation applied to the discrete points. By adjusting this window, the user can effectively mitigate high-frequency localization jitter while ensuring that the tracks remain faithfully aligned with the sub-pixel grid density. Furthermore, the interface provides a “Min Track Length” filter, which serves as a critical data-cleaning step by discarding short, fragmented trajectories that are statistically more likely to represent noise artifacts. The qualitative improvement of this smoothing process is demonstrated by the transition from disjointed point clouds to continuous, laminar flow lines within the phantom structure (Figure 4.8 a-e). While the smoothed tracks faithfully retain the original morphological character of the vessels - preserving the exact path geometry - they exhibit a significant reduction in jaggedness. The flow behavior at the bifurcation becomes clearer and more continuous, accurately representing the laminar nature of the fluid dynamics.

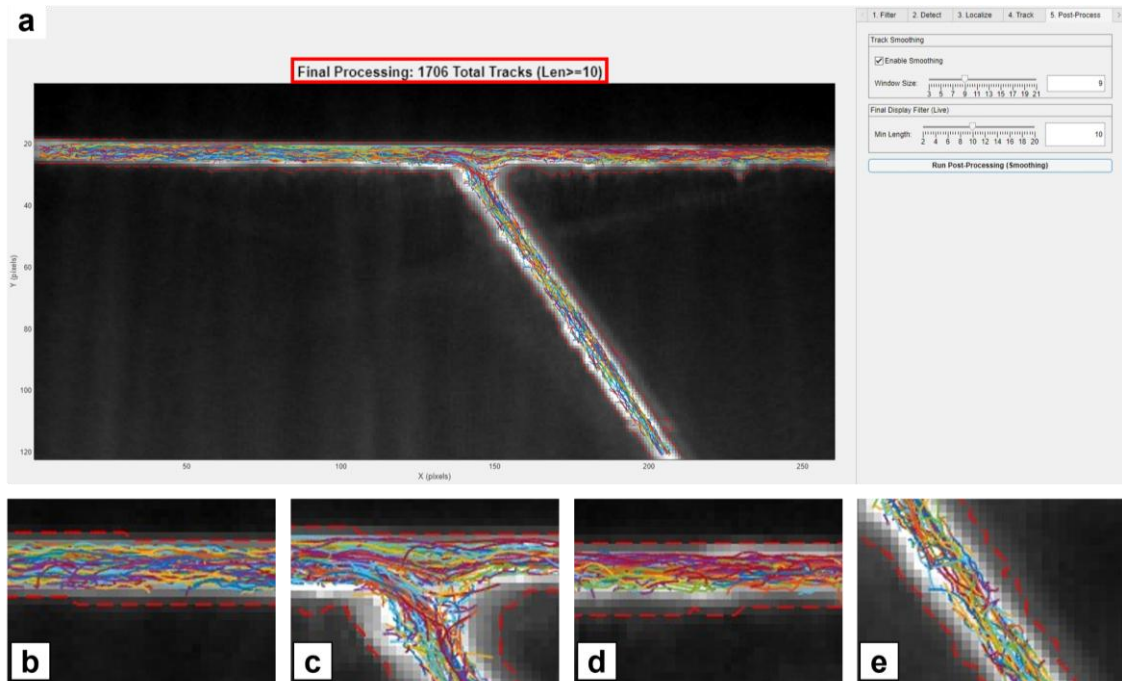


Figure 4.8: Impact of post-processing smoothing and interpolation. (a) Visual display of the smoothed tracks inside the phantom. (b-e) Micro vascular smoothed reconstruction detail (zoomed view).

4.2.7 Super-Resolution Rendering and Visualization

The final stage involves generating super-resolution maps via the “RENDER” tab, providing interactive control over the “Upsampling Factor” to resolve microvascular features below the diffraction limit. All results presented are generated using the computationally efficient Histogram method, ensuring a statistically unbiased distribution

of bubble counts and velocities. By centralizing these controls, the GUI enables a real-time feedback loop for rapid parameter tuning on individual files. This serves as an essential “initial indicator” of final image quality before the complete, heavy-duty processing pipeline is executed across the entire multi-file dataset in MATLAB.

The versatility of this rendering approach is showcased by the four distinct super-resolution outputs generated from a single representative dataset (Figure 4.9). First, the density map utilizes power-law compression to reveal the fine structural boundaries of the 300 μm channels, ensuring that the vessel edges are visible despite the dominant flux in the centerline (Figure 4.9 a). Second, a combined fusion map overlays velocity information onto the density structure, acting as a validation tool to confirm that high-velocity tracks (red/yellow hue) are correctly confined to the vessel lumen, while slower tracks (blue hue) appear near the walls, consistent with fluid dynamics theory. (Figure 4.9 b). Third, the raw velocity map displays the direct arithmetic mean of bubble velocities at each pixel, preserving the unbiased statistical distribution of the flow (Figure 4.9 c). Finally, a filtered velocity map can be generated by applying a conservative Gaussian kernel ($\sigma = 0.6$) to recover a continuous, laminar flow profile, bridging the discrete sampling gaps inherent to particle tracking. Furthermore, this filtering effectively mitigates spatially isolated high-velocity outliers, which typically arise from tracking errors in regions of high bubble density. By smoothing these artifacts, the maximum velocity values are corrected to align with theoretical expectations. Visually, this correction confines peak velocities (rendered in red) to the centerline of the main inlet channel, significantly improving the clarity of the velocity drop observed at the bifurcation (Figure 4.9 d).

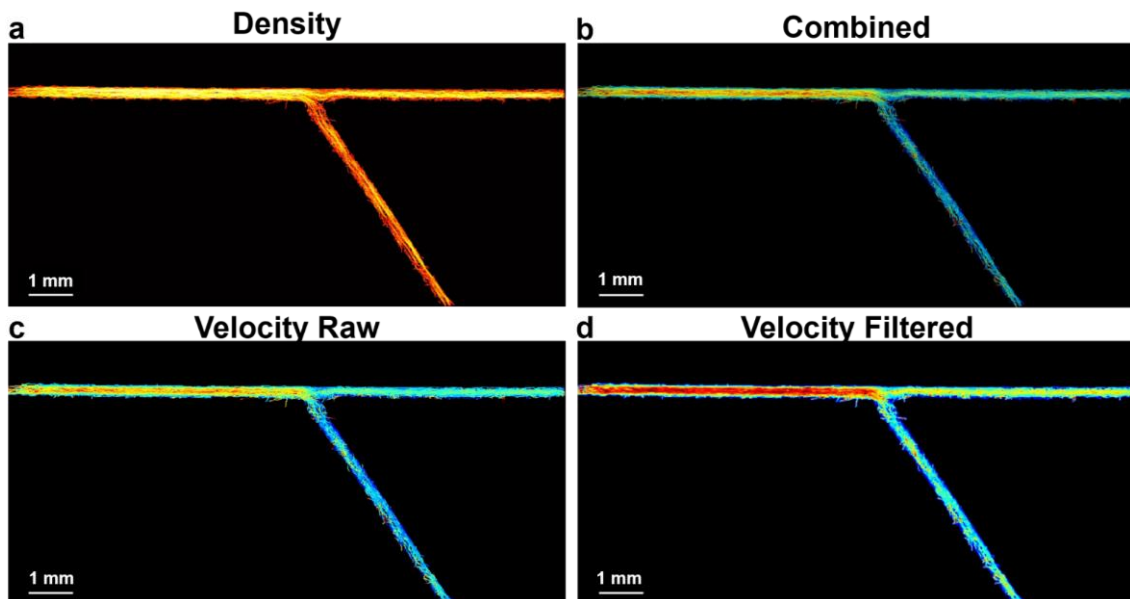


Figure 4.9: Multi parametric super-resolution outputs. (a) Bubbles density map. (b) Dual-Mode fusion (velocity + density). (c) Raw velocity map. (d) Filtered velocity map (gaussian smoothed).

4.3 In Vitro Comparative Analysis: Tracking Algorithm Evaluation on NB

Following the development of the optimization suite, a controlled in vitro experiment was conducted to determine the optimal tracking strategy for sub-wavelength NBs. The primary objective was to evaluate how different tracking logics handle the reconstruction of complex micro-channel geometries under strictly controlled flow conditions.

To this end, lipid-shell NBs were infused into a custom-made gelatin phantom featuring a 300 μm primary channel that bifurcates into a 100 μm secondary branch. A precision syringe pump maintained a steady flow rate of 0.03 ml/min, establishing a laminar flow profile with a theoretical centerline velocity of approximately 11 mm/s. The US acquisition was performed at a transmit voltage of 20V. To ensure a valid comparison, all datasets were processed using identical clutter filtering and localization parameters, with a strict minimum track length filter of 15 frames applied to isolate only robust trajectories.

