



Volumetric chemical imaging by clearing-enhanced stimulated Raman scattering microscopy

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Three-dimensional visualization of tissue structures using optical microscopy facilitates the understanding of biological functions. However, optical microscopy is limited in tissue penetration due to severe light scattering. Recently, a series of tissue-clearing techniques have emerged to allow significant depth-extension for fluorescence imaging. Inspired by these advances, we develop a volumetric chemical imaging technique that couples Raman-tailored tissue-clearing with stimulated Raman scattering (SRS) microscopy. Compared with the standard SRS, the clearing-enhanced SRS achieves greater than 10-times depth increase. Based on the extracted spatial distribution of proteins and lipids, our method reveals intricate 3D organizations of tumor spheroids, mouse brain tissues, and tumor xenografts. We further develop volumetric phasor analysis of multispectral SRS images for chemically specific clustering and segmentation in 3D. Moreover, going beyond the conventional label-free paradigm, we demonstrate metabolic volumetric chemical imaging, which allows us to simultaneously map out metabolic activities of protein and lipid synthesis in glioblastoma. Together, these results support volumetric chemical imaging as a valuable tool for elucidating comprehensive 3D structures, compositions, and functions in diverse biological contexts, complementing the prevailing volumetric fluorescence microscopy.

volumetric imaging | stimulated Raman scattering | tissue clearing | metabolic imaging | cancer metabolism

Three-dimensional structures of biological tissues are closely associated with their functions in both health and disease. Representative examples range from neuroscience and cancer biology, to developmental biology: 3D distributions of neurites determine neuronal wiring of the brain (1–6); 3D interactions between tumor cells and their microenvironment influence tumor growth, invasion, and metastasis (7, 8); and 3D organ morphogenesis reveals key mechanisms of embryonic development (9, 10). Therefore, the ability to visualize structures and functions of tissues in 3D is crucial for enhancing fundamental understanding across biomedical disciplines.

Toward this goal, light microscopy presents an appealing tool to noninvasively probe biological processes with subcellular resolution. In particular, fluorescence microscopy is the method of choice for bio-imaging, offering high sensitivity, molecular specificity, and biocompatibility (11–13). However, its imaging depth is limited to superficial layers of tissues due to inevitable light scattering originating from heterogeneous refractive indices (RIs) within tissues. Typically, fluorescence imaging depth in tissues is limited to ~500 μm (1 mm in rare cases), achieved by two-photon excited fluorescence microscopy (14). A number of advanced techniques have been developed to extend this depth limit, including adaptive optics, longer excitation wavelengths, and higher-order nonlinear excitation (15–19). Unfortunately, these techniques usually require complicated instrumentations or procedures, and the achievable depth extensions are often within a factor of two or three.

Recently, a series of tissue-clearing-based fluorescence microscopies have emerged and demonstrated remarkable results toward volumetric visualization of whole organs (20, 21). Compared with instrumentation-based approaches, these sample-centered methods adopt a fundamentally different strategy by reducing light scattering

with active homogenization of tissue RIs (20, 21). Enabled by tissue clearing, volumetric fluorescence imaging provides a window to peer deep into a variety of tissues (e.g., brain, lung, bones) of different organisms in both physiology and pathology (e.g., Alzheimer's disease, cancer) (7, 8, 22–25).

Complementary to fluorescence microscopy in many aspects, stimulated Raman scattering (SRS) microscopy allows for imaging of chemical bonds in biological samples with subcellular resolution. By harnessing quantum amplification via stimulated emission, SRS microscopy offers bond-selective vibrational specificity with high sensitivity (down to micromolars), fast acquisition (up to video-rate), and general biocompatibility, making it a powerful technique for biomedicine (26–29). Compared with X-ray tomography (30), magnetic resonance imaging (31), and positron emission tomography (32), SRS offers higher spatial resolution and unique bond-selective specificity. However, the typical imaging depth of SRS has been limited to ~100 μm inside highly scattering tissues, such as the brain, because of the scattering loss of laser power and overwhelming background (29). In less scattering tissues, the imaging depth is still limited to 300–500 μm . Therefore, extending SRS microscopy to deep volumetric imaging (millimeter range) would provide holistic chemical information in a host of environments relevant to biomedicine.

Inspired by the success of tissue clearing, we devised an SRS-based volumetric chemical imaging method to generate chemical-specific 3D maps deep into tissues. We note that many of tissue-clearing

Significance

Cells form structures and perform functions through intricate 3D tissue organizations. However, due to tissue scattering, coherent Raman microscopy—a powerful method complementary to fluorescence imaging—suffers from limited imaging depth in tissues. Here, we develop a volumetric chemical imaging method with greater than 10-fold depth increase. We formulate a Raman-tailored tissue-clearing recipe and combine it with advanced Raman microscopies. Equipped with the toolbox of volumetric chemical imaging and analyses, we elucidate complex 3D structures, chemical compositions, and metabolic dynamics in diverse tissues including lipid synthesis throughout tumor spheroids, 3D networks of axons, vasculatures, and cell bodies in brain regions, as well as heterogeneous tumor structures and tumor metabolism.

