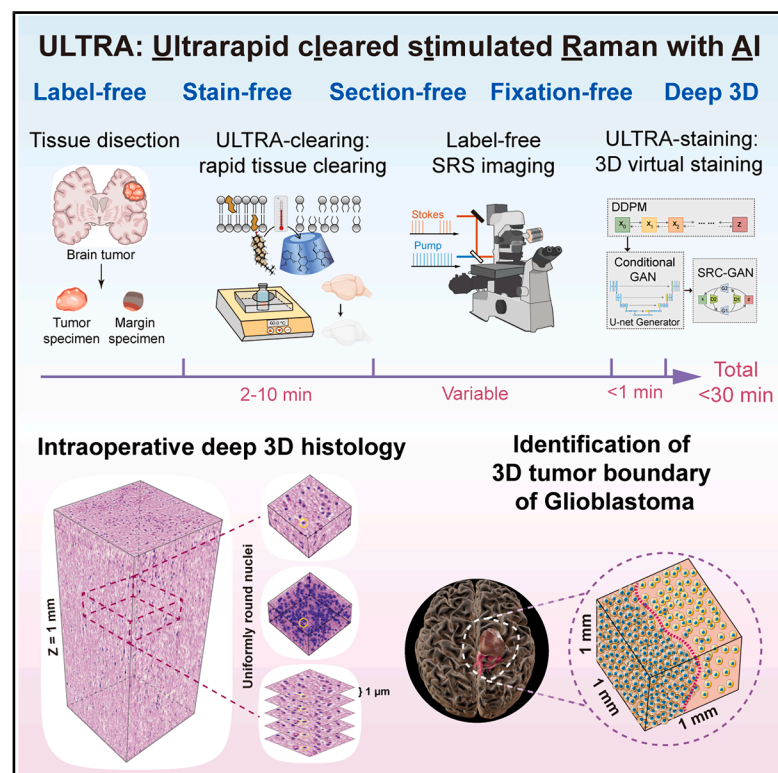


Ultrarapid deep 3D histology enables intraoperative mapping of glioma infiltration

Graphical abstract



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In brief

ULTRA compresses 3D histology from days or hours to within 30 minutes by leveraging the chemical specificity of stimulated Raman scattering (SRS) microscopy through the synergistic development of rapid tissue clearing and unsupervised AI. The platform enables intraoperative visualization of glioma architecture and depth-dependent tumor infiltration margins.

Highlights

- ULTRA clearing enables ultrarapid tissue clearing for label-free volumetric SRS imaging
- ULTRA staining generates FFPE-grade 3D virtual histology from SRS data
- ULTRA compresses 3D histology from days to an intraoperative timescale
- ULTRA uncovers depth-dependent glioma heterogeneity and infiltration margins

Resource

Ultrarapid deep 3D histology enables intraoperative mapping of glioma infiltration

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SUMMARY

Three-dimensional (3D) histology provides volumetric insights into tissue microarchitectures across entire specimens, holding great promise for more accurate prognostication. However, existing methods are too slow for intraoperative consultations. We present ULTRA (ultrarapid cleared stimulated Raman with AI), a label-free, stain-free, fixation-free, and section-free platform that leverages the chemical specificity of stimulated Raman scattering (SRS) microscopy for rapid 3D histological analysis. Through the synergistic development of a one-step tissue-clearing protocol and unsupervised learning algorithms, ULTRA delivers high-resolution, formalin-fixed, paraffin-embedded (FFPE)-grade deep 3D virtual histology within 30 min, covering orders of magnitude more tissue than slide-based methods. In human surgical glioma samples, ULTRA accurately resolves key histological features in 3D and delineates depth-dependent tumor infiltration margins at single-cell resolution. By compressing 3D histology from days or hours to an intraoperative timescale, ULTRA addresses a critical clinical gap and enables more informed surgical decision-making in the operating room.

INTRODUCTION

Tumors are phenotypically heterogeneous and intrinsically three-dimensional (3D) entities.^{1–4} Conventional histological analyses rely on thin tissue sections,⁵ which inherently lose the 3D spatial context of tumor organization and tissue histoarchitectures.^{3,6–9} In addition, because these methods typically focus on a limited number of pre-selected sections, they are prone to sampling bias, limiting a comprehensive understanding of tumor heterogeneity.^{7,10–12} This limitation may compromise the accuracy of cancer diagnosis,^{3,4} as metastatic sites can be easily overlooked when analyzing only a few sections.

To address these limitations, 3D histology has emerged as a powerful tool for capturing 3D tissue information in a more holis-

tic and unbiased manner.^{7,8,13–19} Recent studies have shown that enhanced sampling of heterogeneous tissues, coupled with analysis models based on 3D morphological or molecular features, improves patient prognostication and biomarker discovery beyond what is achievable with traditional 2D histopathology.^{6,8,10,13–18} For instance, whole-tissue imaging has revealed spatially distinct patterns associated with epithelial-to-mesenchymal transition and angiogenesis.¹³ Additionally, a recent deep learning framework further demonstrated that volume-based 3D prognostication outperforms both 2D plane-based analysis and clinical baselines in prostate cancer, underscoring the clinical relevance of 3D histology.¹⁴

Despite its clear potential to improve risk stratification and guide treatment decisions, 3D histology remains underutilized in

intraoperative consultations due to limitations in processing speed. Current 3D histology techniques, through morphological investigation and/or molecular profiling, often rely on labeling protocols that involve lengthy pre-processing, staining, and imaging steps, which typically require several days to complete.^{7,13–15,18,20–22} For example, even the fastest reported workflows take more than 2 days to analyze entire biopsy volumes using light-sheet fluorescence microscopy following small-probe labeling and tissue clearing.^{13,21} Other molecular profiling methods depend on 2D serial sectioning and reconstruction (for example, mass cytometry and cyclic immunofluorescence), making them low throughput and labor intensive for thick samples.^{20,23} By contrast, intraoperative diagnosis demands a turnaround time of 20 to 30 min, which prevents the application of current 3D histology techniques in the operating room (OR).

Label-free imaging is emerging as a promising alternative to conventional histology (Table S1), often termed virtual histology, offering distinct advantages for near real-time assessment of resected tissues.²⁴ Infrared spectroscopic imaging combined with artificial intelligence (AI) enables digital chemical pathology, reducing diagnostic turnaround times and improving patient outcomes.^{25–28} However, this approach is inherently two-dimensional and lacks optical sectioning capabilities. Ultraviolet photoacoustic microscopy (UV-PAM)^{29–31} and microscopy with ultraviolet surface excitation (MUSE)³² have shown potential for histological imaging. Yet, due to the limited penetration depth of ultraviolet light, these methods are restricted to superficial tissue layers. Similarly, quantitative phase imaging (QPI), which leverages refractive index (RI) as an endogenous contrast, is also limited by shallow penetration depth.³³ Optical coherence tomography (OCT) provides depth-resolved imaging,^{34,35} but its limited molecular specificity and insufficient spatial resolution for subcellular structures reduce its utility for detailed pathological evaluation. Autofluorescence imaging and harmonic generation microscopy rely on endogenous signals from biomolecules such as reduced nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide (FAD), collagen, and elastin.³⁶ Nevertheless, these methods often suffer from limited specificity, high background noise, and inter-tissue variability, resulting in reduced accuracy and inconsistent diagnostic performance.

Among label-free modalities, stimulated Raman scattering (SRS) microscopy stands out for its optimal combination of high speed, chemical selectivity, and subcellular resolution.^{37–43} SRS detects the vibrational contrast of chemical bonds, allowing direct visualization of molecular compositions in tissues.^{37,38,44} Two-color SRS microscopy has been used for virtual histology (named stimulated Raman histology [SRH]) by imaging lipid and protein at $\sim 2,850$ and $\sim 2,930$ cm^{-1} , respectively.^{45–47} SRH has demonstrated diagnostic performance comparable to the gold-standard hematoxylin and eosin (H&E) staining.^{45–49} Powered with advanced AI models, SRH enables molecular classification of diffusive gliomas⁵⁰ and detection of glioma infiltration⁵¹ in 2D with fresh surgical tissue, highlighting the potential of SRH contrast as a general-purpose adjunct for guiding brain tumor surgeries. Moreover, due to its nonlinear two-photon excitation, SRS provides good optical sectioning capability comparable to confocal fluorescence imaging.^{37,52} Together, these advantages make SRS an ideal candidate for 3D histology.

Here, we present ULTRA (ultrarapid cleared stimulated Raman with AI), a fast, label-free strategy for 3D histology. ULTRA integrates a rapid tissue-clearing protocol tailored for SRH (ULTRA clearing) and deep learning-based volumetric reconstruction and virtual staining (ULTRA staining) to enable section-free, FFPE-grade (formalin-fixed, paraffin-embedded), millimeter-scale 3D histology within minutes (Figure 1). While the ULTRA framework is broadly applicable to diverse tissue types, this study focuses on its in-depth validation and clinical demonstration in the context of intraoperative glioma assessment, a setting in which accurate margin evaluation remains particularly challenging. Using glioma specimens, we show that ULTRA captures pronounced depth-dependent heterogeneity and resolves diagnostic uncertainty through two representative clinical case studies. By compressing nondestructive 3D histology from hours or days to an intraoperative timescale, ULTRA provides a practical, OR-compatible approach for capturing volumetric tissue architecture and tumor heterogeneity. We anticipate that this capability will enhance intraoperative diagnostic confidence and surgical decision-making in neurosurgery, while also establishing a framework for rapid 3D histology.

RESULTS

Screening for a one-step rapid clearing protocol compatible with SRS

The depth of optical imaging in biological tissues is fundamentally limited by inhomogeneous light scattering, with SRS typically imaging 100–300 μm into tissues.^{52,53} Recent advances in tissue-clearing techniques have enabled fluorescence-based 3D imaging of intact specimens by replacing optically inhomogeneous components with specialized clearing agents that render tissues transparent.^{8,54–56} However, developing a rapid clearing formulation that is compatible with SRS-based 3D histology remains a distinct challenge. Most existing protocols are unsuitable for SRS, as many RI-matching agents generate strong Raman background signals, which can overwhelm the intrinsic vibrational signals from endogenous biomolecules.^{52,57,58} Additionally, current methods are often multi-step and time-consuming, typically requiring several hours to days for complete processing.⁸

To address these issues, we first investigated existing one-step clearing formulations used in fluorescence imaging (Table S2)^{9,59–66} to identify reagents potentially suitable for SRS. Among the candidates, dimethyl sulfoxide (DMSO) emerged as promising due to its role as a penetration enhancer and RI-matching medium.^{59,67} DMSO denatures proteins and facilitates the permeation of both hydrophilic and lipophilic molecules. In addition, its RI ($n_{\text{DMSO}} = 1.479$) closely matches that of proteins ($n_{\text{protein}} = 1.44$) and lipids ($n_{\text{lipids}} = 1.45\text{--}1.48$), making it ideal for both tissue clearing and RI matching.⁶⁷ Additionally, urea, a weak denaturant, was identified for its clearing efficacy—promoting collagen fiber dissociation and enhancing membrane fluidity. Although urea-based aqueous clearing has previously been applied in label-free SRS imaging, it requires over 4 days to clear a 1-mm-thick mouse brain section,⁵⁸ limiting its use for rapid applications.

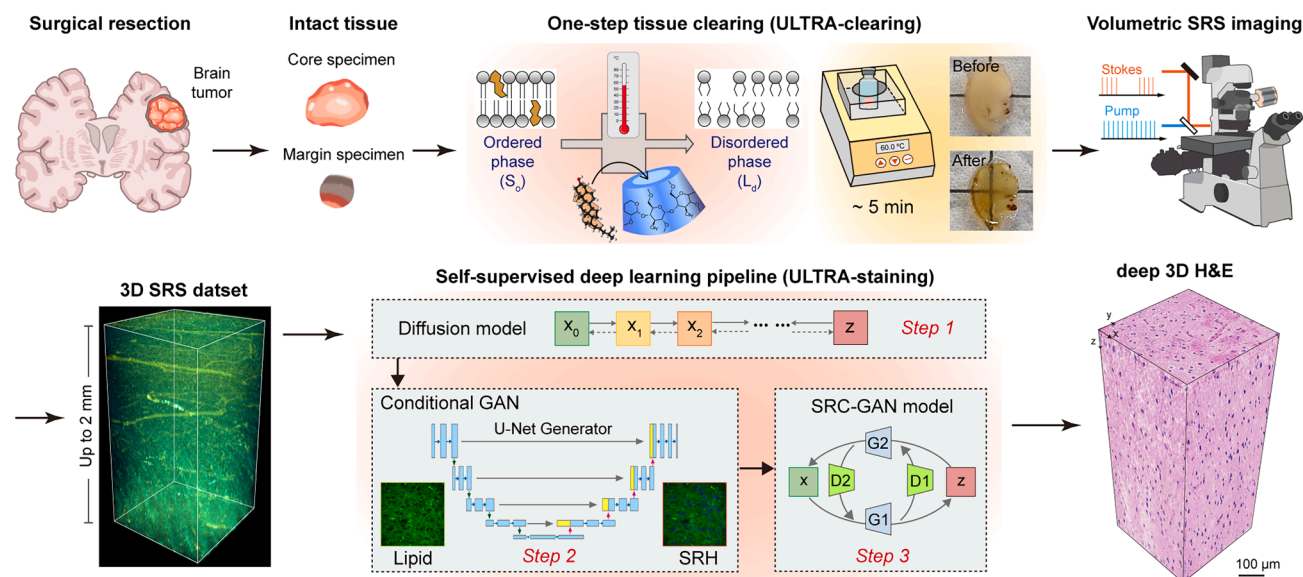


Figure 1. Schematic overview of the ULTRA method for rapid 3D histology

Tissues are first processed with a one-step clearing method (ULTRA clearing) and imaged volumetrically using SRS microscopy at the CH_2 vibrational band ($2,850\text{ cm}^{-1}$). The resulting lipid-channel images are then transformed into FFPE-grade 3D virtual H&E histology using a three-step self-supervised deep learning pipeline (ULTRA staining): a diffusion-model-based depth-aware denoising module, a conditional GAN for predicting protein-channel SRH images, and a modified CycleGAN for virtual H&E staining.