4.3.1 Comparative Analysis of Tracking Performance

The impact of the tracking algorithm on the final super-resolution image is evaluated by comparing the velocity maps generated by five progressively advanced tracking configurations (Figure 4.10). The limitations of basic tracking algorithms are evident in the reconstructions using the NN (Figure 4.10 b-e) and the Standard HT (Figure 4.10 f-i) methods. While both capture the basic boundary layer effect, they fail to resolve the distinct centerline peak characteristic of laminar flow. Instead, the central flow regions appear homogeneous and noisy (“flat” profile), exhibiting frequent velocity fluctuations that lack spatial coherence (Figure 4.10 b-i). Although the Standard HT method improves global track continuity compared to NN, the velocity profile remains blunted, indicating an inability to resolve fine gradients.

Intermediate configurations provide visible improvements in flow visualization. The Standard KT introduces a predictive motion model that smooths velocity estimation, thereby revealing the parabolic nature of the flow (Figure 4.10 j-m). However, tracking noise and “ghost tracks” persist outside the channel boundaries in this configuration. Conversely, the HT method with a SCM utilizes directional and brightness penalties, implemented within the advanced cost calculation engine, to delineate the laminar profile

more clearly. Despite this clarity, the strict rejection criteria inherent to this configuration result in a sparser map, particularly within the bifurcation zone where flow directions are more complex (Figure 4.10 n-q).

Ultimately, the optimal reconstruction is achieved using the KT integrated with the SCM (Figure 4.10 r-u). This configuration combines the advantages of predictive modeling with the strict linkage constraints of the SCM to resolve a fully developed laminar profile. The resulting map demonstrates a dominant, high-velocity core that decays smoothly toward the vessel walls (Figure 4.10 r-u). By effectively eliminating extra-luminal noise and tracking artifacts seen in simpler methods, this integrated approach successfully reconstructs the theoretical parabolic profile required for accurate microvascular analysis.

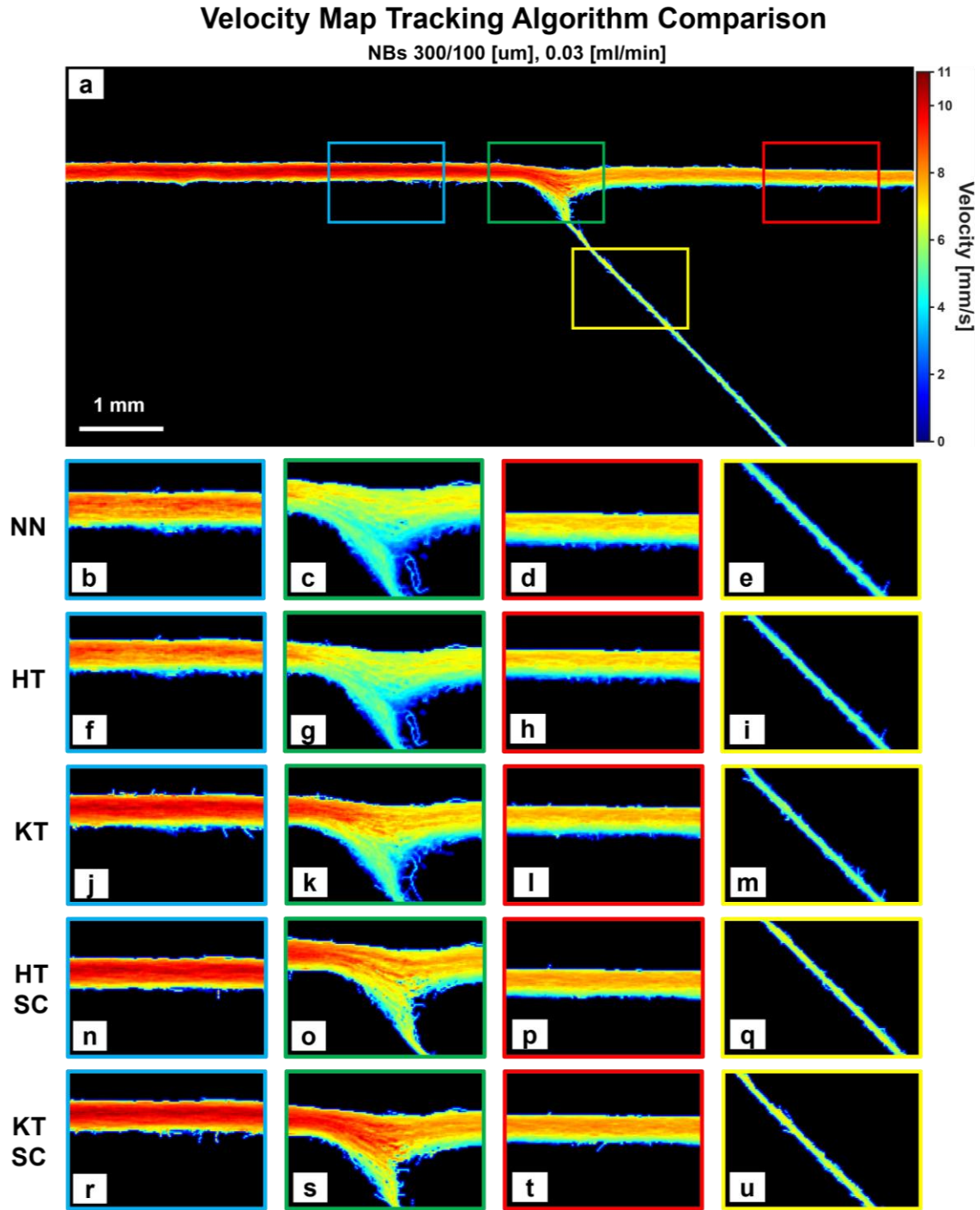


Figure 4.10: Comparative evaluation of ULM tracking algorithms where velocity maps reconstructed using different tracking methods. (a) Velocity map of a gelatin phantom where the colored ROIs depict the magnified areas in (b-u) of different regions, highlighting the impact of the tracking method on vessel continuity, reconstruction density, and the reduction of tracking artifacts. **(b-e)** Nearest Neighbor (NN). **(f-i)** Hungarian Tracking (HT). **(j-m)** Kalman Tracking (KT). **(n-q)** Hungarian Tracking with smart cost matrix (HT SC). **(r-u)** Kalman Tracking with smart cost matrix (KT SC).

4.3.2 Statistical Analysis of Tracking Metrics

While the velocity maps in Section 4.3.1 provide a qualitative assessment of the reconstruction, a quantitative analysis is required to objectively measure the improvement

in tracking fidelity. To this end, statistical distributions of track morphology were computed for the entire dataset with all the tracks.

For this analysis, the minimum track length filter was relaxed to 5 frames (compared to 15 in the visual maps). This relaxation was necessary to expose the raw performance of the tracking algorithms, including their tendency to generate short, fragmented, or erroneous tracks in high-density scenarios. A key biomarker for healthy microvascular flow in phantom channels is laminarity, which implies that trajectories should be smooth and direct. This characteristic is quantified by the Tortuosity Index (τ), the definition and significance of which were detailed in the methodology (Figure 4.11).

The baseline algorithms, NN and Standard HT, exhibited a broad tortuosity distribution with a heavy tail extending up to $\tau = 8$ (Figure 4.11 a, e). Crucially, the peak count at the ideal laminar value ($\tau \approx 1$) was relatively low, at approximately 1300 tracks for both the NN and the Standard HT methods (Figure 4.11 a, e). When the data was filtered for tracks longer than 10 frames, the persistence of high-tortuosity outliers in these baseline methods remained evident, with a median tortuosity value of 1.4 (Figure 4.11 c, g). Significant improvements were observed when introducing the predictive motion models of the KF, which narrowed the distribution tail and increased the frequency of laminar tracks to approximately 3000 and 3500 counts for the raw and filtered datasets, respectively (Figure 4.11 i, k). The most substantial enhancement, however, occurred with the integration of the SCM. By applying directional and brightness penalties to the linking cost, the SCM configurations achieved a sharp peak at the ideal tortuosity value, effectively concentrating the distribution at $\tau \approx 1$ and reaching a peak count of 3000 tracks with HT SC and almost 4500 with KT SC, even before the data was filtered for tracks longer than 10 frames (Figure 4.11 m, q).