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methods developed for volumetric fluorescence imaging alter the tissue composition, and are thus unsuited for comprehensive chemical imaging. Therefore, we identified two Raman-specific criteria, developed a Raman-tailored tissue-clearing strategy, and coupled it with SRS microscopy, including its advanced variations, such as multispectral imaging and deuterium oxide (D_2O) probing coupled with SRS (DO-SRS) of metabolic probes (33) (Fig. 1A). Our method achieves a greater than 10-times depth increase compared with the standard SRS. We further developed a volumetric phasor analysis for 3D clustering and segmentation of multispectral SRS images. With this whole toolkit, we reveal complex 3D tissue structures, compositions, and functions in the context of neurobiology and cancer biology.

Results

Development of Raman-Tailored Reagents for Clearing-Enhanced SRS Imaging. To develop a clearing recipe tailored for SRS microscopy, we identified two Raman-specific criteria that differ from those for volumetric fluorescence imaging. First, the tissue-clearing chemicals should achieve certain “Raman transparency,” defined as the ability to generate optically transparent tissues without introducing interfering Raman signatures in the designated vibrational windows. While a general method to create transparency across the entire Raman spectrum, including the fingerprint region, would be rather challenging, herein we focus on two important spectral windows: the high-wavenumber C-H region ($2,800\text{--}3,000\text{ cm}^{-1}$) and the cell-silent region ($1,800\text{--}2,600\text{ cm}^{-1}$). The vibrational signatures for lipids and proteins are in the high-wavenumber C-H region, repre-

sented by CH_2 ($2,845\text{ cm}^{-1}$) and CH_3 ($2,940\text{ cm}^{-1}$) bands, respectively (34, 35) (Fig. 1B). The interplay between proteins and lipids has important implications in cancer (36, 37) and Alzheimer's disease (38, 39). For the cell-silent window, many emerging vibrational tags reside in this region (40–44), allowing imaging of metabolic dynamics of small metabolites and also super multiplexed imaging (45, 46). Hence, our first criterion requires the clearing reagents to make tissues optically transparent without introducing Raman peaks in the CH region or the cell-silent region. Second, the native chemical structures of examined tissues should be largely preserved after tissue clearing. Here the clearing process should maintain the integrity of both proteins and lipids. This is a rather challenging requirement because many clearing methods [including almost all organic-solvent and hydrogel-embedding-based methods (20, 21)] are optimized for imaging specific proteins by removing lipids to reduce the heterogeneity of RIs.

Based on the two Raman-specific criteria, we developed a clearing recipe for Raman microscopy. We examined existing optical-clearing reagents and identified urea as a promising candidate. In fact, urea is the central component in *Scale/ScaleS* (2, 47). In terms of our first criterion about Raman transparency, urea shows no Raman peaks in a broad window from $1,800$ to $3,000\text{ cm}^{-1}$ (*SI Appendix, Fig. S1A*) by virtue of its unique structure containing only N-H bonds, but no C-H bonds. In contrast, several common clearing reagents—dibenzyl ether [3DISCO (22)], sorbitol [ScaleS (47)], and fructose [SeeDB (5) and FRUIT (48)]—show strong Raman peaks in the CH stretching region ($2,800\text{--}3,000\text{ cm}^{-1}$) (Fig. 1C), causing severe background issues. To examine the second criterion about maintenance of tissue integrity, we adopted the spontaneous Raman