We next examined FOCM and its derivative FOCMS, two one-step clearing recipes that use both urea and DMSO.^{59,68} While urea does not produce background Raman signals in the C–H stretching region (Figure 2A), regular DMSO exhibits strong Raman interference. To resolve this, we substituted DMSO with fully deuterated DMSO ($\text{d}_6\text{-DMSO}$), which shifts its vibrational background away from the target region (Figure 2A). Using a modified FOCMS formulation (30% urea in $\text{d}_6\text{-DMSO}$), we applied the clearing protocol to 1-mm-thick mouse brain sections. After 60 min, the tissue became visually transparent (Figure 2B), and SRS imaging achieved a depth of $940\text{ }\mu\text{m}$, defined by a signal-to-noise ratio (SNR) > 2 (Figure 2C). This streamlined protocol reduced the clearing time from days to just 1 h, representing a significant improvement over conventional urea-based methods.⁵⁸

However, despite improvements in processing speed, this protocol remains insufficient to meet the stringent time requirements for intraoperative histology, where imaging and analysis must be completed within 20–30 min. Further optimization is required to develop a clearing method that is both compatible with SRS and fast enough for intraoperative use.

Development of ULTRA clearing by modulating membrane fluidity

Building on the success of the $\text{d}_6\text{-DMSO}$ /urea protocol, we next aimed to enhance tissue permeability while maintaining maximal chemical preservation, particularly of lipid signals. We hypothesized that by modulating membrane fluidity, the penetration of DMSO and urea into tissues could be improved, thereby accelerating the clearing process. To test this hypothesis, we explored two complementary strategies known to induce membrane disorder: thermal modulation and cholesterol depletion.

First, temperature plays a critical role in regulating the packing of phospholipids within lipid bilayers. Raising the temperature above the phase transition temperature (T_m) of phospholipids introduces conformational kinks in the fatty acid chains, resulting in increased membrane fluidity. In addition, the rate of passive diffusion is temperature-dependent, and elevated temperatures further promote molecular transport. Second, cholesterol contributes to the tight packing of phospholipids, and the human brain is particularly rich in cholesterol. *In vitro* studies have demonstrated that reducing cholesterol content induces a transition to the liquid-disordered (L_d) phase (Figure S1A).⁶⁹ We hypothesize that combining thermally induced membrane disorder with cholesterol depletion establishes a positive feedback loop that enhances membrane permeability and improves tissue-clearing efficiency.

To evaluate the effect of thermally induced membrane disorder, we conducted an orthogonal study by changing either temperature or urea concentration on 1-mm-thick mouse brain sections following a 10-min incubation. While increasing urea concentration moderately enhanced clearing, elevated temperature produced a more pronounced effect (Figure 2D). Notably, two distinct temperature ranges— 25°C – 42°C and 50°C – 60°C —correlated with steep increases in clearing depth. These ranges correspond to the T_m values of two common saturated phosphatidylcholine species: dipalmitoylphosphatidylcholine (DPPC; 16:0, 41°C) and distearoylphosphatidylcholine (DSPC; 18:0, 55°C) (Figure 2E; Table S3). Beyond 60°C , higher temperatures showed minimal additional benefit (Figure 2F), and thus 60°C was selected as the optimal incubation temperature.

To assess whether membrane disorder was indeed enhanced under these conditions, we performed Raman spectral analysis following tissue-clearing treatment with 30% urea in $\text{d}_6\text{-DMSO}$

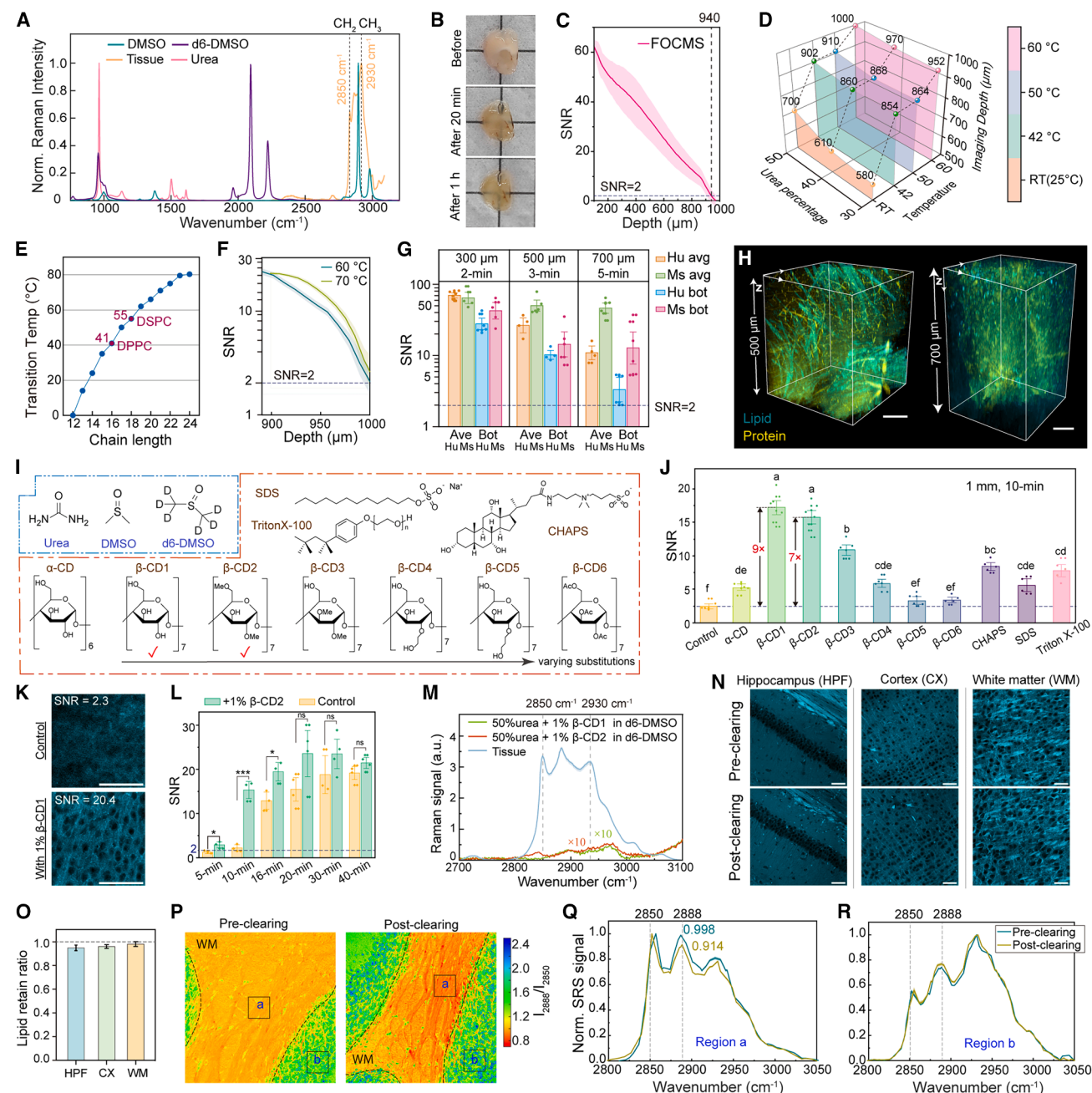


Figure 2. Development of ULTRA clearing for one-step tissue clearing in SRS microscopy

(A) Spontaneous Raman spectra of DMSO, urea, d₆-DMSO, and mouse brain tissue.
 (B) Photographs of 1-mm-thick mouse brain sections before clearing and after FOCMS clearing for 20 min and 1 h.
 (C) Depth-dependent lipid channel SNR in a 1-mm-thick mouse brain section cleared with FOCMS for 1 h.
 (D) Imaging depth achieved under different urea concentrations and temperatures. 1-mm-thick mouse brain slices were cleared for 10 min.
 (E) Correlation between phospholipid phase transition temperature and saturated fatty acid chain length.
 (F) Depth-dependent SNR curves for mouse brain sections cleared with 50% urea at 60 °C or 70 °C for 10 min. Data are mean ± SEM (n = 4).
 (G) Average and bottommost SNRs of mouse and human brain sections with different thicknesses and clearing times. Data are mean ± SD (n = 4, 6, 7, 8).
 (H) Volume-rendered lipid and protein SRS images from cleared mouse and human brain slices.
 (I) Chemical structures of additives used in clearing reagents, including CDs and surfactants.
 (J) Bottommost SNR achieved with different additives in the clearing protocol for 1-mm-thick mouse brain slices. Identical letters indicate no significant difference (p > 0.01).
 (K) Representative lipid-channel SRS images comparing control and 1% β-CD1-treated samples.

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at 60°C. The spectra showed a broadening of the 2,888 cm^{-1} peak and a decreased $I_{2,888 \text{ cm}^{-1}}/I_{2,850 \text{ cm}^{-1}}$ intensity ratio after, consistent with increased membrane disorder^{70,71} (Figures S1B and S1C). Using this protocol, mouse and human brain slices up to 700 μm thick were cleared within 2–5 min, although clearing in human tissue was less efficient (Figures 2G, 2H, S1D, and S1E).

Next, we focused on cholesterol depletion to further enhance tissue clearing. Cyclodextrins (CDs), particularly β -CDs, have been used in protocols like ScaleS and vDISCO to improve tissue permeability and antibody penetration.^{66,72} β -CD is especially effective at extracting cholesterol due to its optimally sized cavity.^{73–75} We screened several β -CD derivatives on 1-mm-thick mouse brain slices following a 10-min incubation. By quantifying the SNR at the bottom of the samples, we identified two derivatives (denoted β -CD1 and β -CD2, structures shown in Figure 2I) that yielded a 7- to 9-fold improvement in clearing performance (Figures 2J, 2K, and S1F). Notably, β -CD derivatives with larger substitutions were less effective, likely due to reduced penetration efficiency within the short treatment time.

Time-course analysis confirmed that β -CD2 significantly accelerated the clearing process (Figure 2L), and a dose-response study showed that 1% β -CD2 ($\sim 8 \text{ mM}$) was sufficient to achieve maximal effect (Figure S1G). Importantly, Raman background analysis indicated that both β -CD1 and β -CD2 maintained signal-to-background ratios (SBRs) > 80 at both 2,850 and 2,930 cm^{-1} , enabling virtually background-free SRS imaging (Figure 2M). By contrast, α -CD, which primarily interacts with phospholipids, and conventional detergents yielded only modest clearing effects, highlighting the critical role of cholesterol removal in enhancing clearing efficiency.

To validate the compatibility of β -CDs with SRS imaging, we confirmed that their use did not alter lipid signals essential for SRH. Comparison of the CH_2 signal at 2,850 cm^{-1} in pre- and post-cleared tissue revealed no significant changes in lipid distribution across subregions (Figure 2N), with $>95\%$ signal preservation observed even after a 1-h clearing treatment (Figure 2O). We further analyzed hyperspectral SRS profiles of mouse cerebellum before and after clearing. Ratiometric images of $I_{2,888 \text{ cm}^{-1}}/I_{2,850 \text{ cm}^{-1}}$, an indicator of alkane chain order, showed reduced signal intensity in white matter regions, which are enriched in cholesterol and myelin (Figures 2P, 2Q, S1H, and S1I). By contrast, granular layer regions, used as a negative control, exhibited minimal spectral changes (Figures 2R, S1H, and S1I). These findings support the hypothesis that ULTRA clearing enhances tissue permeability by inducing membrane disorder while preserving key biochemical signals necessary for accurate SRS-based histological analysis.

In conclusion, we developed a one-step tissue-clearing protocol by harnessing the synergistic effects of thermally and chole-

sterol-depletion-induced membrane disordering. The optimized formulation comprises d_6 -DMSO with 50% urea and 1% β -CD1 or β -CD2, applied at 60°C for 2–10 min depending on tissue type and thickness. ULTRA clearing enables rapid and efficient clearing, preserves authentic SRS contrast, and introduces minimal spectral background, making it well-suited for 3D histology in intraoperative settings.