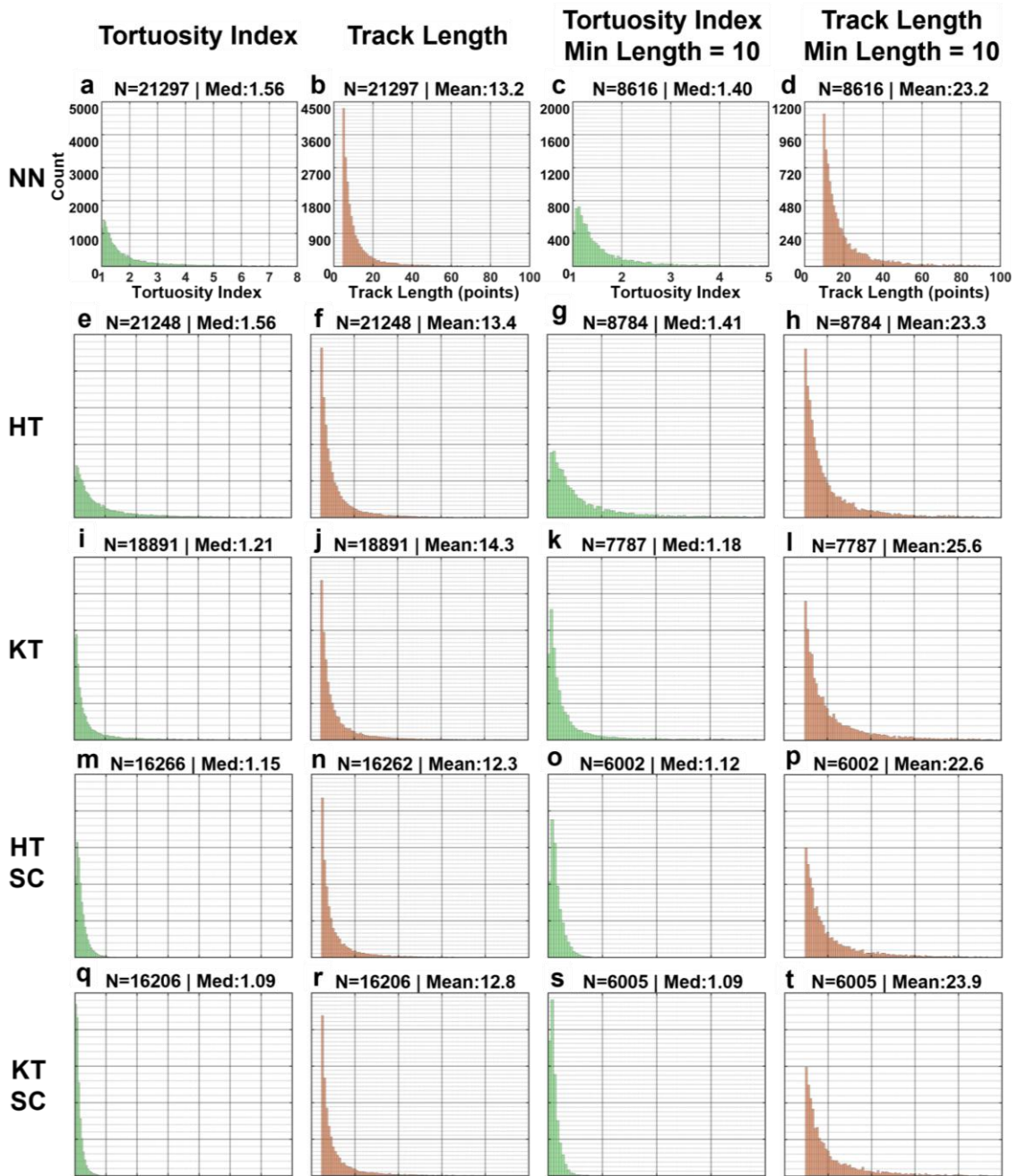


Figure 4.11: Statistical evaluation of tracking fidelity across algorithms. The matrix displays histograms for Tortuosity (Columns 1, 3) and Track Length (Columns 2, 4) for five tracking configurations. (a-d) Nearest Neighbor (NN). (e-h) Hungarian Tracking (HT), (i-l) Kalman Tracking (KT). (m-p) Hungarian Tracking with Smart Cost Matrix (HT SC). (q-t) Kalman Tracking with Smart Cost Matrix (KT SC). Columns 3 and 4 display the same metrics after filtering for tracks with a length > 10 frames. Note the progressive shift towards ideal tortuosity ($\tau \approx 1$) and the reduction in fragmented short tracks as the algorithm complexity increases.

Furthermore, the total number of reconstructed tracks serves as a metric-based indicator for the algorithm's selectivity versus its tendency toward fragmentation. The NN and Standard HT methods produced the highest raw yields, with 21,297 and 21,248 tracks identified respectively (Figure 4.11 b, f). However, given the high tortuosity observed in these datasets, this surplus likely represents fragmented segments, where a single long

vessel is erroneously broken into multiple short tracks and residual noise artifacts (Figure 4.11 d, h). The Standard KT reduced this count to 18,891 tracks (Figure 4.11 j) with a median tortuosity of 1.21, while the inclusion of the SCM resulted in the lowest overall yield of 16,206 tracks for the Kalman configuration (Figure 4.11 r). Despite the lower quantity, this configuration achieved the highest fidelity with the lowest overall tortuosity index of 1.09 (Figure 4.11 q). This reduction, combined with the increase in low-tortuosity tracks, confirms that the advanced algorithms successfully exchange quantity for quality. Finally, by filtering out spurious data and preventing the fragmentation of long vessels, the KT SC method reveals the physiological flow with the highest degree of confidence.

4.4 Comparative In Vitro Analysis of NB vs. MB

4.4.1 Quantitative Hemodynamic Profiling and Velocity Validation

To validate the quantitative accuracy of NB as flow tracers, their performance was compared directly against MB in two distinct microfluidic geometries. The velocity distribution analysis is performed across three key hydrodynamic zones, including the main channel prior to the bifurcation, the distal segment of the main channel, and the bifurcating branch (Figure 4.12).

Symmetric Bifurcation (300-300 μm)

In the symmetric configuration (Figure 4.12 a-h), both contrast agents successfully reconstructed the expected laminar flow profiles. However, the agreement between the measured peak velocities in the pre-bifurcation segment (black profiles) and the calculated theoretical maxima varied as a function of the inlet flow rate. At the lowest flow rate (0.01 ml/min), the measured velocities demonstrated high agreement with the theoretical peak velocity of 3.9 mm/s (Figure 4.12 b, f). This correlation decreased slightly, yet remained comparable, at 0.03 ml/min, where the theoretical peak is 11.7 mm/s (Figure 4.12 c, g). In contrast, at the highest tested flow rate (0.25 ml/min), the measured peak velocities (approximately 60 mm/s) significantly underestimated the theoretical maximum of 97.2 mm/s, despite utilizing a high acquisition FPS of 1000 Hz (Figure 4.12 d, h).

A distinct hydrodynamic behavior was observed post-bifurcation. While the geometry is nominally symmetric, a velocity disparity emerged between the continuing main channel (Blue ROI) and the bifurcating branch (Red ROI). At lower flow rates (0.01 ml/min), the velocities in both branches were comparable. However, at the highest flow rate of 0.25 ml/min (Figure 4.12 d, h), the velocity in the straight channel was notably higher than in

the branching channel. This divergence is likely attributable to the inertia of the fluid at higher Reynolds numbers, which favors the straight flow path over the diverted path.