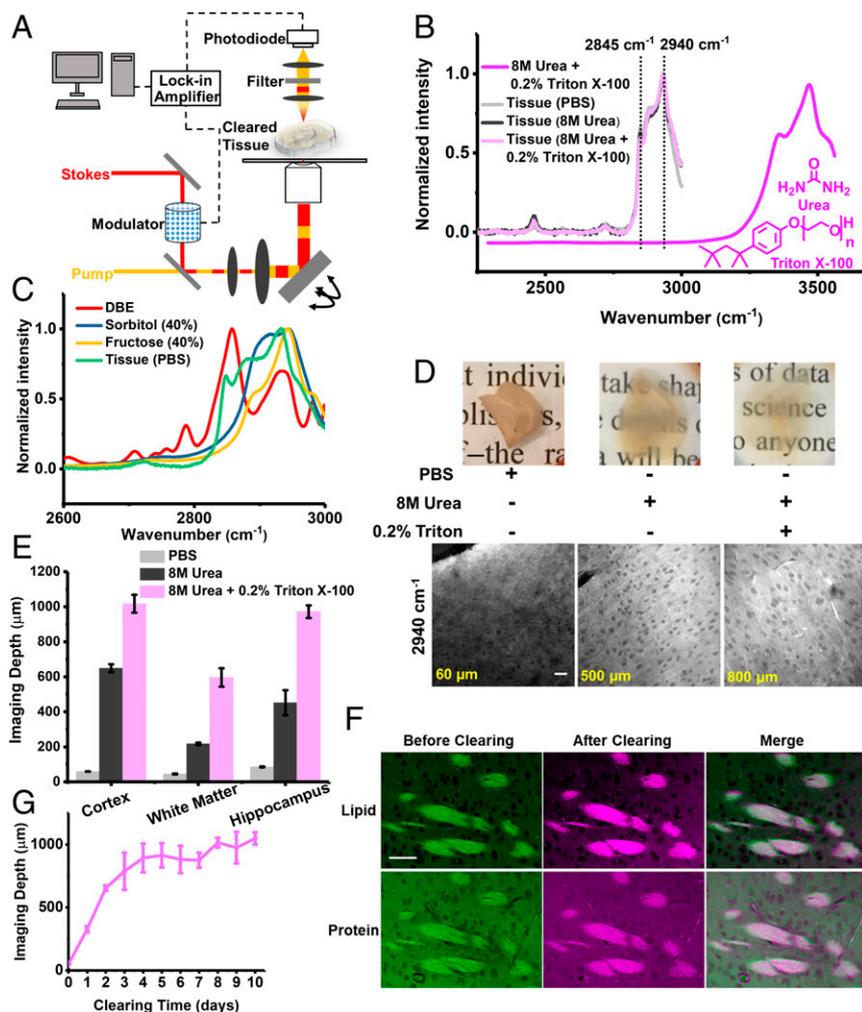


Fig. 1. Development of clearing-enhanced SRS microscopy. (A) Set-up for clearing-enhanced SRS microscopy. (B) Spontaneous Raman spectra of 8 M urea and 0.2% Triton X-100 (dark magenta), mouse brain slices in PBS (light gray), slices cleared with 8 M urea (dark gray), and slices cleared with 8 M urea solution with 0.2% Triton X-100 (light magenta). (C) Spontaneous Raman spectra of dibenzyl ether (red), 40% sorbitol solution (blue), 40% fructose solution (yellow), and mouse brain slices in PBS (green, the same as B). (D) Photos and the corresponding SRS images ($2,940\text{ cm}^{-1}$) close to depth limit (60, 500, and $800\text{ }\mu\text{m}$) of 1-mm-thick brain slices in PBS, cleared with 8 M urea, and with 8 M urea and 0.2% Triton X-100, respectively. [Scale bars: 2 mm in the photos (Upper), 50 μm in the SRS images (Lower).] (E) Quantification of label-free imaging depths for different brain regions in PBS, cleared with 8 M urea, and cleared with 8 M urea and 0.2% Triton X-100. Error bars indicate SEM ($n = 4, 6,$ and 4 for cortex, white matter, and hippocampus in PBS. $n = 4, 3,$ and 3 for cortex, white matter, and hippocampus in 8 M urea. $n = 3$ for each brain region in 8 M urea and 0.2% Triton X-100). The imaging depth limit was defined as the depth with signal-to-background ratio of 0.5. (F) SRS images of regions in the same white matter region before and after tissue clearing (8 M urea with 0.2% Triton X-100). (Scale bars, $50\text{ }\mu\text{m}$.) (G) Quantification of imaging depth with clearing time (8 M urea with 0.2% Triton X-100). Error bars indicate SD ($n = 3$ for each data point).

spectrum as a benchmark because it is highly sensitive to chemical structures. We observed minimum change of characteristic Raman peaks in the CH stretching region for tissues incubated in 8 M urea for 4 d (Fig. 1B, dark gray) compared with those in PBS (Fig. 1B, light gray), indicating preservation of proteins and lipids. Our results agree well with a previous report that urea can preserve the integrity of both protein and lipid ultrastructures at the resolution of electron microscopy (47).

After validating the two Raman-specific clearing criteria, we verified that a high concentration of urea (8 M) was capable of rendering tissues substantially less opaque than the control (Fig. 1D). This effect is likely due to the small size of urea that allows it to penetrate, partially denature, and hydrate the hydrophobic core of high-refractive index proteins (2, 20, 21, 47). We then performed label-free SRS imaging on brain tissues cleared with urea. Indeed, we did not observe background interference from high-concentration urea in either band ($2,845\text{ cm}^{-1}$ or $2,940\text{ cm}^{-1}$). We defined the SRS imaging depth limit as the depth at which the signal-to-background ratio of SRS images at $2,940\text{ cm}^{-1}$ reaches 0.5, below which imaging contrast became too low. By this measure, we found that urea extended the SRS imaging depth for ~ 5 – 10 times at different brain regions (Fig. 1E). Representative SRS images ($2,940\text{ cm}^{-1}$) of 1-mm-thick brain slices are shown in Fig. 1D at depths close to the defined imaging depth limits: $60\text{ }\mu\text{m}$ for the control slice in PBS and $500\text{ }\mu\text{m}$ for the slice treated with 8 M urea. This clearing solution of 8 M urea is well suited for volumetric chemical imaging of multicellular spheroids (Fig. 2).

Improving Raman-Tailored Clearing for Volumetric SRS Imaging. To improve Raman-tailored clearing for imaging deep tissues, we asked whether adding auxiliary reagents could further improve clearing performance. We found addition of 0.2% Triton X-100, a typical detergent, allows substantial depth extension with well-preserved features (axon fibers and cell bodies) in brain tissues (Fig. 1D). This is perhaps due to increased membrane permeability effected by Triton X-100. Note that 0.2% Triton X-100 alone has only marginal effect on imaging depth (*SI Appendix, Fig. S2*).