ULTRA clearing enables rapid and complete transparency of millimeter-scale fixed and fresh tissues

We evaluated the performance of ULTRA clearing across diverse tissue types and conditions. In fixed mouse brain tissue, the protocol cleared 1-mm-thick sections within 10 min, achieving $\text{SNR} > 10$ at the sample bottom (Figures 3A and 3B). Extending the clearing time to ~ 40 min enabled complete transparency of 2-mm-thick sections, allowing label-free SRS imaging from the neocortex to the hippocampus (Figure 3C; Video S1). ULTRA clearing is also compatible with fluorescence labeling, as demonstrated by lectin-based vascular imaging (Figures 3D and S1J; Video S1). Beyond the brain, ULTRA clearing was successfully applied to mouse liver, heart, kidney, and spleen tissues, showing consistent performance across tissues with diverse protein/lipid compositions (Figures 3E and 3F).

To assess its suitability for intraoperative applications, we next applied ULTRA clearing to freshly harvested (unfixed) mouse brain tissue. Despite differences in texture compared with fixed samples, fresh brain tissue was also fully cleared within 10 min using the same protocol (Figure 3G). A direct comparison of clearing performance between fixed and fresh tissues across varying thicknesses revealed comparable levels of transparency and clearing efficiency (Figures 3H and S1K–S1N). In 1-mm-thick fresh tissue, volumetric SRS imaging achieved excellent SNR and subcellular spatial resolution throughout the entire sample depth (Figures 3I, 3J, S1M, and S1N).

Finally, we tested ULTRA clearing on human brain tissue, which poses additional challenges due to the high lipid content of myelin and substantial inter-sample variability. Nonetheless, the same 10-min protocol achieved efficient clearing in all tested human samples, producing high-contrast SRS images (Figures 3K and 3L). These findings highlight ULTRA clearing's potential for 3D tissue visualization during surgery, offering rapid, high-quality histological insights to support timely intraoperative decision-making.

PBDM enables high-quality volumetric SRS image restoration

While SRS imaging has demonstrated great potential in 2D histology—particularly with advances in virtual staining algorithms^{45–47,50,51,76–80}—its application to 3D histological analysis

(L) Bottommost SNR values with or without 1% β -CD2 at different clearing durations. Data were analyzed by two-tailed *t* test. ns, not significant, $p > 0.05$; $^*p < 0.05$; $^{***}p < 0.001$.

(M) Raman spectra of mouse brain slices and clearing solution containing β -CD1 or β -CD2.

(N and O) Lipid-channel SRS images (N) and quantification (O) before and after 1-h clearing of 100- μm -thick mouse brain slices with 1% β -CD2 in 50% urea d_6 -DMSO at 60°C across the hippocampus, cortex, and white matter.

(P) Ratio images of the 2,888/2,850 cm^{-1} signal before and after ULTRA clearing.

(Q and R) Hyperspectral SRS analysis of the region in (P), showing spectral changes after clearing.

Scale bars: 100 μm in (H) and (K), 50 μm in (N).

See also Figure S1.

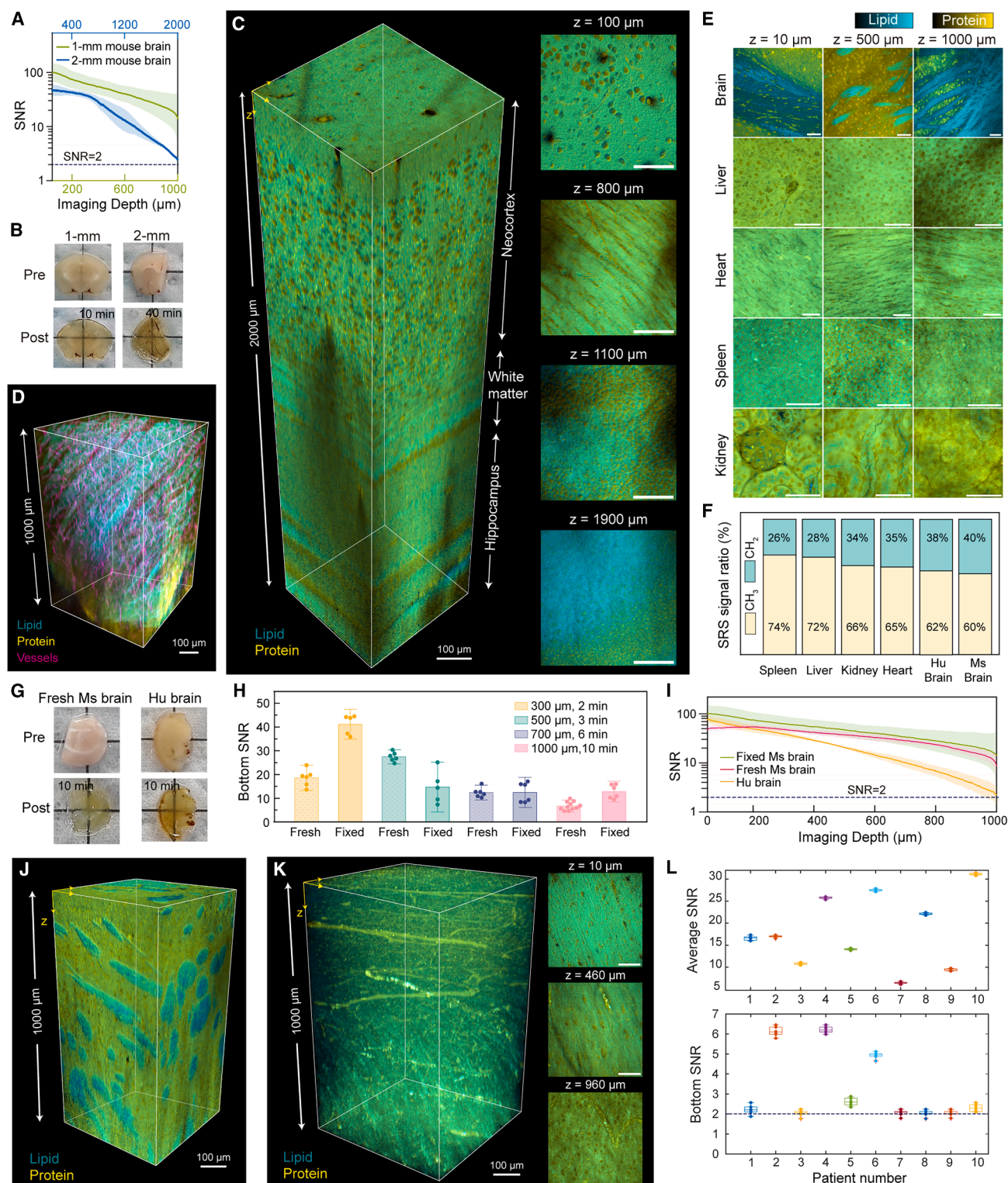


Figure 3. Performance of ULTRA clearing across diverse tissues and conditions

(A) Depth-dependent SNR curve of 1-mm- and 2-mm-thick mouse brain slices after rapid clearing. Data are mean $\pm$ SEM ($n = 4$).

(B) Photographs of 1-mm-thick (10 min) and 2-mm-thick (40 min) mouse brain sections before and after treatment.

(C) Volume-rendered SRS images of a cleared 2-mm-thick mouse brain section.

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remains underexplored. A primary challenge lies in the progressive loss of contrast and spatial resolution with the imaging depth, resulting in inhomogeneous SNRs across the z stack. These inconsistencies introduce artifacts in virtual staining, compromising image quality and reproducibility. Although tissue clearing improves imaging depth, the SRS signal still gradually deteriorates with increasing depth (Figures 3A and 3I), necessitating computational solutions for robust 3D reconstruction.

To address this, we developed a self-supervised deep learning strategy to restore high-resolution, high-SNR images throughout the imaging volume. Diffusion models⁸¹ have recently emerged as state-of-the-art tools for image restoration, offering stable and realistic predictions without excessive smoothing.^{82,83} Here, we adopt a generative diffusion framework to learn a depth-dependent distribution of additive noise. The hierarchical, chain-based structure of the diffusion model is well-suited for capturing the gradual SNR decay (i.e., noise increase) observed in 3D SRS datasets. Once trained, the model operates by iteratively reversing the forward noise-adding process, inferring clean, high-quality images from degraded inputs using the learned noise distribution (Figure 4A). After learning the depth-dependent noise, the model can be applied to restore image quality at arbitrary depths within a 3D image stack (see STAR Methods for details). Importantly, because the model only needs to learn the statistical structure of depth-dependent noise, it can be trained in a fully self-supervised manner using unpaired high-quality SRS images, eliminating the need for paired ground-truth (GT) datasets.

We first trained a conventional diffusion model that uses Gaussian noise on lipid-channel ($2,850\text{ cm}^{-1}$) images from mouse brain tissue. While the model successfully preserved major structures, the reconstructed images exhibited noticeable smoothing and loss of fine detail compared with the GT, which was acquired from the same region of interest (ROI) using higher laser power and longer integration times (Figure 4B). Recognizing that Gaussian noise in diffusion models represents a computational simplification rather than the actual physical noise characteristics of SRS, we hypothesized that a Poisson-based diffusion model (PBDM) would better reflect the shot-noise-dominated nature of SRS signals (see STAR Methods). This noise arises primarily from pump laser fluctuations under radio-frequency modulation. Indeed, PBDM yielded substantially improved reconstructions that closely matched the GT (Figure 4C). Quantitative comparisons based on Pearson correlation coefficients (PCs) and peak SNR (PSNR) further confirmed the superior performance of PBDM over the conventional Gaussian noise model (Figure 4D).

We further evaluated PBDM's performance across input images with varying SNRs. As described above, the imaging depth limit is defined as the depth at which the SNR drops to 2. The trained PBDM model successfully restored images with SNRs as low as 1.8, preserving fine structures and closely matching the GT for both mouse (Figures 4E and 4F) and human (Figures S2A and S2B) brain tissues. PSNR and PC values remained stable across input SNR levels, confirming consistent image quality throughout the imaging depth (Figures 4G and S2C). To assess model stability in the absence of GT, we quantified output uncertainty using the coefficient of variation (CV, the ratio of the standard deviation to the mean) across 10 inferences per real input image (Figure 4H; STAR Methods). The model was considered reproducible when $CV \leq 0.15$, which corresponded to input images with raw SNR ≥ 2 (Figures 4I, S2D, and S2E).

We next applied PBDM to real volumetric SRS datasets from mouse brains. By assigning each depth slice to a specific diffusion timestep based on its raw SNR, the model produced reconstructions with $CV < 0.05$ across a 1.5-mm-thick sample, confirming its robustness for large-volume imaging (Figure 4J). Restored images at different depths demonstrated homogeneous signal levels and near-diffraction-limited resolution, even at the bottom of the specimen, proving the excellent performance of the PBDM (Figure 4K). Additionally, PBDM reduced tissue-clearing time requirements. When applied to a 1-mm-thick brain section cleared for only 5 min, the model produced image quality comparable to that of the standard 10-min-cleared sample (Figures S2F and S2G). These results highlight the effectiveness of PBDM in achieving high-quality restoration of SRS images.

ULTRA staining: A framework for high-quality 3D virtual histology

We next developed a complete framework, termed ULTRA staining, which comprises three sequential deep learning models to transform raw SRS human brain data into high-fidelity, FFPE-grade 3D virtual histology. Step 1 employs the PBDM model to restore image quality across the entire imaging depth (Figure 5A). However, an intrinsic challenge remains. Conventional 2D SRH requires two-color SRS imaging— CH_2 at $2,850\text{ cm}^{-1}$ (lipid dominated) and CH_3 at $2,930\text{ cm}^{-1}$ (protein dominated)—to generate H&E-like stains.^{45–48} Extending this approach to 3D necessitates volumetric acquisition and restoration of both lipid and protein channels, introducing several practical and computational burdens. These may include doubled imaging time (or the need for a dual-pump beam setup), dual

(D) Volume-rendered lipid, protein, and blood vessel images in a 1-mm-thick mouse brain section. Blood vessels were labeled with DyLight 649-conjugated lectin.

(E) Dual-channel SRS images of cleared mouse organ sections (1 mm).

(F) CH_3/CH_2 signal ratios across different tissues.

(G) Photographs of fresh mouse and human brain sections (1 mm) before and after 10-min clearing.

(H) Bottommost SNR values of fresh and fixed mouse brain sections with varying thicknesses.

(I) Depth-dependent SNR profiles of fixed mouse brain, fresh mouse brain, and human brain sections after clearing. Data are mean $\pm$ SEM ($n = 4$).

(J and K) Volume-rendered SRS profiles of fresh mouse brain (J) and human brain (K) sections.

(L) Average and bottommost SNR values of surgically resected human brain specimens (1 mm) from 10 subjects after clearing.

Scale bars, 100 μm .

See also Figures S1 and S7.