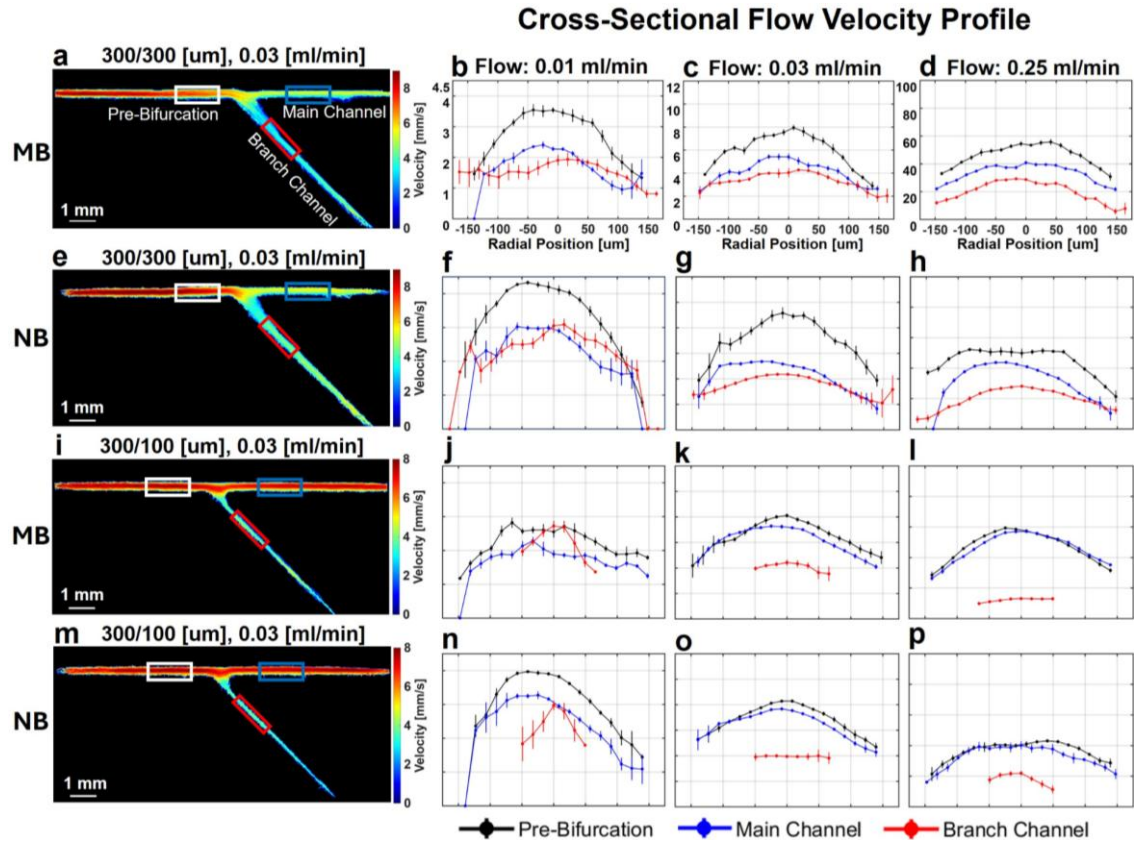


Figure 4.12: Quantitative velocity profiling of MB versus NB in symmetric and asymmetric bifurcation phantoms. The figure presents a comparative cross-sectional analysis across two geometries: (rows a–h) a symmetric 300–300 μm bifurcation and an (rows i–p) asymmetric 300–100 μm bifurcation, comparing MBs (rows a–d, i–l) to NBs (rows e–h, m–p). (a, e, i, m) Super-resolved velocity maps perfused at 0.03 mL/min. ROIs for cross-sectional extraction are marked: main channel pre-bifurcation (white box), main channel post-bifurcation (blue box), and the bifurcating branch (red box). (b, f, j, n) Corresponding cross-sectional velocity profiles at a flow rate of 0.01 mL/min. (c, g, k, o) Corresponding cross-sectional velocity profiles at a flow rate of 0.03 mL/min. (d, h, l, p) Corresponding cross-sectional velocity profiles at a flow rate of 0.25 mL/min. For visual clarity, axis labels and flow rate titles are displayed only on the top subplot of each column (b, c, d). **Legend:** Solid lines indicate the measured ULM velocity profiles, corresponding to the mapped ROIs (Black = Pre-bifurcation, Blue = Post-bifurcation, Red = Bifurcating branch).

Asymmetric Bifurcation (300–100 μm)

The asymmetric phantom presented a high-resistance secondary channel (Figure 4.12 i–p). As expected, the velocity in the main channel post-bifurcation (Blue ROI) remained nearly identical to the pre-bifurcation velocity (Black ROI) across all flow rates. Conversely, the velocity in the 100 μm branch (Red ROI) was markedly lower, consistent with the preferential flow of fluid through the path of least resistance (the wide main

channel). A minor reduction in the main channel velocity was observed downstream, corresponding to the small fraction of mass conservation diverted to the narrow branch.

4.4.2 Comparative Analysis of Microvascular Partitioning and Flow Distribution

Having validated the hemodynamic fidelity of NB through velocity profiling, the next phase of the analysis focused on their perfusion efficiency and microvascular distribution. A central hypothesis regarding sub-micron contrast agents is that their reduced size may facilitate enhanced entry into high-resistance, narrow capillaries compared to larger MB. To test this hypothesis while accounting for the significant differences in native concentration, we applied the localization-based Partitioning Ratio metric (detailed in Section 3.8.4). This approach isolates the physical behavior of the agents from experimental concentration variations, allowing for a direct assessment of their flow-following capabilities at bifurcations. The results of this partitioning analysis, including super-resolved velocity maps for each geometry and pie charts quantifying the fractional distribution between branches, are presented across the various experimental regimes (Figure 4.13).

Overall, the data indicates a strong hemodynamic concordance between NB and MB. In the majority of matched experiments, the fractional distribution of NBs (Figure 4.13 m-u), closely mirrors that of the MBs (Figure 4.13 d-l). This suggests that despite their significantly smaller size, NBs are subject to the same fluid dynamic forces (drag and pressure gradients) that govern MB distribution in these channel sizes 500-100 μm .

Effect of Geometry

The sensitivity of the metric is validated by geometric comparison. In the symmetric phantom experiments, the distribution tends towards equilibrium, whereas in the asymmetric 300-100 μm (Figure 4.13 g-i, p-r) and 500-200 μm (Figure 4.13 j-l, s-u) phantoms, a marked shift is observed for both agents, with a significantly lower percentage of bubbles entering the high-resistance branch. The fact that both MBs and NBs exhibit this shift confirms that the NBs are reliable flow tracers that respect the underlying hemodynamic resistance network.