We next validated the fulfillment of two Raman-clearing criteria with 8 M urea and 0.2% Triton X-100. First, 0.2% Triton X-100 introduced no extra Raman background at the CH imaging-window (Fig. 1B, dark magenta). Second, we found minimum changes of characteristic Raman peaks in the CH region (Fig. 1B, light magenta). We further confirmed the distributions of proteins and lipids in tissues were preserved after clearing, using SRS imaging at different brain regions. The SRS images of the same regions aligned well with each other at the subcellular level before and after clearing (Fig. 1F and *SI Appendix, Fig. S3*). We found a less than 7% increase in protein/lipid ratio comparing the same regions before and after clearing (*SI Appendix*). As a negative control, treating tissues with a high concentration (15%) of Triton X-100 or the 3DISCO method clearly disrupted the lipid structure (4, 22), as indicated by the attenuation of the lipid peak at $2,845\text{ cm}^{-1}$ (*SI Appendix, Figs. S4A and S5A*) and the disappearance of lipid-rich structures (nerve tracts and axon fibers) (*SI Appendix, Figs. S4B and S5B*). While this recipe of 8 M urea and 0.2% Triton X-100 preserved proteins and lipids in tissues, it removed lipids from multicellular spheroids (*SI Appendix, Fig. S6*), possibly because compact tissue structures are more resistant to Triton X-100. Therefore, the basic recipe of 8 M urea is the method of choice for volumetric chemical imaging of spheroids (Fig. 2), while the improved recipe of 8 M urea and 0.2% Triton X-100 was used for tissue samples.

Clearing-Enhanced SRS Imaging with Greater than 10 Times Depth Extension. We then confirmed the optimal clearing time of the improved recipe (8 M urea and 0.2% Triton X-100) on 1-mm-thick coronal brain slices (Fig. 1G and *SI Appendix, Fig. S7*). The clearing effect plateaued after a 4-d incubation (Fig. 1G). As reported previously (2), we observed a slight volume expansion of cleared brain tissues with an average expansion factor of 1.2 ± 0.1 (*SI Appendix*). Such a degree of expansion would allow moderately higher imaging resolution, as is harnessed in expansion microscopy (49).

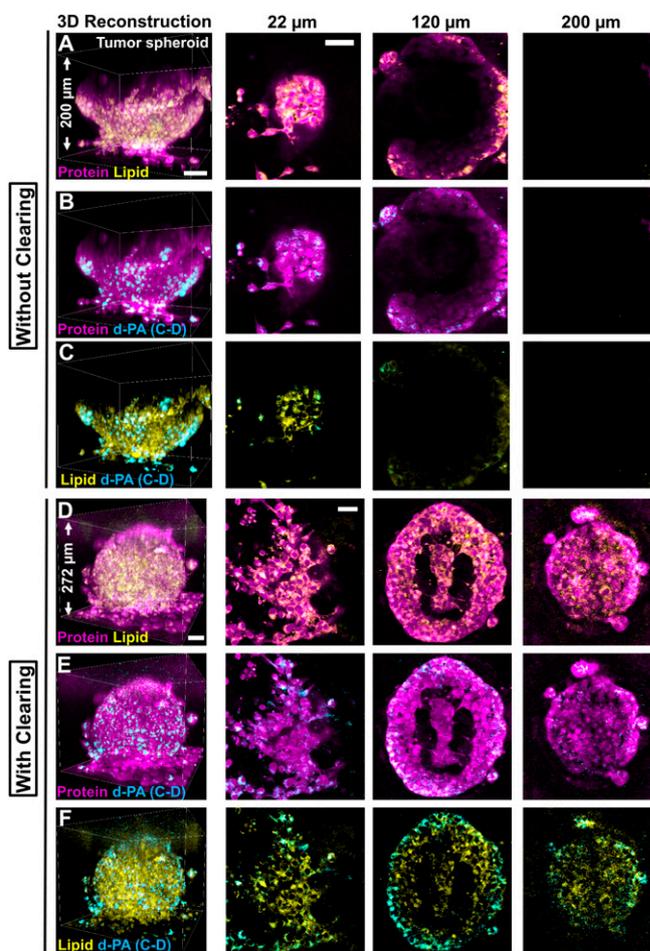


Fig. 2. Clearing-enhanced volumetric chemical imaging of 3D tumor spheroids. (A–C) Three-dimensional reconstructions and representative 2D images (at $z = 22\text{ }\mu\text{m}$, $120\text{ }\mu\text{m}$, and $200\text{ }\mu\text{m}$) of a tumor spheroid of MCF-10A^{Hras} breast cells without tissue clearing. (D–F) Three-dimensional reconstructions and representative 2D images (at $z = 22\text{ }\mu\text{m}$, $120\text{ }\mu\text{m}$, and $200\text{ }\mu\text{m}$) of a tumor spheroid of MCF-10A^{Hras} breast cells with tissue clearing. Protein (magenta) and lipid (green) channels are overlaid in A and D. Protein (magenta) and the d-PA (cyan) channels are overlaid in B and E. Lipid (green) and d-PA (cyan) channels are overlaid in C and F. (Scale bars, $50\text{ }\mu\text{m}$.)

We next determined the depth extension of clearing-enhanced SRS imaging. Remarkably, our improved Raman-tailored clearing recipe increased the depth limit of SRS imaging by 17.2-fold (13.8-fold with correction for expansion) in the cerebral cortex, 13.2-fold (10.6-fold with correction) in the white matter, and 11.4-fold (9.1-fold with correction) in the hippocampus (Fig. 1E). The different depth extensions likely originated from varied chemical compositions among these brain regions. For example, the white matter has more lipids than the cortex and the hippocampus, leading to higher RI heterogeneity, severer scattering, and thus a shallower imaging depth. In summary, we have achieved label-free, dual-color volumetric chemical imaging approximately 1-mm deep in highly scattering tissues.