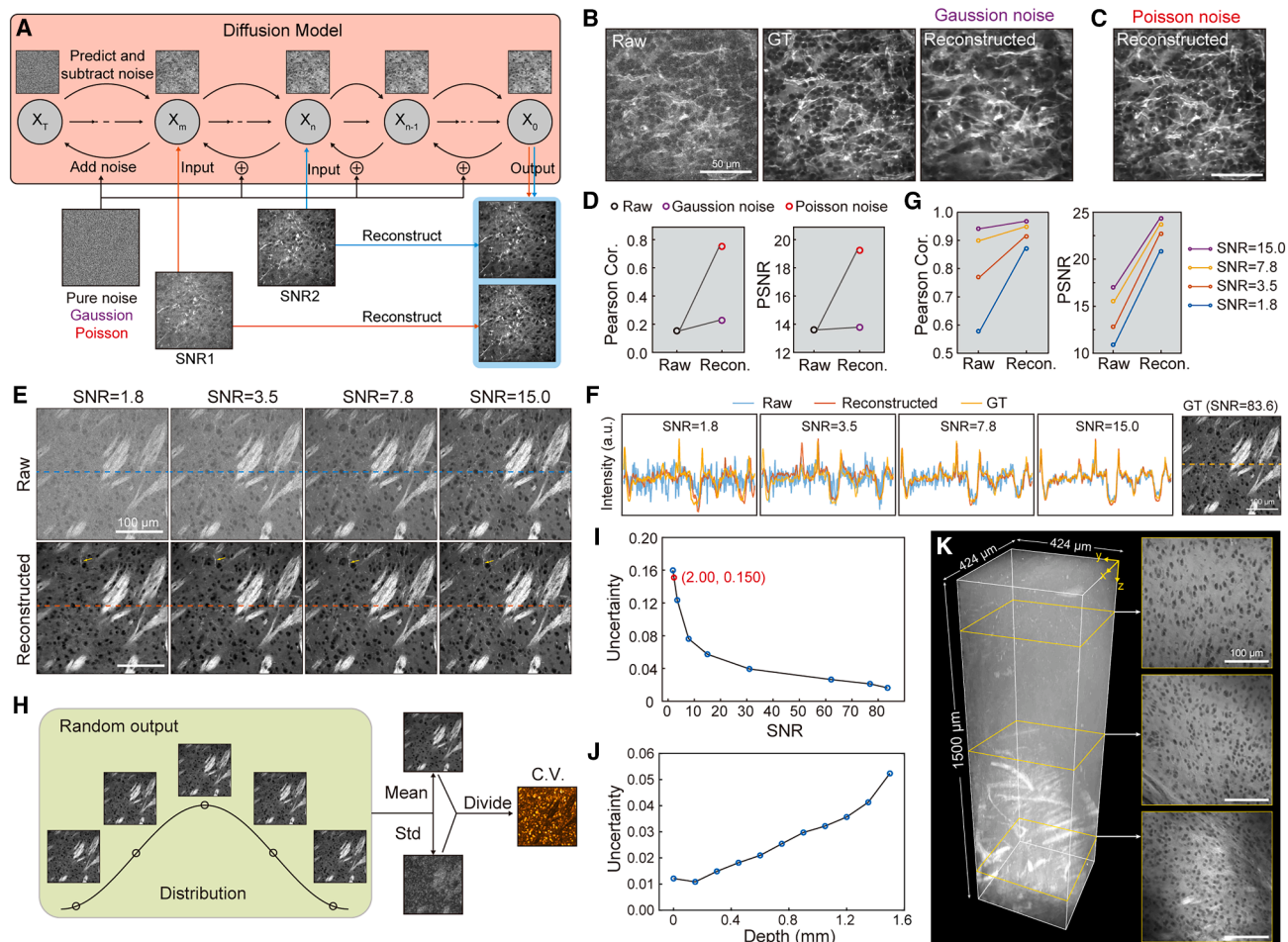


Figure 4. Principle and validation of the PBDM

(A) Schematic of the self-supervised diffusion model framework.
 (B and C) Comparison of diffusion model reconstruction using Gaussian noise (B) or Poisson noise (C), alongside raw and ground-truth (GT) SRS images.
 (D) Quantitative comparison of Gaussian- and Poisson-noise-based diffusion models using Pearson correlation and PSNR relative to GT images.
 (E) Raw SRS images and corresponding PBDM reconstruction under different SNR conditions.
 (F) Intensity profiles along the indicated lines in raw, reconstructed, and GT images.
 (G) PBDM performance across varying raw image SNR levels, evaluated by Pearson correlation and PSNR.
 (H) Conceptual illustration of uncertainty estimation in PBDM.
 (I) Model uncertainty as a function of raw image SNR.
 (J) Depth-dependent model uncertainty in a 1.5-mm-thick mouse brain dataset.
 (K) Volume-rendered PBDM reconstruction of the 1.5-mm-thick mouse brain dataset.
 Scale bars, 50 μm in (B) and (C); 100 μm in (E), (F), and (K).
 See also Figure S2.

diffusion model training, and the accumulation of random errors from independently trained diffusion models.

To address this challenge, we introduced an alternative strategy as step 2 in the ULTRA-staining pipeline. Rather than acquiring two separate channels, we propose that the protein channel can be computationally inferred from the 2,850 cm^{-1} channel to synthesize a virtual 3D SRH volume (Figure 5A, step 2). Although the SRS signal at 2,850 cm^{-1} primarily reflects lipid content, it also contains features associated with proteins. To exploit this, we constructed a conditional generative adversarial network (conditional GAN) with

a U-Net-based decoder architecture for lipid-to-protein channel prediction (Figure 5B, step 2).⁷⁸ The model was trained using paired 2D SRS images (see STAR Methods). We validated the model on 256 independent field of views (FOVs), with representative results shown in Figure 5C. The predicted protein images showed strong agreement with GT protein images acquired at 2,930 cm^{-1} from the same FOVs. Quantitative evaluation using PC and PSNR further confirmed the model's predictive accuracy (Figure 5D), demonstrating its ability to generate high-fidelity SRH images directly from single-channel lipid data.

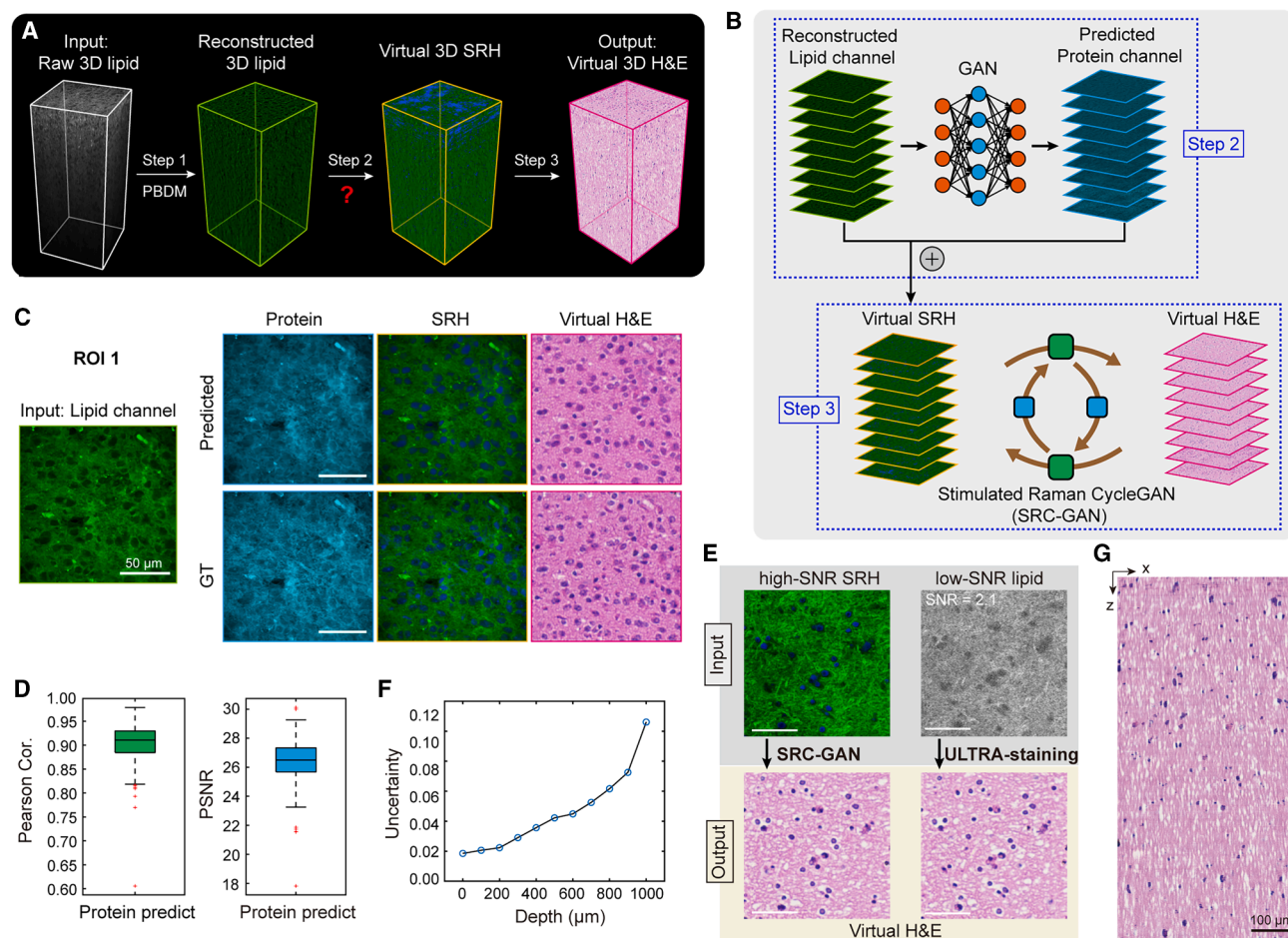


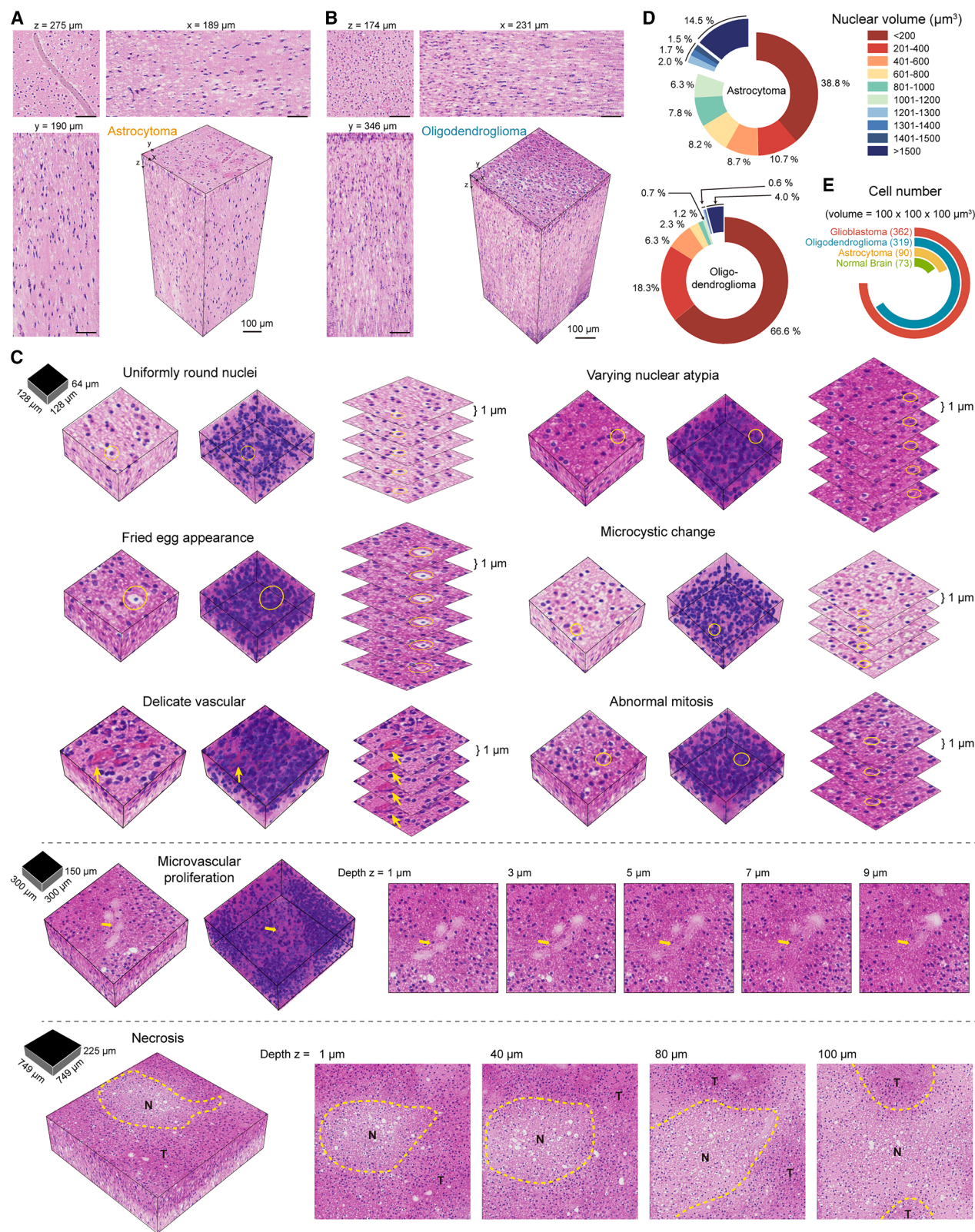
Figure 5. Development of ULTRA staining for high-quality 3D virtual histology

(A) Workflow of ULTRA staining from raw volumetric SRS lipid images to virtual 3D H&E histology.
(B) Schematic of step 2 and step 3 of ULTRA staining: conditional GAN-based protein prediction and SRC-GAN-based virtual H&E staining.
(C) Comparison between predicted and GT images, corresponding SRH images, and derived virtual H&E images.
(D) Quantitative evaluation of protein prediction accuracy using Pearson correlation and PSNR across 400 image groups.
(E) Comparison of virtual H&E images generated from high-SNR SRH data or low-SNR lipid input processed through ULTRA staining.
(F) Depth-dependent uncertainty analysis of PBDM on rapidly cleared human brain tissue.
(G) Reconstructed x-z cross-sectional image of a 1-mm-thick human brain section acquired with 1 μ m z-steps.
Scale bars, 50 μ m in (C) and (E); 100 μ m in (G).
See also Figure S2.