Relative Perfusion distribution

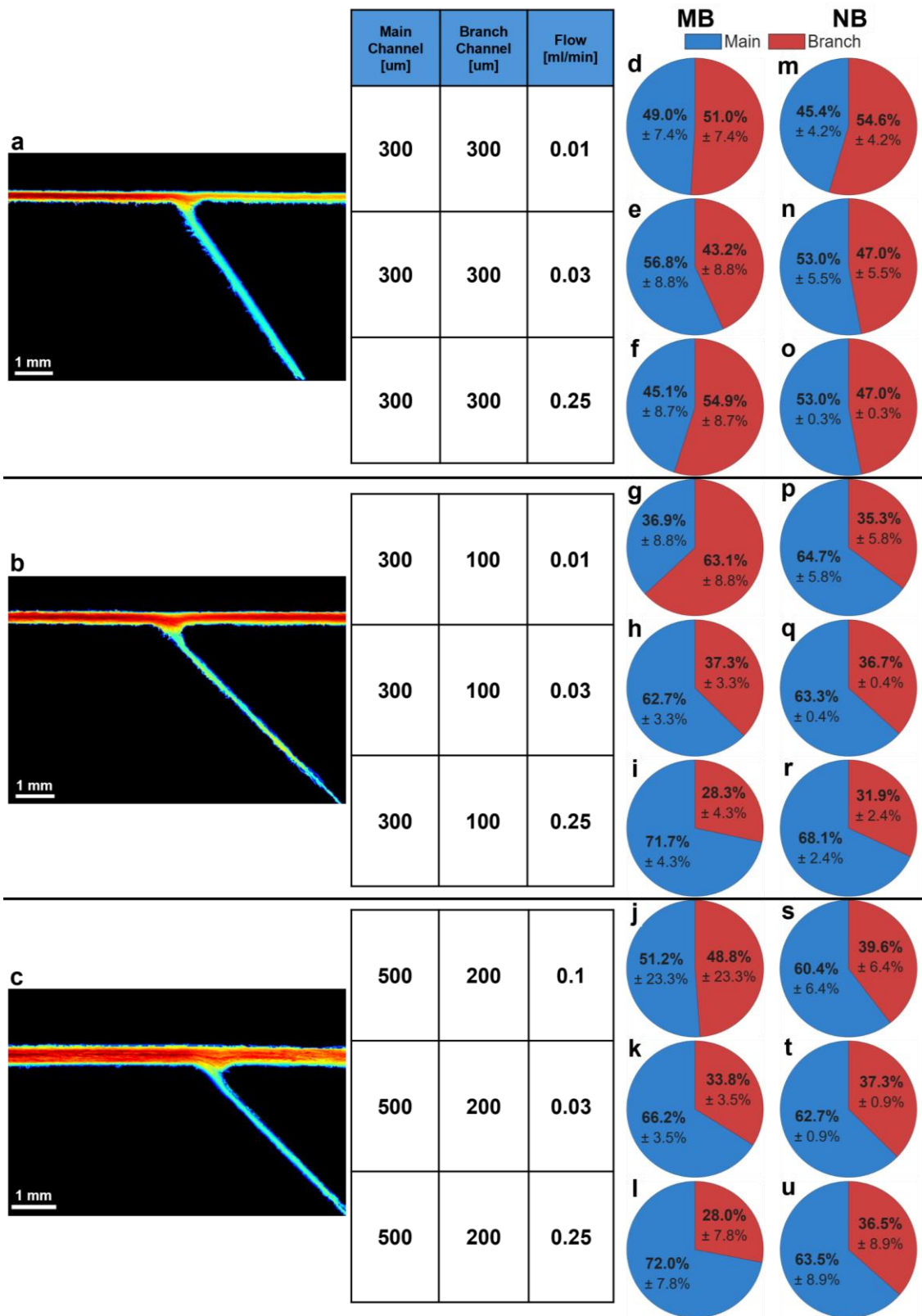


Figure 4.13: Comparative vascular partitioning of MB versus NB. (a-c) Super-resolved velocity maps representing the three distinct bifurcating phantom geometries. A central table details the specific structural parameters and inlet flow rates corresponding to each row. (d-l) Pie charts quantifying the relative distribution of detected MBs between the bifurcating branch (red slice) and the continuing main channel (blue slice). (m-u) Corresponding pie charts displaying the fractional flow distribution for NBs across the identical experimental regimes. Values in all pie charts represent the mean $\pm$ standard deviation, calculated by partitioning each acquisition into three independent temporal segments ($n=3$). Note: Displaying the percentage of total accumulated localizations normalizes for absolute concentration differences between the agents, isolating the effect of agent type on flow partitioning dynamics.

While the general trends remain consistent across most experimental conditions, certain pairwise comparisons exhibit localized divergence. For instance, a notable difference in the partition ratios between the two agents is observed when comparing the initial MB distribution in a specific flow regime (Figure 4.13 l) to the corresponding NB results (Figure 4.13 u). A similar discrepancy is found in the high-resistance comparisons where the MB fractional flow (Figure 4.13 g) deviates from the NB profile (Figure 4.13 p). Given the high consistency observed in the majority of the datasets, these specific discrepancies are likely attributable to transient experimental fluctuations during the acquisition window, such as minor pressure variations in the pump system or temporary partial occlusions, rather than an intrinsic difference in the physical behavior of the contrast agents.

4.5 ULM Pipeline Validation on Benchmark In Vivo Data

Before applying the optimization pipeline to the specific challenges of the rat kidney model, the system's performance was validated on the benchmark in vivo rat brain dataset described in Section 3.9. This step was crucial to verify that the core algorithms could correctly resolve complex, hierarchical vascular structures in a standard biological environment. To evaluate the intrinsic stability of the tracking engine, the raw IQ data was processed using the ULM pipeline with a baseline. Specifically, a standard SVD clutter filter (Ranks 5-800) was applied, followed by a temporal Butterworth bandpass filter (50-250 Hz) to isolate MB harmonic signatures. Bubble centers were identified using Gradient-based Radial Symmetry, and trajectories were reconstructed using a standard KT. Notably, this simplified approach, omitting advanced cost matrices or post-tracking QC, was intentionally selected to demonstrate the robust performance of the fundamental tracker on high-quality input data.

The results of this validation process highlight the significant resolution enhancement achieved by the pipeline across four progressive stages (Figure 4.14). Initially, the

temporal average of the raw image sequence is dominated by strong echogenic reflections from static tissue structures, making it impossible to distinguish the vascular network or individual contrast agents (Figure 4.14 a). However, the application of SVD filtering successfully isolates the blood pool from the surrounding clutter, although the resulting image remains physically constrained by the wavelength of the US. At this stage, the visualization is diffraction-limited, resulting in a blurred map where closely spaced vessels in the cortex appear fused and cannot be spatially resolved (Figure 4.14 b).

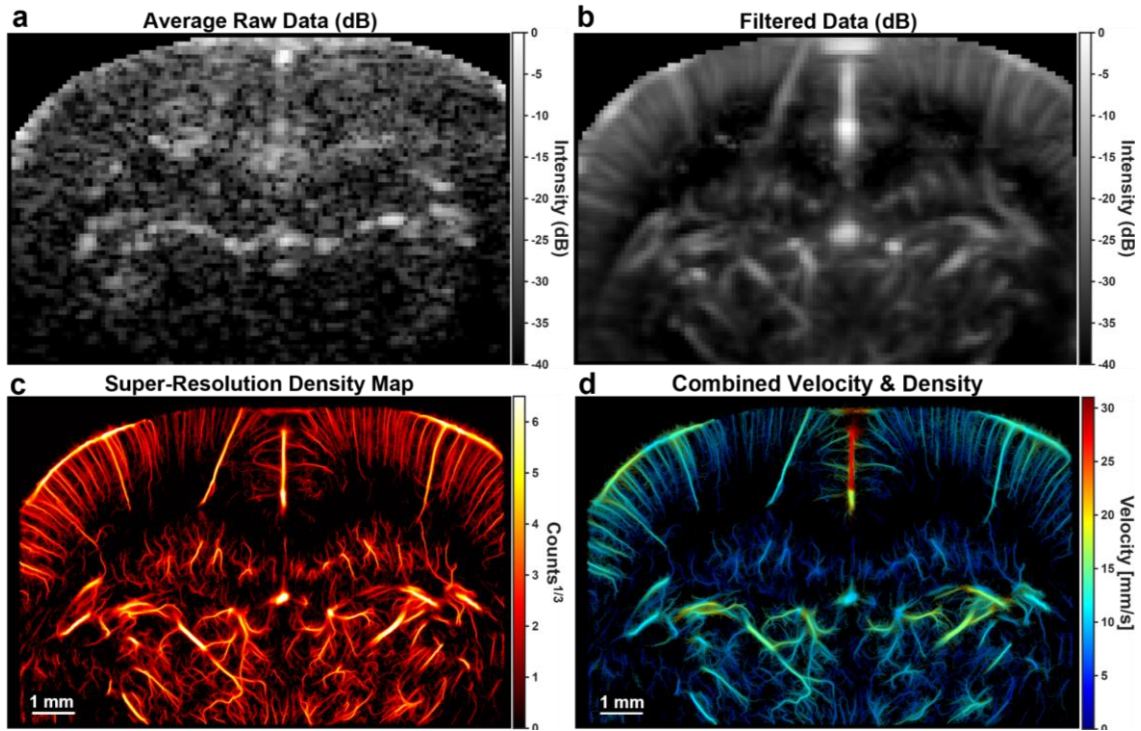


Figure 4.14: Benchmark In Vivo data reconstruction validation. (a) Temporal average of raw IQ B-mode data. (b) SVD-filtered image showing isolated but diffraction-limited vascular segments. (c) Super-Resolution Density Map, reconstructed vascular architecture. (d) Dual-Mode Fusion, combined visualization where brightness represents vessel density and color encodes flow velocity (saturated at 30 mm/s).

The transition to the ULM Density Map represents the final reconstruction output after localization, tracking, and accumulation on a high-definition grid with an upsampling factor of 10. The algorithmic processing successfully separates adjacent microvessels that were blurred in the diffraction-limited image (Figure 4.14 b), revealing the discrete angio-architecture of the capillary bed at scales ten times smaller than the theoretical resolution limit (Figure 4.14 c). Finally, the combined visualization modulates brightness by vessel density and encodes flow velocity with a saturation limit of 30 mm/s. This functional layer correlates structure with physiology, clearly differentiating the high-velocity feeding arterioles, rendered in red and yellow, from the slower, diffusive flow in the microvascular network, rendered in blue (Figure 4.14 d). Ultimately, this progression

validates the tracker's accuracy and confirms its ability to produce a coherent velocity gradient that aligns with the expected hemodynamic profile of the rat.

4.6 In Vivo Renal Vascular Mapping: Comparative Analysis of NB and MB Perfusion

The performance of the ULM pipeline was evaluated in vivo using a rat kidney model. Figure 4.15 displays the TMIP maps for both MBs and NBs. These images represent the cumulative vascular signal over the entire acquisition duration, generated from the clutter-filtered and motion-corrected IQ data.