Clearing-Enhanced SRS Imaging of Whole Tumor Spheroids. With the recipe of 8 M urea, we aimed to achieve volumetric imaging of multicellular tumor spheroids. Tumor spheroids are widely used in vitro models of solid tumors. Compared with 2D cultures, tumor spheroids are considered to better mimic the 3D tumor microenvironment and are thus increasingly used to study tumor cell physiology and response of tumors to radiotherapy and chemotherapy in a 3D multicellular context (50, 51). In addition to the two-color label-free imaging, we further introduced a third

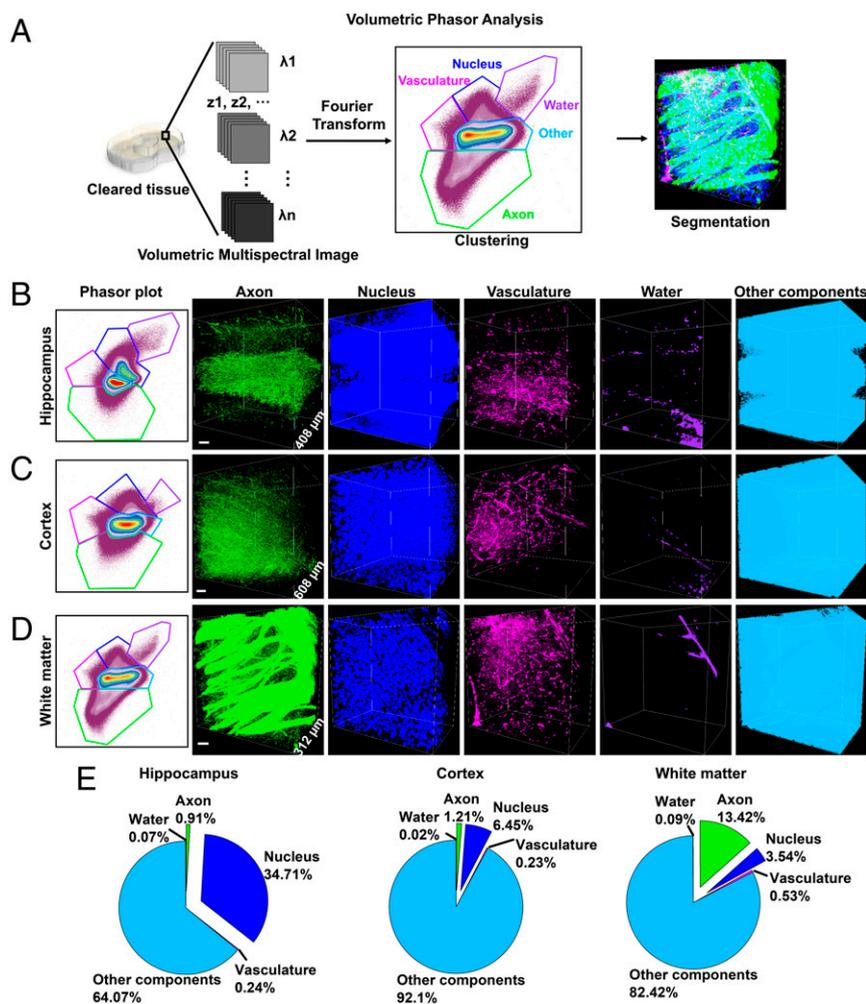


Fig. 4. Volumetric phasor analysis. (A) Schematic process of volumetric phasor analysis. Volumetric phasor analysis provides segmentation of various regions in mouse brains: the hippocampus (B), the cerebral cortex (C), and the white matter (D). For B–D, the phasor plot is shown (Left) and followed by volumetric images of segmented species. “Other components” include extracellular matrix and cellular structures that are not resolved by SRS. (E) Volume fraction of tissue components in the hippocampus (B), the cerebral cortex (C), and the white matter (D). (Scale bars, 50 μm .)

volume to phasor points on a single phasor plot (Fig. 4A). Therefore, clustering and segmentation is performed collectively for the entire imaging volume, resulting in systematic and holistic segmentation of tissue components.

We demonstrated volumetric phasor analysis on mouse brain tissues. To implement this analysis, we first acquired volumetric multispectral images by taking z-stack images across 13 different wavenumbers covering 2,810–3,090 cm^{-1} (SI Appendix). Second, we generated a reference phasor plot by selectively cropping out individual regions of axons, vasculature, nuclei, or extracellular matrix and transforming these regions onto a phasor plot. Indeed, each structure was identified as a separable cluster on the reference phasor plot (SI Appendix, Fig. S9). Third, we transformed the whole stack of volumetric multispectral images onto a phasor plot (Fig. 4B–D) and clustered this phasor plot according to the reference phasor plot. Fourth, we projected these clusters back to the volumetric image and evaluated the clustering results. As shown in Fig. 4B–D and SI Appendix, Fig. S10, we reliably segmented axons, nuclei, vasculature, water, and other components (including extracellular matrix and cellular structures that were not resolved by SRS) in the cerebral cortex, the hippocampus, and the white matter. The volume fractions of tissue components showed that the hippocampus contained more nuclei densely packed in the dentate gyrus than the cortex and the white matter, while the white matter contained more axons as nerve tracts than the other two brain regions (Fig. 4E). These results indicate volumetric phasor analysis achieves rigorous, chemically specific segmentation for a large number of species in 3D.