Building on this foundation, we introduced a modified stimulated Raman cycleGAN (SRC-GAN) model as step 3 of the ULTRA-staining pipeline (Figure 5B; STAR Methods).⁷⁹ SRC-GAN is a semi-supervised deep learning model we previously developed to generate FFPE-grade, H&E-style virtual histology from two-color SRH images of fresh surgical specimens.⁷⁹ When applied to both predicted and GT SRH images (Figure 5C), SRC-GAN produced virtual H&E outputs that closely resembled one another (right row in Figure 5C), demonstrating the feasibility of generating H&E-like images directly from single-channel lipid data.

To evaluate the robustness and compatibility of the ULTRA-staining multimodel architecture, we applied the pipeline to SRS lipid images with varying SNRs. The resulting virtual H&E images remained highly consistent across input SNRs down to

~2 (Figure S2H), confirming the stability of the pipeline. Furthermore, virtual H&E images generated from single-channel lipid inputs closely resembled those derived from high-SNR, two-color SRH images processed by SRC-GAN alone (Figures 5E and S2H), demonstrating the reliability of ULTRA staining. We further applied ULTRA staining on real 3D datasets acquired from 5-min-cleared, 1-mm-thick human brain tissues. In step 1, depth-wise uncertainty analysis yielded CV values below 0.11 across the imaging volume, indicating high robustness and reproducibility of PBDM on human brain data (Figure 5F), comparable to results in mouse brain samples (Figure 4). In steps 2 and 3, the reconstructed 3D H&E-style images exhibited continuous and morphologically coherent features along the z axis, supporting the spatial fidelity and stability of ULTRA staining (Figure 5G).



(legend on next page)

ULTRA reveals subtype-specific 3D histological features of gliomas

Gliomas, the most common primary malignant brain tumors, exhibit substantial histological and molecular heterogeneity. Accurate classification and grading of gliomas are critical for prognosis, treatment planning, and clinical decision-making. We demonstrated that the ULTRA platform enables high-resolution 3D visualization of histological features across different glioma subtypes.

We applied ULTRA to 1-mm-thick brain tumor core specimens from patients diagnosed with various glioma subtypes. Direct acquisition of high-resolution 3D virtual H&E images of these tumor cores revealed distinct and rich histological features (Figures 6A and 6B; Videos S2 and S3A–S3C). By analyzing selected regions within the complete 1-mm sample, we identified key 3D histological features for different glioma subtypes. All critical clinical histological markers of gliomas were captured, but now in a true 3D manner (Figure 6C). These included uniform round nuclei, a fried-egg appearance (cytological hallmarks of oligodendrogliomas), nuclear atypia, along with microcystic changes (characteristic features of astrocytoma), delicate vasculature, and abnormal mitosis (critical indicators for glioma grading), as well as microvascular proliferation and necrotic regions (histopathological characteristics of glioblastomas [GBMs]).

Furthermore, 3D unfolding analysis of stacked images provided a comprehensive spatial representation of these features. The 3D morphology of fried-egg-like structures and the distinct nuclear morphological variations were clearly visualized, reinforcing the accuracy and reliability of the results (Figure 6C). In contrast to conventional 2D histology, where critical features may be missed due to sampling bias, ULTRA 3D histology enables complete and unbiased assessment of complex tissue architectures (Figures S3D and S3E).

ULTRA not only captures distinguishing histological features but also enables comprehensive and quantitative characterization of nuclear heterogeneity. Quantitative comparison between ULTRA-generated virtual H&E images and gold-standard H&E sections from matched patient samples yielded highly consistent cell density measurements (Figure S3F), establishing the quantitative reliability of ULTRA. Nuclear volume is a well-established morphological metric that correlates with tumor grade, proliferation, and prognosis.^{84,85} For instance, oligodendrogliomas typically have small, uniform, round cells, whereas astrocytomas exhibit larger, irregular nuclear morphologies.⁸⁶ High-grade astrocytoma often contains enlarged, atypical cells with increased nuclear volume. Using ULTRA 3D histology, we performed 3D nuclear segmentation across all collected specimens. Our analysis revealed that grade 3 astrocytoma ex-

hibited significant nuclear volume heterogeneity, with individual nuclei markedly larger than those in oligodendrogliomas and GBMs (Figures 6D and S3G). By contrast, oligodendroglioma and GBM displayed significantly increased cell densities (Figure 6E), with extensive necrotic regions prominently observed in GBM (Figure 6C). Normal brain tissues, by comparison, showed a markedly sparser cellular distribution and larger nuclear volume compared with tumor tissues (Figures S3A and S3G).

ULTRA facilitates intraoperative identification of 3D tumor infiltrative margins

The primary goal of brain tumor surgery is to maximize tumor resection while preserving critical brain functions. Studies have shown that extended resection improves survival, surgical outcomes, and recurrence rates.^{86–89} Preoperative magnetic resonance imaging (MRI) is essential for surgical planning and navigation⁸⁷; however, the FLAIR (fluid-attenuated inversion recovery)-hyperintense edema region is biologically heterogeneous and cannot reliably distinguish infiltrated tissue from reactive or non-tumor brain tissue (Figure 7A). To mitigate the risk of neurological injury, intraoperative neurophysiological monitoring (IONM) is routinely employed (Figure 7A), but it provides functional rather than histological information and cannot differentiate tumor from normal tissue. In parallel, standard 2D histology offers only limited sampling and lacks the ability to capture tumor invasion in three dimensions, restricting accurate assessment of diffuse glioma infiltration. Together, these limitations highlight the need for high-resolution, intraoperative 3D imaging approaches that can directly assess tumor infiltration, thereby improving margin evaluation, surgical precision, and ultimately patient outcomes.

We demonstrated the feasibility of ULTRA in unveiling the 3D infiltrative margin in GBM. Using a tissue specimen resected intraoperatively from the tumor margin, we applied the ULTRA pipeline to analyze a volume of $\sim 1 \text{ mm}^3$ with a $5 \mu\text{m}$ z-step size (Figures 7B and S4A; Video S3). From planar images, ULTRA's performance on large-volume tissues is comparable to standard FFPE pathology (Figures 7C and S4A). The total processing time, including ULTRA clearing, SRS imaging, and ULTRA staining, was approximately 30 min. This duration is comparable to frozen section analysis while offering vastly richer 3D contextual information. Importantly, this duration is not a fixed requirement but scales with the tissue volume interrogated. When only a localized region or partial depth of a specimen is required for clinical decision-making, imaging time can be reduced substantially, bringing the total workflow into the range of only a few minutes. ULTRA therefore compresses

Figure 6. 3D virtual histology reveals diagnostic features of brain tumor subtypes

(A and B) Volume-rendered and representative cross-sectional virtual H&E images of a 1-mm-thick astrocytoma (A) and oligodendroglioma (B) samples. (C) ULTRA revealed key 3D diagnostic features, including cellular and nuclear atypia, microcystic changes, abnormal vasculature, microvascular proliferation, and necrosis. Left: translucent virtual H&E rendering preserving tissue architecture. Right: minimum-intensity projection highlighting the nuclei distribution. (D) Distribution of 3D cell volumes in astrocytoma and oligodendroglioma samples. (E) Quantification of cell density within $100\text{-}\mu\text{m}^3$ volumes across astrocytoma, oligodendroglioma, and GBM samples. Scale bars, $100 \mu\text{m}$. See also Figure S3.

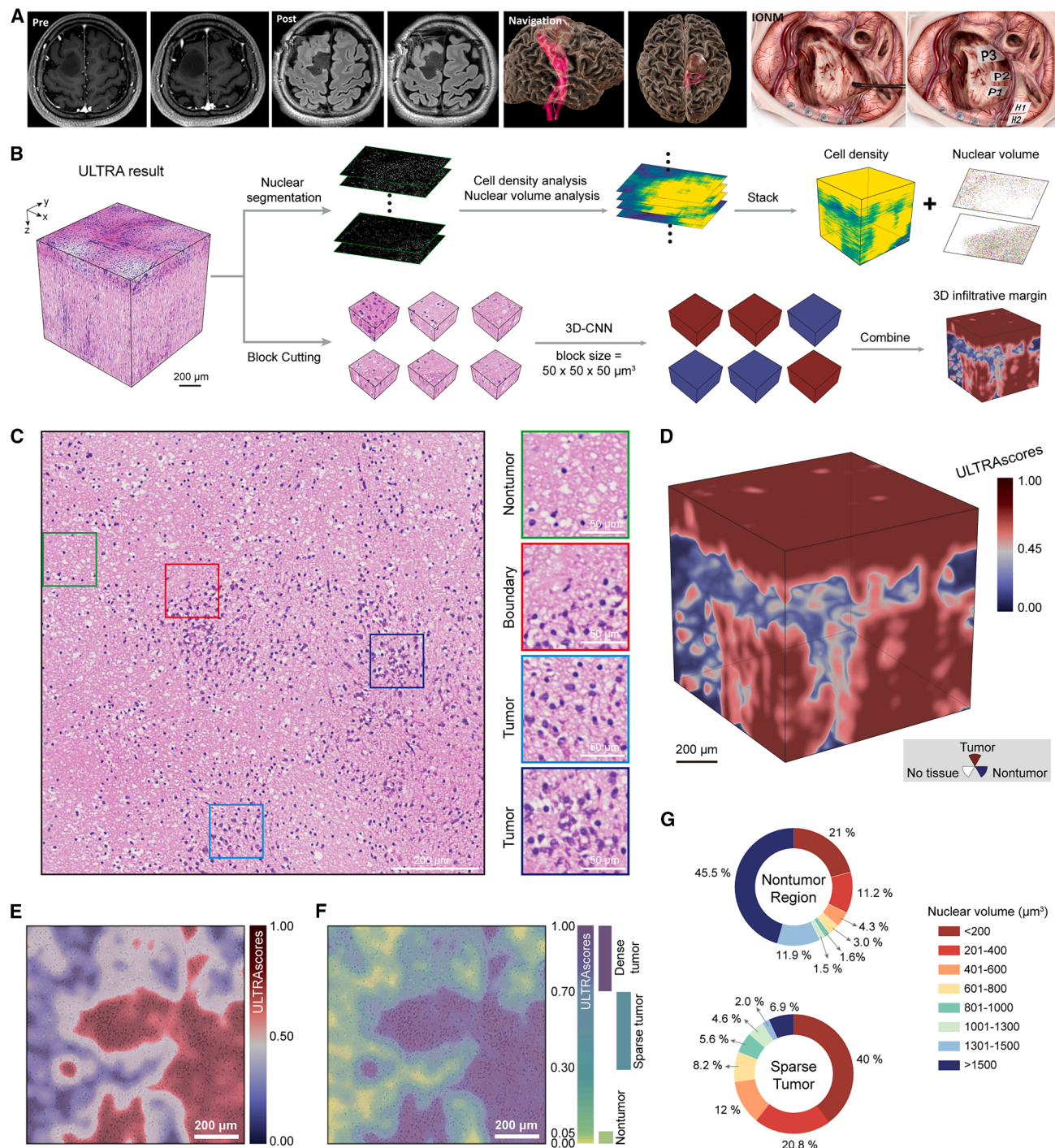


Figure 7. ULTRA enhances the delineation of glioma diffusion boundaries

(A) Schematic of electrophysiological guidance during glioma surgery integrating MRI navigation and IONM.

(B) Workflow for glioma boundary analysis and 3D infiltrative margin segmentation.

(C) Representative single-plane image at a depth of $z = 400 \mu\text{m}$ with magnified views of selected regions.

(D) 3D glioma diffusion margin visualized using ULTRAScore derived from a 3D-CNN classification model.

(E and F) Representative single-plane ULTRAScore maps (E) and 3D segmentation maps (F) overlaid on the ULTRA-derived virtual H&E image.

(G) Cell volume distribution in non-tumor and sparse tumor regions.

Scale bars: $50 \mu\text{m}$ in the right panel of (C), $200 \mu\text{m}$ elsewhere.

See also [Figures S4–S6](#).

nondestructive 3D pathology from hours or days to an intraoperative timescale.