The TMIP map for the MB data reveals the macrovascular structure of the kidney, with

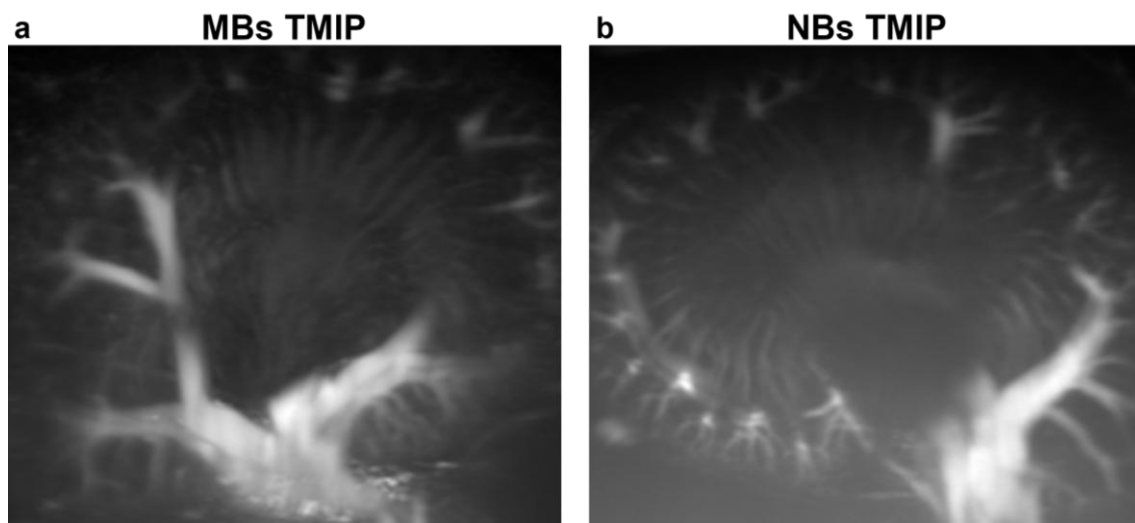


Figure 4.15: In vivo Temporal Mean Intensity Projection (TMIP) maps of the rat kidney vasculature. (a) MB data and (b) NB data. The maps represent the diffraction-limited vascular density following respiratory gating, motion correction, and SVD clutter filtering. Note the different anatomical cross-sections due to variations in kidney orientation between experimental sessions.

high signal intensity localized in the primary renal artery and its segmental branches (Figure 4.15 a). The vascular boundaries are well-defined, showing the distribution of MBs within the larger vessels of the renal hilum and the interlobar arteries. In comparison, the NB map demonstrates successful perfusion of the sub-micron agents throughout the renal parenchyma, including the cortex and the medullary regions (Figure 4.15 b). While the signal intensity in the NB map is more diffuse compared to the MB dataset, it captures a dense network of smaller vascular structures.

It is observed that the imaging planes for the two datasets are not identical due to variations in the kidney's anatomical orientation during the separate experimental sessions. Specifically, the MB data captures a longitudinal cross-section emphasizing the main arterial supply (Figure 4.15 a), while the NB data reflects a different plane providing

a broader view of the cortical perfusion (Figure 4.15 b). Despite these differences in orientation, both maps show a clear suppression of background tissue clutter and a lack of motion-induced blurring in the vessel outlines.

5. Discussion

The development of the ULM processing pipeline and the associated “ULM Master GUI” allowed for a systematic comparison between NB and MB. The following sections interpret the experimental results, focusing on the technical trade-offs of the signal processing stages and the physical phenomena observed during flow validation.

5.1 In Vitro Flow Validation and Spatial Resolution

The in vitro experiments focused on quantifying the accuracy of the localized tracks against the known geometry of the microfluidic phantom. First, the pipeline successfully resolved the major bifurcations and varying channel diameters, with the resulting ULM maps showing high agreement with the physical dimensions of the phantom. Moreover, the tracking results for both agents demonstrated that the pipeline could maintain track continuity even in regions of high curvature.

However, a nuanced interpretation of these resolution metrics is necessary. It is important to note that in these phantom experiments, the super-resolution processing did not reveal structural details that were inherently invisible to B-mode imaging, as the smallest channels were already near the diffraction limit. Nevertheless, these experiments serve a crucial proof-of-concept purpose; they validate that despite their significantly lower acoustic cross-section and challenging SNR, NBs can be successfully detected, localized, and tracked using the high-sensitivity pipeline and interactive optimization platform developed here. Consequently, this establishes the foundational feasibility for future in vivo efforts, providing the necessary methodology to bridge the gap toward the biological realization of sub-micron ULM imaging.

5.2 Signal Isolation and SVD Filtering Optimization

The first stage of the pipeline involved the isolation of bubble signals from stationary tissue clutter. The results indicate that the optimal SVD thresholding range is not a static parameter but varies based on the specific experimental conditions.

Contrary to the hypothesis that NBs, due to their smaller scattering cross-section, would consistently require higher singular value cutoffs to be separated from noise, the analysis showed no definitive trend in SVD thresholds between the two agents. This suggests that at the acoustic pressures used in these experiments (maintained at a constant voltage for

both agents), the primary challenge for signal isolation is not the agent size itself, but the presence of quasi-stationary artifacts.

A recurring observation was the “stuck-bubble” phenomenon, where agents adhered to the gelatin channel walls. These bubbles exhibit high temporal correlation, causing their signal to overlap with the tissue subspace in the SVD spectrum. The interactive GUI was instrumental in finding a compromise: a threshold low enough to retain slow-moving bubbles while high enough to suppress most of the stationary gelatin reflections. However, the inability to separate these signals often led to partial detections or stationary artifacts that required further mitigation during the tracking stage.

5.3 Evaluation of Tracking Algorithms

The comparison of tracking methodologies (Section 4.3) highlighted the limitations of proximity-based linking in ULM. While NN and standard HT algorithms generated high track counts, the high tortuosity indices and short track lengths revealed significant fragmentation.

The KT SC demonstrated the highest fidelity by incorporating a kinematic motion model. By predicting the bubble's future position based on its current velocity, KT maintained track continuity even when NBs were administered at high concentrations. To ensure objectivity, the “Max Linking Distance” (search radius) was automated based on the experimental flow rate and FPS. The success of the KT SC algorithm in producing trajectories with a tortuosity index near 1.0 confirms that predictive modeling is essential for reconstructing laminar flow, especially when high bubble density increases the probability of false associations (ID-swaps).

5.4 Hemodynamic Accuracy and Velocity Underestimation

The validation of the ULM pipeline against theoretical parabolic flow models revealed a high degree of accuracy at low volumetric flow rates; however, a consistent underestimation of peak velocities was observed at the highest tested rate of 0.25 ml/min. First, it is essential to consider the mechanical properties of the experimental environment, as the gelatin-based phantoms exhibit significant elasticity compared to rigid microfluidic structures. Furthermore, observations during high-pressure flushing procedures indicated visible dilation of the channels, suggesting that the internal pressure generated at higher flow rates induces a radial expansion of the vessel. According to the continuity equation ($Q = V \cdot A$), such an increase in the cross-sectional area (A) for a fixed volumetric flow rate (Q) necessarily results in a proportional decrease in flow

velocity (V). Consequently, the observed discrepancy likely represents the actual physical velocity within a dilated channel rather than a failure of the tracking logic.

Moreover, this physical interpretation is reinforced by the systematic nature of the results across both contrast agent types and all phantom geometries. While the acquisition rate of 1000 FPS was theoretically sufficient to resolve inter-frame displacements of approximately 2 pixels, well within the search gates of the tracking algorithms, the persistence of the underestimation suggests that the bottleneck is not algorithmic. In addition, the use of custom-built SVD filtering and optimized tracking parameters ensured that motion blur and localization jitter were minimized. Therefore, the results validate the pipeline's ability to capture real-time hemodynamic changes, even when those changes are driven by the structural deformation of the vessel itself.

5.5 Comparative Utility of NBs vs. MBs

The experimental results demonstrate that the performance of NBs mirrored that of the MB controls with exceptionally high fidelity across all tested geometries. Specifically, in both symmetric and asymmetric phantom structures, the NBs reconstructed parabolic velocity profiles that were virtually indistinguishable from those obtained using MBs. Furthermore, the strong concordance in partitioning ratios and peak velocity measurements confirms that NBs can function as reliable hemodynamic tracers in vessels ranging from 100 to 500 μm .

Given that both agents were tested under identical acoustic voltage settings, the parity in performance is particularly noteworthy. Traditionally, sub-micron agents suffer from a significant Rayleigh scattering penalty, which can limit their SNR compared to larger MBs. However, the sensitivity of the custom-developed ULM pipeline, particularly the interactive SVD clutter filtering and the high-precision localization algorithms, proved sufficient to overcome these physical limitations. Consequently, NBs can be used interchangeably with MBs for quantitative microvascular mapping at these scales without a loss in accuracy or resolution.