Clearing-Enhanced SRS Imaging of Tumors with Volumetric Phasor Analysis.

We applied the two-color label-free volumetric imaging to capture 3D chemical information in tumor xenografts. Glioblastoma multiforme is the most aggressive brain cancer with a median survival time of ~ 15 mo from diagnosis (55). To investigate its 3D structure, we performed large-area mosaic imaging up to 700 μm into the glioblastoma xenograft in mouse brain (Fig. 5A). The high-protein, low-lipid distribution of the tumor allowed delineation of tumor margins (Fig. 5B) (36, 37). Single-plane images at a series of depths through the same tumor (Fig. 5A, C, and D and SI Appendix, Fig. S11) revealed distinct features of normal tissues, infiltrating glioma, and dense glioma (36, 37). Dense glioma has a high level of proteins and a low level of lipids. In contrast, normal brain tissues are lipid-rich, containing axon fibers and low density of cell bodies. Infiltrating glioma at the tumor margins (Fig. 5C and D, arrows, and SI Appendix, Fig. S11B and C) show lipid-rich areas of brain parenchyma with clusters of infiltrating glioblastoma cells. We validated this method of tumor demarcation by immunolabeling with a human-specific vimentin antibody, which specifically stained human-sourced tumor cells but not mouse cells (SI Appendix, Fig. S12). The z-stack results indicate the margins of the tumor differ at different imaging depths, as the size of the tumor gradually expands with more complex structures from 200 to 700 μm (Fig. 5A–D and SI Appendix, Fig. S11). We mapped out the protein/lipid ratios ($S_{\text{protein}}/S_{\text{lipid}}$, the signal of the protein channel over that of the lipid channel) of the whole coronal slices as a function of depth (Fig. 5E and F and SI Appendix, Fig. S13). This single parameter indicates chemical heterogeneity, both between tumor and normal tissues and within tumor tissues. These results render volumetric chemical imaging a capable tool for capturing