To visualize the 3D margin of GBM, we first segmented the tissue into likely tumor and likely normal regions using cellularity analysis applied to stacked 2D image slices (Figures 7B, S4B, and S4C; see STAR Methods for details). In conventional pathology, increased cell density is one of the first visual indicators of glioma presence,^{84–86} supported by our analysis in Figure 6E. A reconstructed 3D density map (Figure S4D) revealed a gradual transition from high-density tumor regions to infiltrated parenchyma, capturing the hallmark diffuse invasion pattern of GBM. This approach enabled direct 3D annotation of tumor and normal regions (Figures S4E and S4F). Building on this, we trained a 3D convolutional neural network (3D-CNN) model¹⁴ using the cellularity map to infer final labels of the infiltrative tumor margin (Figure 7D). The model outputs a continuous ULTRAScore, representing the probability that a given 3D tissue block contains tumor (see STAR Methods). The full pipeline was implemented as an end-to-end graphical software platform, termed ULTRA-3D Studio.

The resulting ULTRAScore maps, visualized in both volumetric (Figure 7D; Video S3) and planar images (Figure 7E), delineate diffuse and irregular tumor borders and reveal pronounced heterogeneity in tumor distribution across imaging depths (Figures S4G and S4H). Importantly, ULTRA also detected single-cell infiltration at the invasive margin (Figure 7C, right), a level of resolution typically beyond the reach of standard clinical imaging modalities such as MRI. Based on predefined ULTRAScore thresholds, we further segmented the tissue into dense tumor, sparse infiltrative tumor, and normal brain regions (Figure 7F). Notably, nuclear volume distributions measured within the sparsely infiltrative tumor region (Figure 7G) show a tumor-biased but intermediate profile relative to GBM tumor cores and normal brain tissue (Figure S3G), characterized by enrichment of larger nuclei but a lower overall frequency than in the tumor core. This graded nuclear phenotype quantitatively captures the transitional nature of glioma invasion at the margin.

To illustrate the limitations of single-plane assessment, independent pathologists reviewed individual 2D sections from the same volumetric dataset. Both failed to identify the tumor in sections spanning the ~200–350 μ m depth range despite tumor presence in adjacent planes, resulting in miss rates of 20% and 23%, respectively (Figure S4I). These findings highlight the limitations of isolated 2D assessment. By contrast, our infiltration model leverages volumetric histological patterns through a 3D-CNN, and direct benchmarking confirmed superior performance of 3D relative to 2D classification (Figures S4J–S4M). Together, these results demonstrate that the added value of ULTRA derives from access to complete volumetric information.

We further evaluated ULTRA in MRI-defined FLAIR edema regions, where radiographic signal cannot reliably distinguish infiltrated tumor from non-tumor brain tissue. In two specimens collected entirely from the edema zone, ULTRA revealed substantial tumor burden characterized by elevated nuclear density and glioma-associated morphology (Figures S5A–S5D), demonstrating that radiographically ambiguous edema regions may harbor extensive histological tumor infiltration. Notably, these pathological features were heterogeneously distributed across

imaging depths and detectable only at specific z-planes (Figures S5E and S5F), underscoring the risk of false-negative interpretation from limited 2D sampling.

We next evaluated ULTRA in regions affected by navigation drift during the later stages of resection. Two specimens were obtained from non-eloquent cortex that appeared normal on neuronavigation and were negative on neurophysiological monitoring. ULTRA nevertheless revealed clear tumor involvement in both specimens (Figures S6A–S6D). In one specimen, pronounced depth-dependent heterogeneity was observed (Figure S6E). Although intraoperative MRI can partially mitigate navigation shift, its implementation is associated with substantial cost, workflow disruption, prolonged operative time, and increased infection risk. ULTRA therefore provides a complementary specimen-based approach for resolving navigation-related uncertainty intraoperatively.

DISCUSSION

In intraoperative histology, frozen section analysis remains the standard approach, yet it is inherently limited by 2D sampling and technical artifacts such as freezing-induced distortion, imprecise sectioning, and variable staining quality, which together reduce diagnostic accuracy relative to FFPE histology.⁹⁰ Furthermore, intraoperative evaluation typically relies on only two to three tissue sections, increasing the risk of sampling bias and interpretive variability.^{1,3,8} ULTRA overcomes these limitations by providing section-free, stain-free volumetric histology, preserving tissue microarchitecture and restoring the third spatial dimension within an intraoperative time frame. Notably, ULTRA is compatible with brief mild fixation at the time of excision to stabilize tissue morphology, and spatial orientation can be maintained using standard intraoperative marking methods such as ink or suture tags. Morphological distortion is validated to be negligible in brain tissue (Figures S7A and S7B). Together, these features position ULTRA as a practical adjunct to frozen section analysis, offering enhanced tissue coverage, reduced sampling bias, and richer spatial context to support intraoperative decision-making.

We demonstrated the utility of ULTRA in glioma classification, grading, and tumor margin assessment (Figures 6 and 7), highlighting its ability to resolve key 3D histological features of gliomas. The organ-agnostic nature of ULTRA clearing extends its applicability beyond brain tumors^{50,91,92} to various tissue types and diseases where SRH has shown promise, including breast,^{93–96} gastric,^{78,97,98} prostate,^{80,99} and pancreatic¹⁰⁰ cancers, as well as kidney disease.¹⁰¹ This positions ULTRA as a powerful platform for constructing disease-relevant 3D morphological atlases. ULTRA-derived 3D datasets provide rich inputs for training AI models,^{14,102} supporting digital pathology such as automated tumor grading and tumor margin delineation in a 3D manner.

Because ULTRA is nondestructive, it preserves 100% of the tissue for downstream molecular analyses. Macromolecular integrity was validated by RNA analyses (Figures S7C–S7E), immunofluorescence (Figures S7F and S7G), and immunohistochemistry (Figure S7H). Hence, integration with multiplexed protein imaging or spatial transcriptomics (ST) enables

comprehensive morphomolecular profiling. In gliomas, ULTRA can be coupled with 2D or 3D immunohistochemistry to assess key diagnostic markers such as *IDH* mutation, 1p/19q codeletion, and *MGMT* promoter methylation, enhancing classification accuracy. Coupling ULTRA with ST provides a powerful means to map tissue heterogeneity with cell-type specificity.^{1,103–105} Furthermore, recent developments in machine learning-based virtual ST, which infer spatial gene expression from H&E-stained images,¹⁰⁶ offer a cost-effective strategy for generating large-scale 3D transcriptomic maps when integrated with stain-free ULTRA.

Commercial SRS microscopes are now available from multiple companies worldwide (Table S4), and these commercialization efforts have already enabled clinical deployment of SRH in neurosurgical operating rooms.^{76,107} The ULTRA pipeline is fully compatible with existing platforms. Importantly, because ULTRA requires only a single lipid SRS channel, instrumentation costs can be substantially reduced using dual fixed-wavelength laser beams.

Overall, continued technical refinements and more rigorous clinical validation will help pave the way for incorporating ULTRA into standard clinical workflows.

Limitations of the study

First, ULTRA exhibits intrinsic point-spread-function (PSF) anisotropy, which can introduce modest elongation along the *z* axis when imaging structures whose true dimensions are smaller than the axial PSF. AI-based isotropic reconstruction frameworks may help computationally mitigate such PSF-induced distortions.¹⁰⁸ Second, the diffusion-based deep learning model used in ULTRA staining is slow. In some cases, organ- or tissue-specific tuning may be necessary to maximize performance. However, as datasets expand and models generalize, this challenge is likely to diminish. Third, although ULTRA utilizes all cost-effective reagents, the availability of SRS imaging systems is currently limited to research and specialized clinical centers. Broader adoption of ULTRA will benefit from ongoing progress in hardware simplification, cost reduction, and user-friendly interfaces. Fourth, rigorous validation of spatial fidelity across mechanically and biochemically heterogeneous organs remains necessary before extending ULTRA to broader tumor 3D histology.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Lixue Shi (shilixue@fudan.edu.cn).

Materials availability

This study did not generate any new, unique reagents.

Data and code availability

Representative image datasets have been deposited at Zenodo (<https://doi.org/10.5281/zenodo.21132396>). All other raw data generated in this study are available from the lead contact upon request. All original code has been deposited at GitHub (<https://github.com/Zhijie-Liu/Ultrarapid-Deep-3D-Histology>).

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AUTHOR CONTRIBUTIONS

Conceptualization: L.S. and M.J.; methodology: Z.L., Y.L., and L.S.; software: Z.L.; investigation: Y.L., Mingqian Wei, Mian Wei, Z.L., Y.S., and T.W.; resources: L.C. and Z.L.; formal analysis: Z.L., Y.L., L.C., X.L., and H.C.; visualization: Y.L., Z.L., L.S., and L.C.; writing – original draft: L.S.; writing – review & editing: all authors; supervision: L.S. and M.J.; project administration: L.S. and M.J.; funding acquisition: L.S., M.J., and L.C.

DECLARATION OF INTERESTS

L.S. and Y.L. are inventors on several inventions related to ULTRA tissue-clearing methods.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
NeuN	MedChemExpress	Cat# HY-P80241; RRID: AB_3102373
GFAP	MedChemExpress	Cat# HY-P80140; RRID: AB_3102091
Iba1	MedChemExpress	Cat# HY-P80501; RRID: AB_3102574
Myelin Basic Protein	Abcam	Cat# Ab7349; RRID: AB_305869
Human IDH1 R132H (H09)	Dianova	Cat# DIA-H09; RRID: AB_2335716
Ki-67 (RM360)	Dianova	Cat# 31-1246-00; RRID: AB_2783599
Olig2 (Clone 211F1.1)	Sigma-Aldrich	Cat# MABN50; RRID: AB_10807410
Goat anti-Rat IgG H&L (Alexa Fluor® 594)	Abcam	Cat# Ab150160; RRID: AB_2756445
Goat anti-Rabbit IgG H&L (Alexa Fluor® 647)	Abcam	Cat# Ab150083; RRID: AB_2714032
Biological samples		
Gliomas patient brain tissue samples	Huashan Hospital	N/A
Chemicals, peptides, and recombinant proteins		
4% Paraformaldehyde Fix Solution	Sangon Biotech	Cat# E672002-0500
PBS buffer	Sangon Biotech	Cat# E607008-0500
Urea	Sangon Biotech	Cat# A610148-0500
Dimethyl Sulfoxide	HySeal	Cat# A55833
Dimethyl Sulfoxide-D6	Adamas	Cat# 38114L
Methyl-beta-Cyclodextrin	Adamas	Cat# 19185A
Heptakis(2,6-di-O-Methyl)-beta-Cyclodextrin	Adamas	Cat# 61213A
beta-Cyclodextrin	Adamas	Cat# 81437A
alpha-Cyclodextrin	Adamas Life	Cat# 10047G
(2-Hydroxypropyl)-beta-Cyclodextrin	Adamas	Cat# 19184A
Hydroxyethyl-beta-Cyclodextrin	Adamas Life	Cat# 112067A
Triacetyl-beta-Cyclodextrin	Adamas	Cat# 39917F
3-[(3-Chloamidopropyl)dimethyl-ammonium]-1-propanesulfonate	Sangon Biotech	Cat# A600110-0005
Triton X-100	Sangon Biotech	Cat# A110694-0100
Sodium dodecyl sulfate	Sangon Biotech	Cat# A600485-0100
Bovine serum albumin	Sigma-Aldrich	Cat# SRE0096
TRIzol Reagent	Invitrogen	Cat# 15596026
Normal goat serum	Jackson ImmunoResearch	Cat# AB2336990
Deposited data		
Raw and analyzed data	This manuscript	Zenodo: https://doi.org/10.5281/zenodo.21132396
Experimental Models: Organisms/Strains		
Mouse: C57BL/6J	Shanghai Jihui Laboratory Animal Care Co.,Ltd	Cat# SHJH-M-01002
Software and algorithms		
Custom code	This manuscript	Github: https://github.com/Zhijie-Liu/Ultrarapid-Deep-3D-Histology
Custom ULTRA 3D Studio	This manuscript	Github: https://github.com/Zhijie-Liu/Ultrarapid-Deep-3D-Histology/releases/tag/Ultra3D_v1
MATLAB	Mathworks	http://www.mathworks.com/products/matlab/
Python	V3.11.7	http://www.python.org/

(Continued on next page)

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Napari	V0.5.5	https://napari.org/
Numpy	V1.26.4	http://www.numpy.org
Pillow	V10.40.0	https://pillow.readthedocs.io/en/stable/
OpenCV	V4.5.5	http://opencv.org
Pandas	V2.2.2	https://pandas.pydata.org
Matplotlib	V3.9.2	https://matplotlib.org
Pytorch	V2.7.1	https://pytorch.org/
SciPy	V1.13.1	http://www.scipy.org/
Torchvision	V0.22.1	https://docs.pytorch.org/vision/stable/
CuPy	V13.6.0	https://cupy.dev/
PyQt5	V2.4.1	https://pypi.org/project/PyQt5/
Diffusers	V0.34.0	https://huggingface.co/docs/diffusers/index
GraphPad Prism	GraphPad V9.2.0	https://www.graphpad.com/
Origin	OriginLab V9.7	https://www.originlab.com/
Fiji	OpenSource	https://imagej.net/software/fiji/
Other		
Picosecond OPO laser	Applied Physics & Electronics	picoEMERALD
Inverted laser-scanning microscope	Evident (Olympus)	FV3000
Objective	Evident (Olympus)	XLPLN25XWMP2
Confocal Raman microscope	Renishaw	inVia

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Laboratory Animal Resources Center at Fudan University (protocol DSF-2024-004). Wild-type C57BL/6J mice, including both juvenile (3–5 weeks old) and adult (3 months old) animals, were used in this study. Both male and female mice were included, and no sex-associated differences were observed in the experimental outcomes. Mice were housed under specific pathogen-free (SPF) conditions, with up to six animals per cage, in a temperature- and humidity-controlled facility under a regular 12-h light-dark cycle, with ad libitum access to food and water.