5.6 In Vivo Pipeline and Contrast Agent Performance

The clarity of the TMIP maps obtained from the rat kidney provides a direct validation of the pre-processing stages of the custom ULM pipeline. The absence of spatial blurring in the vascular structures confirms that the two-stage motion correction strategy was effective. Specifically, the gating module successfully removed frames corrupted by large-scale respiratory motion, while the subsequent cross-correlation registration

compensated for the fine-scale pulsatile displacements caused by the cardiac cycle. This stability is a prerequisite for achieving the high-contrast projections seen in the results. Furthermore, the successful isolation of the bubble signals from the renal parenchyma demonstrates the efficacy of the SVD filtering implementation. The renal environment presents a high clutter-to-signal ratio, yet the interactive threshold optimization allowed for the removal of stationary tissue signals without losing the transient dynamics of the contrast agents. The detection of NBs in the deeper cortical layers is particularly significant, as it confirms that these agents maintain sufficient stability and echogenicity within the systemic circulation to map complex organ vasculature.

The variation in the captured imaging planes between the MB and NB sessions highlights the flexibility of the processing framework, as the algorithms performed consistently regardless of the anatomical orientation. Although these preliminary maps are diffraction-limited and do not yet reach sub-pixel resolution, they establish that the data is sufficiently stabilized and filtered for the final localization and tracking stages. These findings suggest that both MBs and NBs are viable for *in vivo* ULM, with NBs showing potential for providing a more detailed representation of microvascular perfusion in the peripheral regions of the kidney.

5.7 Study Limitations

Despite the successful validation of the ULM pipeline and the performance of NBs as flow tracers, several inherent limitations must be acknowledged. These constraints span from the fundamental signal processing assumptions to the physical geometry of the experimental models and the subjective nature of the optimization process.

First, while SVD filtering serves as a computationally efficient cornerstone of the ULM pipeline [14], its performance is fundamentally constrained by several idealized physical assumptions that are frequently violated in practice. The primary challenge lies in the assumption of tissue stationarity; non-rigid physiological motion in the *in vivo* model causes clutter energy to “leak” from the low-rank subspace into higher-order components, leading to spectral leakage that obscures the bubble signal [17]. Furthermore, the physical coupling between bubbles and moving vascular walls violates the mathematical requirement for subspace independence, which can manifest as “ghosting” artifacts. This is further complicated by the “slow-flow conflict”, where bubbles in the microvasculature exhibit temporal dynamics nearly identical to stationary tissue, leading to their unintended suppression [16]. Consequently, the efficacy of SVD filtering remains strictly dependent

on the implementation of high-quality motion correction prior to subspace separation, as it is not a standalone solution for complex biological environments.

Moreover, certain limitations of the current *in vitro* model must be addressed regarding its physiological relevance. The custom phantom fabrication process reaches a resolution limit at approximately 100 μm , which is significantly larger than the true capillary scale [15]. At this dimension, standard MB still flow freely without steric hindrance, meaning the unique advantage of NB, their ability to navigate sub-10 μm capillaries, could not be fully demonstrated in this specific model. In addition, the current fabrication method utilizes square-profile channels that do not perfectly replicate the circular cross-sections of biological microvasculature, potentially influencing local flow velocity profiles and wall shear stress distributions [21]. While the study establishes NB as accurate tracers for super-resolution imaging, their full potential for visualizing perfusion in the finest vascular networks and their superior circulatory stability compared to microbubbles remain to be fully explored in platforms that more closely mimic the sub-wavelength capillary regime [62].

Finally, the interpretation of the localized data and the role of the processing interface introduce systemic variables. A primary physical constraint involves the interpretation of the PSF during the localization of NBs; given their sub-wavelength dimensions and high concentration requirements, it remains challenging to definitively distinguish between a single agent and a localized cluster of bubbles [57]. Furthermore, the performance of the developed ULM pipeline is intrinsically tied to user expertise. While automated optimization might be desirable, the high variability of US data, driven by differences in acquisition systems and experimental conditions, necessitates a “user-in-the-loop” approach. Because there is often no single “globally optimal” parameter set, the fidelity of the final super-resolution maps is strongly correlated with the operator’s ability to tune parameters empirically [13]. This reliance on expert calibration is a deliberate design choice to accommodate the diverse nature of ULM data, yet it limits the accessibility of the platform for non-expert users and emphasizes the role of the system as a research-centric optimization tool rather than a fully automated diagnostic solution.

5.8 Future Research Directions

The current ULM framework provides a robust foundation, yet several avenues for technical and experimental enhancement remain. First, while CNC-machined aluminum molds provided a reliable method for creating microchannels, they reached a physical fabrication limit at approximately 100 μm . Future work could overcome this constraint

by utilizing advanced manufacturing techniques, such as powder-based 3D printing or selective laser melting (SLM) [24], [87], to achieve the higher resolutions necessary for true capillary-scale modeling [15]. Moreover, the current phantom design, which forms square cross-sections by joining a patterned gelatin half with a planar surface, could be refined to better mimic the circular geometry of physiological vessels [15]. Despite this geometric simplification, the current method remains highly valuable for its versatility in simulating complex vascular pathologies, such as aneurysms and stenoses, which are central to ongoing research in the laboratory [15].

Furthermore, a significant transition toward 3D US imaging is proposed to address the inherent limitations of 2D tracking. In the current 2D implementation, MBs frequently enter and exit the FOV along the elevation plane (y-axis), resulting in fragmented trajectories and loss of continuity [26], [48], [57], [88], [89]. Transitioning to a 3D acquisition scheme would allow for the continuous monitoring of bubble kinetics in a volumetric space, thereby providing a more comprehensive understanding of flow dynamics [48], [59], [62]. In addition, 3D imaging would fundamentally improve the motion correction stage for in vivo kidney data. Since tissue displacement in a living system occurs across all three spatial dimensions [27], [30], [90], a 3D correction algorithm would enable more precise registration and higher quality super-resolution maps by accounting for out-of-plane motion that is currently unaccounted for in the 2D pipeline [27], [57], [90], [91].

Finally, the contrast-to-noise ratio (CNR) and localization precision could be significantly bolstered by shifting processing further upstream to the RF level through the implementation of adaptive beamforming and coherence-based filtering. Specifically, transitioning from standard delay-and-sum (DAS) to adaptive methods such as Minimum Variance (MV) or Capon beamforming could suppress off-axis interference and sharpen the PSF, directly improving the accuracy of the subsequent localization stage [47], [84], [92]. Furthermore, the integration of coherence-based metrics, such as the Coherence Factor (CF), Generalized Coherence Factor (GCF), or Phase Coherence Factor (PCF), provides a powerful mechanism for suppressing incoherent noise and sidelobes by weighting the beamformed signal based on the phase alignment of the received echoes [67], [93]. Advanced spatial coherence techniques, such as Short-Lag Spatial Coherence (SLSC) imaging, could further improve bubble detectability in low-SNR environments, such as deep-tissue imaging, by focusing on the spatial correlation of the wavefront rather than just its magnitude [11], [29], [67]. These RF-level optimizations would

fundamentally increase the bubble-to-clutter ratio before any clutter filtering is applied, leading to more robust tracking and higher-fidelity super-resolution maps [47], [92]. Beyond anatomical mapping, future research should prioritize the functional quantification of renal microstructures, specifically the counting and morphological characterization of glomeruli [32]. Given that glomeruli represent the primary filtration units of the kidney and consist of dense capillary tufts, the sub-micron scale of NBs provides a critical advantage for resolving these intricate functional units where traditional MBs may be limited [5], [33]. The application of the developed pipeline toward “sensing ULM” (sULM) frameworks could facilitate the automated quantification of glomerular density, potentially serving as a non-invasive diagnostic biomarker for renal pathologies such as focal segmental glomerulosclerosis or for differentiating between renal pseudotumors and malignant growths [94]. Moreover, the modularity of the processing framework allows for its expansion to emerging classes of contrast agents, such as gas vesicles (GVs) [8], [33], [75], [95]. These genetically encodable, protein-shelled nanostructures offer distinct acoustic properties and the potential for molecular targeting, presenting a promising frontier for combining super-resolution imaging with the monitoring of specific gene expressions or cellular functions [8], [33], [75], [95].