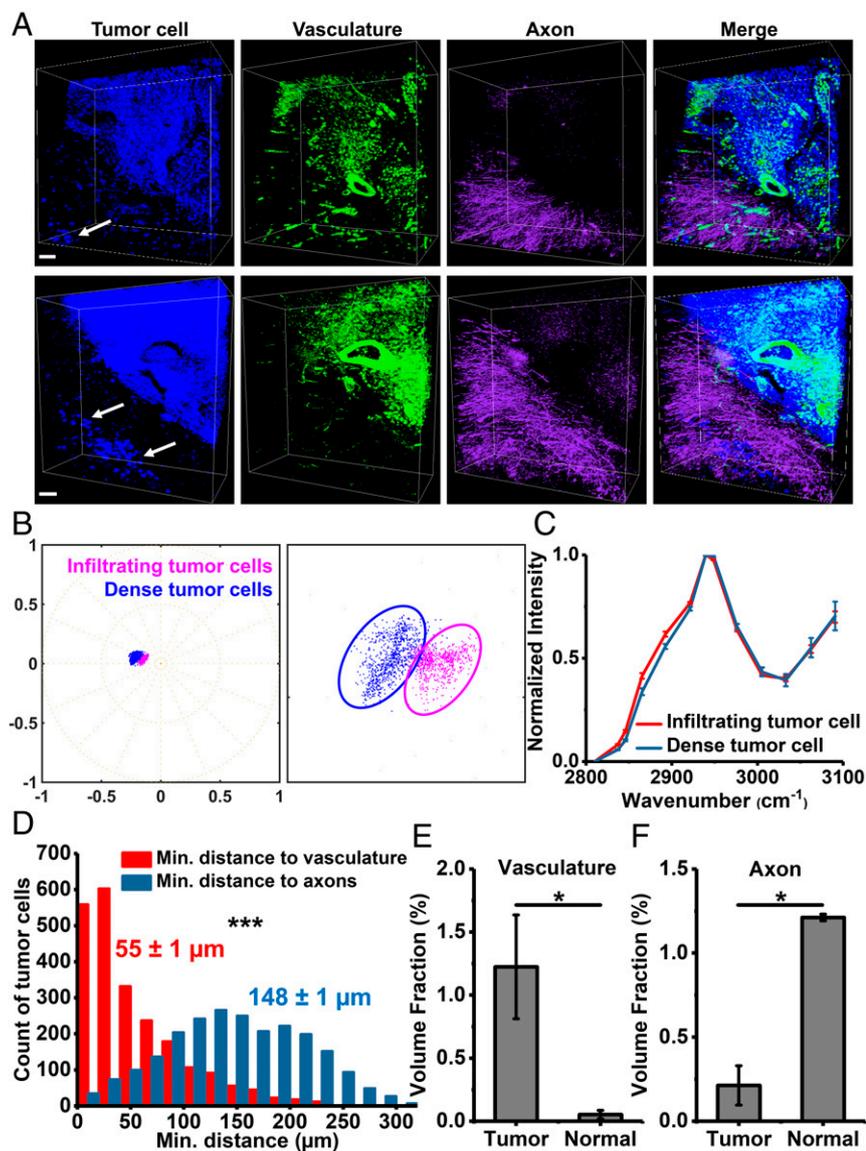


Fig. 6. Segmentation and quantification of brain glioblastoma tissues using volumetric phasor analysis. (A) Volumetric images of segmented tumor cells, vasculature, and axons in two different regions of brain glioblastoma. Arrows indicate infiltrating tumor cells. (Scale bars, 50 μm .) (B) Phasor plots of infiltrating tumor cells ($n = 6$ cells) and dense tumor cells ($n = 6$ cells). The plot (Right) is a zoom-in of the Left. (C) SRS spectra of infiltrating tumor cells ($n = 6$ cells) and dense tumor cells ($n = 6$ cells). Error bars are SD. (D) Histograms of the minimal distances from individual tumor cells ($n = 2,265$) to vasculature and axons. Mean \pm SEM are shown near the histograms. (E) Volume fraction of vasculature in tumor ($n = 5$) and normal ($n = 3$) tissues. Error bars are SEM. (F) Volume fraction of axons in tumor ($n = 3$) and normal ($n = 3$) tissues. Error bars are SEM. Statistical significance was determined by pair-sample two-tailed t test. * $P < 0.05$ and *** $P < 0.001$.

spindle-shaped cells that are narrow and elongated and located far away from tumor margins (arrowheads in Fig. 7A). Infiltrating tumor cells were also sphere-shaped with high vimentin expression similar to sphere-shaped dense tumor cells (Fig. 7B and C). These results contradict with canonical phenotypes of mesenchymal cells—high vimentin expression and spindle-cell morphology, indicating that EMT of these tumor cells is a gradual process with multiple intermediate or hybrid states instead of an “all-or-none” process (52, 63). Detailed quantification with DO-SRS (Fig. 7C) revealed: (i) infiltrating tumor cells exhibited higher activity of lipid synthesis (indicated by CD_L) than both sphere-shaped and spindle-shaped dense tumor cells; (ii) sphere-shaped dense tumor cells showed higher activity of lipid synthesis than spindle-shaped ones; (iii) infiltrating tumor cells and two types of dense tumor cells had similar levels of newly synthesized proteins (indicated by CD_P); and (iv) infiltrating tumor cells showed higher CD_L/CD_P ratio than both sphere-shaped and spindle-shaped dense tumor cells, which likely contributes to higher lipid-to-protein ratio of infiltrating tumor cells observed earlier (Fig. 6C). These results indicate enhanced lipid synthesis is central to the metabolic reprogramming of infiltrating tumor cells, which agrees with an emerging role of lipid metabolism in glioblastoma pathology (57–59). Infiltrating tumor cells were more similar—in cellular morphology, vimentin expression, and metabolic activities—to

sphere-shaped dense tumor cells than to spindle-shaped dense tumor cells (Fig. 7C), suggesting an infiltration process that dense tumor cells acquire the capacity to invade normal tissues through EMT and enhanced lipid synthesis.

Next we studied the metabolic basis of angiogenesis in tumor. Based on the high-volume fraction of vasculature in tumor (Fig. 6E), we hypothesized that tumor-associated vasculature had high activities of protein and lipid synthesis for sustained angiogenesis. To test this hypothesis, we conducted volumetric DO-SRS imaging on glioblastoma with Raman-tailored tissue clearing. As previously reported (33), tumor tissues exhibited higher activity of protein synthesis (CD_P) than normal tissues (Fig. 8A). What's more, tumor vasculature was highlighted in CD_P channel, indicating highly active protein synthesis in tumor vasculature (Fig. 8A). We then segmented tumor and normal vasculature (SI Appendix) and compared their metabolic activities and chemical composition. As shown in Fig. 8B, tumor vasculature had much higher signals in CD_P and CH_P and slightly higher signals in CD_L and CH_L than normal blood vessels. These data indicate: (i) active angiogenesis takes place in the tumor, (ii) metabolic reprogramming for tumor angiogenesis involves a major increase in protein synthesis and a minor increase in lipid synthesis, and (iii) tumor vasculature has a different chemical composition from normal vasculature. Based on high metabolic activity and