Human participants

This study was approved by the Ethics Committee of Huashan Hospital (Approval No. KY2022-762), and written informed consent was obtained from all participants prior to sample collection. Human tissue specimens were collected from patients undergoing surgical resection of brain tumors. A total of 17 patients (8 males and 9 females; age range, 35–75 years) were included in the study. Histopathological diagnoses included astrocytoma (n = 4), oligodendroglioma (n = 5), glioblastoma (n = 5), childhood diffuse hemispheric glioma (n = 1), and low-grade glioma (n = 2).

METHOD DETAILS

Tissue preparation and sectioning

For mouse tissue preparation, mice were deeply anesthetized with isoflurane and euthanized via cervical dislocation. Brains were carefully removed and, where necessary, fixed in 4% paraformaldehyde (PFA) in PBS at 4°C for 24 h. For human tissue, fresh samples were maintained at 4°C and transported to the imaging laboratory within 2 h of resection. Both mouse and human tissue samples were sectioned into coronal slices ranging from 300 μ m to 2 mm (for mice) or 300 μ m to 1 mm (for humans) in thickness using a vibratome (VT1000S, Leica Biosystems).

Vessel staining

Lycopersicon esculentum (tomato) lectin conjugated with Dylight 649 (100 μ L, Vector Laboratories) was administered via tail vein injection into wild-type C57BL/6J mice (2 months old). Ten minutes post-injection, the mice were euthanized by cervical dislocation. Whole brains were harvested and fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at 4°C for 24 hours. Following fixation, the brains were coronally sectioned into 1 mm-thick slices using a vibratome (VT1000S, Leica). For 100 μ m-thick human tissue slices, permeabilization was performed with 400 μ L of 0.3% Triton X-100 for 1 hour at room temperature, followed by

three 5-minute washes with 500 μ L of PBS. Blocking was carried out with 400 μ L of 5% bovine serum albumin (BSA) for 2 hours, followed by three additional 5-minute PBS washes (500 μ L each) and a final rinse with 200 μ L of 2% goat serum. Samples were then incubated with a staining solution containing 10 μ g/mL DyLight 649-conjugated lectin in 200 μ L of 2% goat serum at 4°C for 24 hours in the dark. Post-staining, slices were washed three times with 500 μ L of PBS for 5 minutes each.

Tissue clearing

For tissue clearing, FOCMS reagent was prepared by dissolving 30% (wt/vol) urea in deuterated dimethyl sulfoxide (d_6 -DMSO). Mouse brain slices (1 mm thick) were immersed in this reagent at room temperature and subsequently mounted in the same medium for stimulated Raman scattering (SRS) microscopy.

For brain specimens from both mice and humans with thickness ranging from 300 to 700 μ m, an ULTRA-clearing solution was prepared by dissolving 50% (wt/vol) urea in d_6 -DMSO. Samples were immersed in the clearing solution and incubated in a 60°C metal bath for 2–5 minutes—specifically, 2 minutes for 300 μ m, 3 minutes for 500 μ m, and 5 minutes for 700 μ m sections—followed by mounting in the same solution for SRS imaging.

For thicker brain tissues (1–2 mm), also from mice and humans, an enhanced ULTRA-clearing reagent was prepared by dissolving 50% (wt/vol) urea and 1% β -CD1(hydroxyethyl- β -cyclodextrin) in d_6 -DMSO. Samples were incubated at 60°C for 5–40 minutes, with 5–10 minutes for 1 mm and up to 40 minutes for 2-mm sections. After incubation, tissues were mounted in the same clearing solution for SRS microscopy.

Sample preparation and RNA quality assessment

To evaluate RNA integrity following tissue clearing, freshly dissected whole mouse brains were hemisected along the midline. Human brain tissue blocks were thawed after storage at -80°C for 24 h and divided into two approximately equal pieces. For each specimen (mouse or human), one part was treated with PBS as a control, while the paired part was subjected to the ULTRA-clearing protocol. Total RNA was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. RNA concentration and purity were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). RNA integrity was assessed using an Agilent 5300 Bioanalyzer (Agilent Technologies). Samples with an RNA quality number (RQN) $\geq$ 6.5, an OD260/280 ratio of 1.8–2.2, an RNA concentration $\geq$ 30 ng/ μ L, and a total RNA yield $\geq$ 1 μ g were used for library preparation.

mRNA sequencing library preparation and sequencing

Poly(A)+ mRNA was enriched from total RNA, fragmented, and reverse transcribed to generate strand-specific cDNA libraries following standard procedures. Libraries were quality assessed using a Qubit 4.0 fluorometer (Thermo Fisher Scientific) and sequenced on the DNBSEQ platform (MGI Tech). Library preparation and sequencing were performed by Majorbio Biotech (Shanghai, China).

Histological processing and immunohistochemistry

To evaluate the compatibility of the ULTRA-clearing protocol with standard pathological diagnosis, human brain tumor tissue blocks were divided into two approximately equal pieces. One piece was treated with PBS (control), while the corresponding piece was subjected to the ULTRA-clearing protocol as described above. Following treatment, both the control and ULTRA-cleared tissue samples were fixed in 10% neutral buffered formalin and embedded in paraffin (FFPE) according to standard histological procedures. Sectioning (4 μ m thick) and immunohistochemical staining were performed by the Department of Neuropathology at Capital Medical University using automated stainers. Sections were stained for the following clinical markers: IDH1 R132H, OLIG2, and Ki-67.

Spontaneous Raman spectroscopy

Spontaneous Raman spectra were acquired using an upright confocal Raman microscope (inVia, Renishaw). Samples were excited with a 532 nm laser (50 mW) through a 50 $\times$ air objective (NA 0.5, N Plan, Leica). Raman spectra were collected with an acquisition time of 1 s per acquisition and averaged over 20 accumulations. For tissue samples, spectra were acquired from at least five randomly selected regions and averaged to obtain a representative spectrum.

Stimulated Raman scattering (SRS) microscopy

For the SRS imaging, we utilized an inverted laser-scanning microscope (FV3000, Olympus) designed for enhanced near-infrared (near-IR) transmission. The system was equipped with a 25 $\times$ water-immersion objective (XLPlan N, 1.05 N.A., MP, Olympus) optimized for near-IR light. A picoEMERALD system (Applied Physics & Electronics) provided the necessary synchronized pump and Stokes beams. The pump beam had a tunable wavelength range of 700–990 nm, with a pulse width of 4.3 ps and a repetition rate of 80 MHz. The fixed-wavelength Stokes beam (1032.3 nm, 6.4 ps pulse width, 80 MHz repetition rate) was modulated at 20 MHz by an electronic optical modulator, with a modulation depth of >95%. After passing through the sample, the forward-propagating pump and Stokes beams were collected using a high N.A. oil condenser (N.A. = 1.4). To ensure accurate signal detection, two high O.D. bandpass filters (Semrock FF01-945/SP-25) blocked the Stokes beam and allowed only the pump beam to pass through. This transmitted pump beam was then directed onto a large-area Si photodiode (S3590, Hamamatsu), reversed biased by a 68-V DC supplier (Guwei Electron, G3606D) for detection of the stimulated Raman loss signal. The photodiode's output current

was processed and demodulated by a lock-in amplifier (HF2LI, Zurich Instrument) at 20 MHz for shot-noise-limited sensitivity. Finally, the demodulated signal was fed into the FV3000 software (FV31S-SW, Olympus), where it was used to generate images during the laser scan.

For lipid and protein signal acquisition, the laser power was set as $P_{\text{pump}} = 20$ mW, $P_{\text{Stokes}} = 160$ –200 mW. The time constants of the lock-in amplifier were chosen as 1 μ s. Pixel dwell time was 2 μ s with pixel sizes of about 0.25–1 μ m. For volumetric imaging, the step size in z was 1 μ m or 5 μ m.

For correlative imaging of fluorescence and SRS, fluorescence channel was acquired on the microscope employing a 640-nm continuous-wave (CW) laser for excitation. Following fluorescence imaging, the system was switched to SRS mode to acquire label-free SRS signals.

Quantification of SRS imaging depth

Imaging depth was evaluated using z -series images acquired at the 2850 cm^{-1} Raman peak. Signal-to-noise ratios (SNRs) were quantified across different z -planes using Fiji. For each depth, the average pixel intensity of the image was taken as the signal. Noise estimation was based on a corresponding IR-off image, where the mean intensity defined the baseline offset and the standard deviation represented the noise level. SNR was calculated using the formula:

$$\text{SNR} = \frac{\text{Signal} - \text{Offset}}{\text{Noise}}.$$

In SRS microscopy, photon noise is dominated by the shot noise of the pump beam and is therefore largely independent of the SRS signal intensity under constant excitation conditions. Total noise comprises both photon noise and electronic noise. The maximum valid imaging depth was defined as the deepest z -plane at which the SNR remained ≥ 2 , corresponding to the point where the total noise reached half of the in-focus SRS signal.

Construction of the ULTRA-staining pipeline

Overview: The ULTRA-staining pipeline comprises three sequential deep learning models to transform raw SRS data into high-fidelity, FFPE-grade 3D virtual histology. In Step 1, a depth-aware denoising module based on a generative diffusion model PBDM restores raw lipid images by learning a depth-dependent noise distribution. The forward process is implemented with a U-Net that predicts additive Poisson noise conditioned on imaging depth, while a Denoising Diffusion Probabilistic Model (DDPM) reverses the degradation. This self-supervised framework eliminates the need for external noise modeling and improves signal-to-noise ratio (SNR) across the 3D stack. In Step 2, a deep conditional GAN (cGAN) predicts the protein channel from the restored lipid channel. Trained on 6000 paired lipid–protein image fields, the generator captures nonlinear mapping between modalities, while a fully convolutional discriminator enforces structural realism. In Step 3, a modified Stimulated Raman CycleGAN (SRC-GAN) performs virtual H&E staining by learning bidirectional translation between two-color SRH images and true H&E-stained tissue. A semi-supervised training strategy combining paired and unpaired data enables robust domain generalization. Together, the ULTRA-staining pipeline enables generating H&E-like deep 3D images directly from single-channel lipid volumetric data.

Step 1. Diffusion model for depth-dependent noise restoration.

Preliminary

Diffusion models consist of two phases: a forward process that progressively adds noise to input images from the dataset, and a reverse process that methodically removes the additive noise. By alternating between two steps, diffusion models learn the underlying data distributions and can subsequently generate new samples. In this work, we adopt the generative framework to model a depth-dependent distribution of additive noise that increases with imaging z -position. Once this depth-dependent noise is learned, the model can be used to restore and enhance image quality at arbitrary depths within a 3D image stack.

Additive noise model

Formally, the observed signal at imaging depth z , denoted as $\tilde{x}^z$, is modeled as:

$$\tilde{x}^z = \hat{x}^z + \varepsilon(z)$$

where $\hat{x}^z$ is the underlying noise-free signal, and $\varepsilon(z)$ is the additive noise depending on depth. The noise term is defined as:

$$\varepsilon(z) = \frac{b}{a(z)} * \delta.$$

Here, $a(z)$ is a depth-dependent attenuation function that monotonically decreases with increasing depth z , b is a constant scalar, and δ represents fixed system noise. As depth increases, signal attenuation leads to lower signal-to-noise ratios due to increased additive noise.

To restore the original signal, we aim to estimate the noise term using a learnable function $\varepsilon_\theta(z) \approx \varepsilon(z)$. The denoised signal can be recovered via a simple linear subtraction:

$$\hat{x}^z \approx \tilde{x}^z = \tilde{x}^z - \varepsilon_\theta(z)$$

where $\tilde{x}^z$ denotes an intermediate result.

Diffusion model

In a diffusion model, the forward process adds noise to a clean image x_0 through a sequence of steps indexed by t . This process is formally defined as:

$$x_t = \sqrt{\bar{\alpha}_t}x_0 + \sqrt{1 - \bar{\alpha}_t}\varepsilon$$

where $\bar{\alpha}_t$ denotes the cumulative product of a variance schedule $\{\alpha_1, \alpha_2, \dots, \alpha_t\}$. This formulation is mathematically equivalent to our additive noise model, provided that ε is replaced with a learnable noise function $\varepsilon_\theta(x_t^z)$, where z denotes the depth of the input image. Rewriting the forward process under this assumption gives:

$$x_t^z \sim x_0^z + \frac{\sqrt{1 - \bar{\alpha}_t}}{\sqrt{\bar{\alpha}_t}}\varepsilon_\theta(x_t^z)$$

This implies that a depth-aware forward process can be learned by modeling the distribution of additive noise $\varepsilon_\theta(x_t^z)$ conditioned on the given x_t^z .