6. Conclusion

This thesis has successfully established and validated a comprehensive ULM processing framework, uniquely tailored to evaluate and compare the hydrodynamic performance of sub-micron NBs against standard MBs. First, by transitioning from a traditional algorithmic approach to a modular, GUI-driven platform, this work addressed the inherent signal processing challenges associated with the low acoustic cross-section and weak SNR of NBs. Furthermore, a major technical contribution of this research is the development of the “ULM Master GUI”, which served as the primary engine for parameter optimization. Because the precise localization of sub-micron agents operating at the critical threshold of detection requires meticulous calibration, the integrated pipeline, utilizing SVD with adaptive thresholding, sub-pixel localization via radial symmetry, and predictive KF tracking, significantly improved detection sensitivity. Specifically, the implementation of KT with a SCM effectively minimized trajectory fragmentation and maintained a tortuosity index near 1.0, even under the high-concentration conditions required for adequate NB detection.

Experimental validation confirmed the viability of NBs as accurate and deterministic flow tracers. Moreover, in vitro experiments utilizing custom-fabricated gelatin phantoms with

complex microvascular bifurcations demonstrated that NBs yield hemodynamic mapping accuracy comparable to traditional MBs. In addition to retrieving theoretical parabolic velocity profiles, microvascular partitioning analysis revealed that the fractional distribution of NBs across bifurcating channels faithfully reflects the structural resistance of the branching network. Consequently, this confirms their reliability in representing true flow dynamics in complex geometries. Furthermore, complementary in vivo imaging of a rat brain model validated the framework's capacity to resolve complex, hierarchical vascular networks, simultaneously capturing high-velocity arcuate arteries and sparse, slow-flow peritubular capillaries. Building upon this validation, preliminary in vivo renal vascular mapping in a rat model demonstrated the pipeline's effectiveness in challenging, high-clutter environments. The resulting TMIP maps confirmed that NBs maintain sufficient systemic stability and echogenicity to resolve dense cortical microvascular networks, while the integrated SVD-filtering and motion correction successfully isolated bubble dynamics from the renal parenchyma.

Despite these technical advancements, several limitations must be acknowledged. From a signal processing perspective, SVD filtering fundamentally assumes tissue stationarity, an idealized condition that is frequently violated by non-rigid physiological motion in vivo. This violation leads to spectral leakage and exacerbates the “slow-flow conflict”, where the dynamics of MB in small capillaries overlap with stationary tissue signals, risking the unintended suppression of valid bubble echoes. Furthermore, the in vitro phantoms were limited to a minimum channel size of $100\ \mu\text{m}$. Because this scale exceeds true capillary dimensions, the theoretical advantage of NBs, specifically their ability to navigate highly restrictive vascular beds without steric hindrance, could not be fully visualized in this specific phantom model. Additionally, while the initial in vivo renal maps successfully demonstrate contrast agent perfusion and stability, they currently remain diffraction-limited, representing a necessary preliminary stage before full super-resolution localization and tracking can be achieved in living tissue.

Addressing these limitations establishes a clear trajectory for future research. Transitioning from 2D to volumetric 3D ULM will be critical for resolving out-of-plane motion artifacts, while upstream RF level optimizations could further enhance the bubble-to-clutter ratio. Moreover, applying “sensing ULM” (sULM) techniques and exploring emerging agents like gas vesicles (GVs) could eventually allow for the direct functional quantification of intricate structures such as renal glomeruli. Ultimately, this thesis demonstrates that with highly sensitive, interactively optimized signal processing, sub-

micron NBs serve as robust and quantitatively accurate contrast agents for super-resolution US, providing a vital stepping stone for future investigations into microvascular pathologies.

7. References

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תקציר

לוקליזציית אולטרסאונד מיקרוסקופית (ULM) היא שיטת דימות בסופר רזולוציה, המאפשרת לעקוף את מגבלת העקיפה האקוסטית כדי למפות כלי דם זעירים ברמה הנימית, אשר לא ניתן לדמות באמצעות אולטרסאונד סטנדרטי. בשיטה זו עוקבים אחרי בועיות גז בודדות המהוות חומר ניגוד שמוזרק למחזור הדם. כיום, מיקרו-בועיות בגודל מיקרומטרי הן הסטנדרט המקובל ליצירת ניגודיות אקוסטית, אך גודלן (1-10 מיקרומטר) עלול להקשות על חדירתן וזרימתן בתוך רשתות הנימים הצרות ביותר. ננו-בועיות, הקטנות משמעותית ממיקרומטר, מציעות פתרון אפשרי לשיפור היכולת להגיע לנימים עדינים אלו, אך השימוש בהן מציב אתגרים משמעותיים. האתגרים ברובם נובעים מגודלן הקטן ומכך שהאות המוחזר מהן חלש מאוד, מה שדורש מתמרי אולטרסאונד רגישים במיוחד ושיטות עיבוד אותות מתקדמות כדי להתגבר על רעשי הרקע. עבודה זו מציגה, למיטב ידיעתנו, את ההדגמה הראשונה לשימוש בננו-בועיות כחומר ניגוד בלוקליזציית אולטרסאונד מיקרוסקופית. התזה מבססת תשתית מחקרית לדימות בעזרת ננו-בועיות, תוך התמקדות בשיטות עיבוד אותות רגישות ובניתוח השוואתי מול מיקרו-בועיות. המחקר בוחן את יחסי הגומלין שבין דיוק האיכון, אמינות המעקב אחר הבועיות ודיוק מיפוי המהירויות במבנים גיאומטריים ובקצבי זרימה שונים. כדי להתמודד עם אתגרי הדימות של ננו-בועיות, פותחה בסביבת מטלאב מערכת עיבוד מלאה מקצה לקצה. חלק מרכזי בעבודה זו הוא פיתוח ממשק משתמש גרפי מקיף, המאפשר לצפות בתוצאות בזמן אמת ולבצע אופטימיזציה של הפרמטרים בכל שלב בתהליך העיבוד. המערכת משלבת שיטה לסינון רעשי רקע מהרקמה (על בסיס פירוק לערכים סינגולריים), ולאחריה לוקליזציה בדיוק גבוה (תת-פיקסל). שלב המעקב בוצע תוך השוואה בין אלגוריתם "השכן הקרוב ביותר" לבין מסנן קלמן מתקדם, במטרה לשמור על רציפות מסלולי הזרימה גם בתנאים של צפיפות בועיות גבוהה. ניסויים שבוצעו במעבדה על פנטומים המדמים כלי דם, הראו כי ננו-בועיות משיגות דיוק במיפוי הזרימה המשתווה לזה של מיקרו-בועיות, ושני סוגי חומרי הניגוד הצליחו לשחזר בצורה מהימנה את פרופילי המהירות התיאורטיים. בנוסף, בדיקת חלוקת הזרימה בהסתעפויות של כלי דם הראתה כי ננו-בועיות מתנהגות בדומה למיקרו-בועיות, וחלוקת חומר הניגוד בין הענפים השונים שיקפה את ההתנגדות של מבנה הרשת. כמו כן, נמצא כי שימוש במסנן קלמן שיפר משמעותית את רציפות המסלולים ושמר על מדד פיתוליות נמוך ותקין, גם בריכוזים הגבוהים הנדרשים לזיהוי ננו-בועיות. יכולות המערכת והדיוק האלגוריתמי אומתו גם בניסויים על מודלים של חיות. התשתית תוקפה באמצעות נתונים של מוח חולדה עם מיקרו-בועיות, שהניבו מפות סופר-רזולוציה באיכות גבוהה והדגימו את יכולת הקוד לשחזר רשתות וסקולריות צפופות ומורכבות בדיוק מירבי. במקביל, במיפוי כלי דם בכליה של חולדה, איששו התוצאות המבוססות על היטלי עוצמה ממוצעים כי ננו-בועיות שומרות על יציבות מספקת בתוך הגוף ומאפשרות להבחין ברשתות כלי דם צפופות בגבול הדיפרקציה האקוסטית. שילוב של סינון רעשים ותיקון תנועה דו-שלבי הצליח להעלים את אותות הרקמה ולייצר היטלים אלו מבלי לטשטש את גבולות כלי הדם. מחקר זה מבסס שיטה חסונה לניתוח מיקרוסקופיית לוקליזציה, ומוכיח כי ננו-בועיות הן חומר ניגוד יעיל ומדויק למיפוי זרימה ברזולוציה גבוהה. התוצאות מצביעות על כך ששימוש בכלי אופטימיזציה אינטראקטיביים הוא חיוני למעבר מוצלח ממיקרו-בועיות סטנדרטיות לחומרים קטנים יותר, ובכך פותח את האפשרות למחקרי כלי דם עתידיים מגוונים.

אוניברסיטת תל-אביב

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בית הספר להנדסה ביו-רפואית

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גריגורי שפירו

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