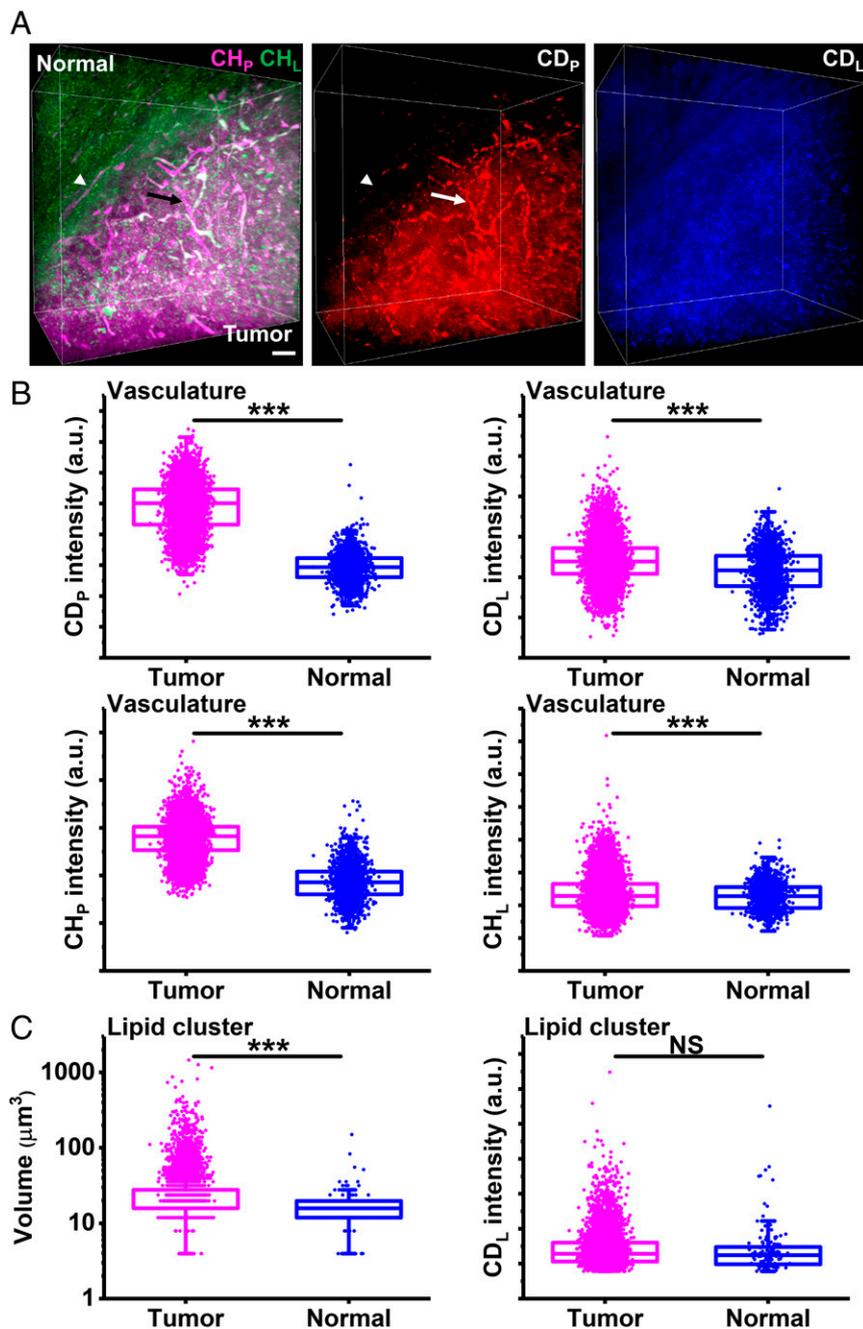


Fig. 8. Metabolic volumetric chemical imaging of glioblastoma. (A) Three-dimensional reconstruction of label-free SRS and DO-SRS images. The arrow indicates a blood vessel in the tumor. The arrowhead indicates a blood vessel in the normal tissue. (Scale bar, 50 μm .) (B) Intensities of CD_p , CD_l , CH_p , and CH_l of tumor vasculature ($n = 7,588$) and normal vasculature ($n = 1,605$) from one mouse. (C) Volume and CD_l intensities of clusters of newly synthesized lipids in the tumor ($n = 6,894$) and those in the normal tissue ($n = 136$) from one mouse. Statistical significance was determined by two-sample two-tailed *t* test. *** $P < 0.001$. NS, not significant.

chemical information of sample composition, identifying and distinguishing molecules, such as lipids, proteins, DNA, water, sugars, cholesterol, and phosphates (29). The spatial distributions, chemical subtypes (e.g., saturated vs. unsaturated lipids), and detailed conformations (e.g., α -helices vs. β -sheets of proteins) of these species are crucial for their functions. Second, label-free SRS enables probing chemical structures in tissues without exogenous labels, free from the difficulties of probe delivery for fluorescence microscopy. Third, the emerging bioorthogonal chemical imaging platform with vibrational tags (such as DO-SRS) allows visualization of metabolic dynamics of biomolecules, such as proteins, lipids, and nucleic acids, inside tissues and organisms (33, 40, 41). Therefore, volumetric chemical imaging by clearing-enhanced SRS microscopy can be a valuable tool for characterizing 3D structures, compositions, dynamics, and functions in diverse biological contexts.

Our technique could be further optimized on several fronts. In terms of next-generation clearing reagents, more chemicals could be

screened to achieve increased depth extension. Instrumentation-wise, SRS microscopes can be constructed for simultaneous multi-channel imaging (69–72), hence allowing high-throughput volumetric chemical imaging. In addition, the clearing-enhanced SRS could be combined with Bessel-beam SRS tomography for fast 3D imaging (73). Regarding applications, the Raman-clearing strategies could be tailored for specialized imaging purposes. For example, by combining SRS with hydrogel-based clearing [e.g., CLARITY (3)], chemical imaging of both protein distributions and protein secondary structures in whole organs may be achievable. Taken together, clearing-enhanced SRS will find broad use for comprehensive understanding of structure–function relationships in 3D tissue context.

Methods

Samples were immersed in the clearing solution (8 M urea or 8 M urea supplemented with 0.2% Triton X-100) for more than 4 d at room temperature and were then mounted in the clearing solution for SRS microscopy. The animal experimental

protocol (AC-AAAQ0496) was approved by the Institutional Animal Care and Use Committee at Columbia University. Full details are given in [SI Appendix](#).

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