Subsequently, the reverse process of the diffusion model can be used to progressively denoise the image. It is defined as:

$$x_{t-1}^z = \frac{1}{\sqrt{\alpha_t}}\left(x_t^z - \frac{1 - \alpha_t}{\sqrt{1 - \bar{\alpha}_t}}\varepsilon_\theta(x_t^z)\right) + \sigma_{t-1}\omega.$$

Where x_{t-1}^z is the denoised estimate at the previous step, ω is random noise sampled from a Poisson distribution, and σ_{t-1} is the noise level at step $t-1$.

Implementation details

Diffusion architecture. We employ a diffusion model to reconstruct raw SRS images acquired from tissue-clearing-enhanced samples into 3D stacks with standardized signal-to-noise ratio (SNR). As a generative model, the diffusion process learns to generate clean images from noise through a Markov chain, where a predefined noise schedule (i.e., a fixed sequence of diffusion coefficients) governs the gradual corruption and recovery of the signal between the original image x_0^z and pure noise $\varepsilon_\theta(z)$.

In our implementation, the forward process is modeled using a U-Net architecture, a fully convolutional neural network, which predicts the depth-dependent noise $\varepsilon_\theta(x_t^z)$ conditioned on the noisy image x_t^z at a given depth z . This enables accurate modeling of noise that varies systematically with imaging depth.

The reverse process is implemented using a simplified Denoising Diffusion Probabilistic Model (DDPM) sampler, which progressively removes the predicted depth-dependent noise from the input. This iterative denoising yields a restored image at each z -position with improved SNR, effectively recovering high-quality 3D histological information.

Training and Novelty. We first trained a neural network to simulate the forward diffusion process by introducing noise into a clean dataset, followed by training the reverse process to systematically match the introduced noise. In the forward process, high-quality SRS images, approximated as noise-free signals, were used as input. The model was trained to predict depth-dependent noise that matches randomly generated ground truth (GT) noise. As both the forward and reverse steps are learned from the data itself, we refer to our approach as a self-learning generative method.

Unlike conventional diffusion models that use Gaussian noise, we adopted random Poisson noise during training. This choice is motivated by the fact that Poisson noise more accurately reflects the inherent noise characteristics of SRS imaging systems, which are dominated by photon-counting statistics. Empirically, we observed that using Poisson-distributed noise improved the fidelity of signal degradation and recovery in the training pipeline.

To justify the substitution of Gaussian noise with Poisson noise, we note that a key requirement for acceleration of diffusion-based models is that the additive property of the noise distribution must be preserved. Specifically, if two independent random variables drawn from the same distribution are added together, the result should also obey that distribution. This property holds for both Gaussian and Poisson distributions:

- Gaussian distribution:

If $X_1 \sim \mathcal{N}(\mu_1, \sigma_1^2)$ and $X_2 \sim \mathcal{N}(\mu_2, \sigma_2^2)$, then

$$X_1 + X_2 \sim \mathcal{N}(\mu_1 + \mu_2, \sigma_1^2 + \sigma_2^2)$$

- Poisson distribution:

If $X_1 \sim \text{Poisson}(\lambda_1)$ and $X_2 \sim \text{Poisson}(\lambda_2)$, then

$$X_1 + X_2 \sim \text{Poisson}(\lambda_1 + \lambda_2)$$

This additive property ensures that Poisson noise is compatible with the probabilistic framework of diffusion models, making it a suitable alternative to Gaussian noise for modeling SRS signal degradation.

Inference.

During inference, a DDPM sampler inputs a noisy image x^z and a sampled diffusion timestep t . The U-Net model then predicts the corresponding depth-dependent noise $\varepsilon_\theta(x_t^z)$. Finally, the signal is progressively restored using the predefined reverse diffusion process, yielding an enhanced image $\hat{x}^z$ with improved signal quality.

To accelerate this process, a Denoising Diffusion Implicit Model (DDIM) is employed, which achieves faster inference with minimal performance trade-offs by effectively skipping steps in the reverse diffusion trajectory. Specifically, DDIM enables inference in fewer iterations by computing multiple steps in one. The update rule for a single DDIM step is defined as:

$$x_{t-n}^z = \sqrt{\frac{\bar{\alpha}_{t-n}}{\bar{\alpha}_t}} \left(x_t^z - \sqrt{1 - \bar{\alpha}_t} \varepsilon_\theta(x_t^z) \right) + \sqrt{1 - \bar{\alpha}_{t-n} - \sigma_{t-n}^2 \varepsilon_\theta(x_t^z)} + \sigma_{t-n} \omega$$

where x_{t-n}^z is the denoised estimate obtained after skipping n steps in the reverse diffusion trajectory, ω is random noise sampled from a Poisson distribution, and σ_{t-n} is the noise level at step $t-n$. By replacing DDPM with DDIM, the inference speed is improved approximately by a factor of n .

Step 2. Deep Conditional-GAN for protein channel prediction.

A deep Conditional Generative Adversarial Network (Conditional-GAN) was employed to predict the protein channel from the lipid channel. This model was trained on a large dataset of paired lipid-protein images (6000 field-of-views) to learn the transformation between the two modalities.

Conditional-GAN consists of two components:

1. Generator-Converts the lipid channel into the corresponding protein channel.
2. Discriminator-Evaluates whether the generated protein channel is realistic or artificially synthesized by the generator.

Generator Architecture

- Uses 3×3 convolutional kernels.
- Accepts a 512×512 input and produces a 512×512 output.
- Comprises three down-sampling layers, three up-sampling layers, and a middle residual structure.
- The residual structure enhances the model's nonlinear mapping capability, improving transformation accuracy.

Discriminator Architecture

- Uses 1×1 convolutional kernels.
- Composed of a fully convolutional network with only down-sampling layers.
- The output size remains 512×512 due to the use of 1×1 kernels.
- The output serves as a judgment mask, which is used to compute the model's loss function during training.

Step 3. 3D H&E virtual staining.

A modified Stimulated Raman CycleGAN (SRC-GAN) architecture is adopted for H&E-style virtual staining, as described in our previous work.⁷⁹ The model comprises two generators and two discriminators that learn the bidirectional mapping between two-color SRH images and corresponding H&E-stained images. The generators are responsible for translating images between the two domains—one converting SRH to virtual H&E and the other converting H&E back to SRH. The discriminators evaluate the authenticity of the generated images in each domain, guiding the generators to produce more realistic and structurally consistent outputs. During training, a semi-supervised learning strategy is employed, consisting of both supervised and unsupervised steps, as described in our previous work.⁷⁹ The training data for virtual staining include two distinct domains: SRH images and corresponding H&E-stained images. In the supervised step, paired data are used to directly guide the translation between domains. In the unsupervised step, unpaired data help the model learn domain characteristics and improve generalization. Once training is complete, the two generators enable bidirectional translation between the SRH and H&E domains, with one generator capable of converting SRH images into virtual H&E-stained images.

Nuclear segmentation of 3D virtual staining dataset

The virtual H&E-stained image stack was imported into Fiji for nuclear segmentation. The Trainable Weka Segmentation plug-in was employed to perform pixel classification. Regions of interest (ROIs) were manually annotated into two categories: nuclei and background. The segmentation model was then trained iteratively, with ROIs adjusted based on segmentation feedback to ensure accurate and complete delineation of cell nuclei. Upon completion, the resulting binary mask stack was saved for downstream analysis.

Nuclear volume statistics of glioma

In each processing iteration, a batch of 100 three-dimensional (3D) binary images, obtained from nuclear segmentation, with a lateral field size of $424.26 \times 424.26 \mu\text{m}$ and a z-step of $1 \mu\text{m}$, is loaded using the tiff file library and then converted into CuPy arrays to leverage GPU acceleration. Connected component analysis is performed with `cupyx.scipy.ndimage.label` to assign unique labels to individual nuclei. To mitigate noise, nuclei with volumes smaller than 60 voxels ($\sim 50 \mu\text{m}^3$) were excluded from the nuclear analysis. This minimum volume threshold serves to suppress small artifacts and non-nuclear signals and is a tunable parameter that can be adjusted depending on the specific applications. The pixel coordinates are transformed into physical units (μm) based on the specified resolution. A triangular mesh representation of each nucleus is generated via the `marching_cubes` algorithm, and volumetric measurements are computed with the `trimesh` library. Computational efficiency is enhanced using `ThreadPoolExecutor` for parallel processing. Finally, the volume distribution of the nuclei is analyzed and reported, and the labeled nuclei are visualized using the `napari` platform (version 0.5.5).

Image visualization

For 3D image reconstruction, attenuation correction was applied using the Attenuation Correction plugin in Fiji, with the morphological opening radius set to 0.5 for 512×512 -pixel frames and 1 for 1024×1024 -pixel frames. After correction, volumetric visualization was performed using `napari` (version 0.5.5). For visualization of the restored lipid and predicted protein channels, a two-color coding scheme was applied to generate stimulated Raman histology (SRH) images. The process included three steps: (1) the predicted protein channel was inferred from the restored lipid channel using a trained deep conditional GAN model; (2) the two channels were merged to form an overlaid SRS image, referred to as SRH. By repeating this process across all image slices, a stacked 3D SRH volume was constructed. (3) the stacked 3D SRH volume was translated into virtual H&E-stained images using a trained CycleGAN model;

3D tumor segmentation and margin identification

The RGB image of the original virtual H&E-stained sample and the binary mask from nuclear segmentation were imported into the analysis pipeline for further processing. A 384×384 -pixel sliding window (with an 8-pixel step size, pixel size = $0.414 \mu\text{m}$) was used to systematically scan the mask image. Within each window, connected components were identified, and the number of valid cells (with an area $> 50 \mu\text{m}^2$) was counted. Based on the resulting cell count matrix, regions containing more than 35 cells per window were labeled as 1 in the binary mask, while all other areas were set to 0. To refine the segmentation, the tumor boundary mask was smoothed using a median filter with a kernel size of 255. For enhanced visualization, regions in the tumor boundary mask with a value of 1 were assigned a pink color (RGB: 242, 84, 116), while regions with a value of 0 were assigned green (RGB: 0, 255, 0), generating an RGB mask. A Gaussian blur with a kernel size of 255 was then applied to soften the mask boundaries. Finally, the blurred mask was overlaid onto the original image with a weight ratio of 0.9:0.2, producing a high-contrast superimposed tumor boundary segmentation image. This 2D analysis-based mask was utilized as annotated labels for the training process below.

Next, a 3D Convolutional Neural Network (CNN) was employed to segment the tumor area of volumetric histology, this model was trained on paired histology blocks and annotated labels (20064 blocks) to learn the spatial feature of tumor.

The 3D CNN consists of three components:

1. Six Convolutional layers.
2. An Adaptive Pooling layer.
3. A Fully Connected layer.

Convolutional layer

- Uses 3×3 convolutional kernels.
- Accepts a $10 \times 120 \times 120 \times 3$ input and produces a $256 \times 1 \times 15 \times 15$ output.
- Comprises six down-sampling layers, six batch normalization layers, three activation layers and three max pooling layers.

Adaptive Pooling layer

- Controls the dimensions of output
- Accepts a $256 \times 1 \times 15 \times 15$ input and produces a $256 \times 1 \times 1 \times 1$ output.

Fully Connected layer

- Gives the result of classification.
- Accepts a 256 input and produces a 2 output.

The model outputs a two-dimensional vector, where the classification is determined by comparing the two elements: if the first element is greater than the second, the corresponding block is labeled as 0; otherwise, it is labeled as 1. To visualize the 3D segmentation, a sliding cube algorithm was implemented. A $10 \times 120 \times 120$ voxel block was moved across the volume with a stride of 24

pixels in the lateral (x–y) directions and 2 pixels in the axial (z) direction. This resulted in each smaller sub-volume of size $2 \times 24 \times 24$ (defined by the stride) being sampled in 125 overlapping blocks. The ULTRAscore for each sub-volume was computed as the average across these overlapping predictions. Finally, colormaps were applied to the ULTRAscore values to generate a spatially resolved 3D visualization of the score distribution. For visualization and segmentation (e.g., dense tumor, sparse infiltration, normal brain), thresholds are applied empirically based on the distribution of scores and corresponding histologic features.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis and data quantification were performed using MATLAB (version 2024b), GraphPad Prism (version 9.2.0.322), Origin 2020 (version 9.7), and Python (version 3.11). Statistical details of experiments can be found in the figure legends, figures, and Results, including the statistical tests used, exact value of *n*. Data are generally presented as mean $\pm$ standard deviation (SD) or mean $\pm$ standard error of the mean (SEM), as indicated in the figure legends. Comparisons between two groups were analyzed using Student's *t*-test, while multiple group comparisons were performed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Statistical significance was defined as **p* < 0.05, ***p* < 0.01, ****p* < 0.001, and *****p* < 0.0001; ns indicates not significant (*p* > 0.05). For imaging-based quantification, a minimum of 10 randomly selected fields of view were analyzed across at least three independent biological replicates.