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# Invited Article: Visualizing protein synthesis in mice with *in vivo* labeling of deuterated amino acids using vibrational imaging

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Proteins are one of the major components of biological systems, and understanding their metabolism is critical to study various biochemical processes in living systems. Despite extensive efforts to study protein metabolism such as autoradiography, mass spectrometry, and fluorescence microscopy, visualizing the spatial distribution of overall protein metabolism in mammals at subcellular resolution is still challenging. A recent study from our group reported imaging newly synthesized proteins in cultured mammalian cells, tissues, or even in mice using stimulated Raman scattering (SRS) microscopy coupled with metabolic labeling of deuterated amino acids (dAA). However, our previous method of dAA administration via drinking water, albeit convenient, is insufficient for *in vivo* studies. This is due to poor labeling efficiency and limited access to many important organs such as the brain, pancreas, or tumor. In this study, we have significantly improved and optimized the in vivo administration method by intra-carotid arterial injection of dAA in mice and obtained imaging contrast of protein metabolic activity in many more organs and tissues, such as cerebral and cerebellar cortex and hippocampal regions in the mouse brain. We also imaged newly formed proteins in the choroid plexus and pancreas at different time points, illustrating the metabolic dynamics of proteins in these important secretory organs. In addition, we visualized the metabolic heterogeneity of protein synthesis in colon tumor xenografts, which can be used to distinguish tumor and normal tissues. In summary, this combination of a new dAA administration technique and SRS imaging platform demonstrates an effective tool for the in vivo study of complex protein metabolism in mammals, in both physiological and pathological states. © 2018 Author(s). All article content, except where otherwise noted, is licensed under a Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/). https://doi.org/10.1063/1.5028134

# INTRODUCTION

The living animal's protein biomass not only provides architectural support for cells but also is critical in maintaining their biochemical processes. At the proteome scale, proteins are metabolized to keep a dynamic homeostasis and to ensure functionality of the body.<sup>1</sup> Therefore, the study of protein metabolism is very important in revealing the structural and functional status inside a cell or an organ. A tool that enables the visualization of the complex protein distribution in a single cell or in organ tissues will largely assist biologists or medical researchers to quickly determine whether a tissue is in normal or pathological conditions. For example, amino acids have been shown to be the major source of biomass in proliferating cells, especially in cancer cells.<sup>2</sup> The rates of protein synthesis and degradation differ drastically among normal and cancer tissues; therefore, the complex



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spatial distribution and temporal dynamics among tissues become direct and important information to identify the tumor boundary and intra-tumor heterogeneity.

Various methods have been applied to obtain protein metabolic information. In autoradiography, radioactive amino acids provide vigorous analysis for either protein synthesis or degradation.<sup>3,4</sup> The main drawback of emulsion-based autoradiography is the qualitative nature of the results: silver grains are difficult and tedious to quantify. Mass spectrometry with stable isotope-labeled amino acids in cell culture (SILAC) offers a quantitative approach for proteomics, but it lacks spatial resolution.<sup>5,6</sup> A fluorescence-based technique, namely, bioorthogonal noncanonical amino acid tagging (BONCAT), utilizes unnatural amino acids containing reactive chemical groups that can be subsequently conjugated to fluorophores via click chemistry.<sup>7–10</sup> However, this method is not yet sufficiently robust, noninvasive, or quantitative for imaging in live cells or live animals.<sup>11,12</sup>

Our group has recently reported a live imaging technique to visualize nascent proteins using stimulated Raman scattering (SRS) microscopy coupled with deuterated amino acids (dAA) integrated by the cells' and tissues' native translational machineries.<sup>13</sup> The newly formed proteins enrich the carbon-deuterium (C—D) chemical bond that can be detected by SRS free from other endogenous chemical bonds. To visualize newly synthesized proteins in mice, the previous study administered 2.5 mg/ml of dAA via drinking water for 12 days and observed C—D signals in liver and intestine tissues but no other organs.<sup>14</sup> We reasoned that drinking water administration has relatively poor efficiency and low speed of dAA labeling, which accounts for the absence of the nascent protein signal in many other organs.<sup>15</sup>

Herein, we developed a new administration method that delivers dAA directly into the blood stream by infusion via the carotid artery to minimize the organ bias and enhance the labeling efficiency. We demonstrated the intra-arterial *in vivo* injection method in mice to probe the complex metabolic dynamics in both healthy and tumor xenograft models. We imaged and quantified the spatial and temporal distributions of newly synthesized proteins in various brain regions, pancreas, livers, and xenografted tumors. There are three major advantages of this method. First of all, following oral administration, the dAA must travel by a long route along the gut lumens; therefore, bypassing the digestion system reduces the amount of very expensive dAA that is required. Second, the direct injection of dAA into the blood stream quickly distributes the dAA evenly to every organ in the body that has blood vessels and speeds up the labeling process in organs such as the brain. Third, the fast delivery of a relatively large amount of dAA allows us to demonstrate time-dependent imaging of protein synthesis dynamics in different organs, especially secretory organs such as the pancreas and choroid plexus. Overall, the new administration method enables the dAA-coupled SRS technique to be a more powerful tool for visualizing more complex protein metabolism *in vivo*.

#### METHODS

#### Stimulated Raman scattering microscopy

Spatially and temporally overlapped pulsed pump (tunable from 720 to 990 nm, 5–6 ps, 80 MHz repetition rate) and Stokes (1064 nm, 6 ps, 80 MHz repetition rate, modulated at 8 MHz) beams (modified picoEMERALD system, Applied Physics & Electronics, Inc.) were coupled into an inverted laser-scanning microscope (FV1200 MPE, Olympus) optimized for near-IR throughput. The pump laser power (W1) and Stokes laser power (W2) were set at W1 = 200 mW and W2 = 400 mW, respectively, for imaging the CH<sub>2</sub> and CH<sub>3</sub> channels, and W1 = 400 mW and W2 = 600 mW, respectively, for imaging the C—D channel. A 25× water objective (XLPlan N, 1.05 N.A., MP, Olympus) with both a high near-IR transmission and a large field of view was used for imaging. The forward-going pump and Stokes beams were collected in transmission by a high N.A. oil condenser. A high O.D. bandpass filter (890/220, Chroma) was used to completely block the Stokes beam and to transmit the pump beam only onto a Si photodiode for detecting the stimulated Raman loss signal. The output current from the photodiode was terminated, filtered, and demodulated by a lock-in amplifier at 8 MHz to ensure shot-noise-limited detection sensitivity. The demodulated signal was fed into an analog channel of FV1200 software (FluoView 4.1a, Olympus) to form image

092401-3 Shi, Shen, and Min

during laser scanning. The dwell time was set to 100  $\mu$ s, each frame contained 512 pixels × 512 pixels, and the total imaging time was ~26 s per image.

# Cell culture

LS174T cells were purchased from ATCC (Manassas, VA) and cultured in Eagle's Minimum Essential Medium (EMEM from ATCC) that was supplemented with 10% fetal bovine serum (Thermo Fisher) in a humidified 5% CO<sub>2</sub> atmosphere at 37 °C. Cultures were harvested for tumor xenograft when the cells reach confluency.

# Animals

Wild type  $3\sim4$  month old adult (C57BL/6J) and nude mice were both obtained from The Jackson Laboratory (Farmington, CT) and were maintained and bred at the Columbia University animal facility. For ex vivo tissue experiments, mice injected with dAA were anesthetized with isoflurane and sacrificed. Various organs and tissues were harvested, fixed with 4% formaldehyde overnight, and then cut into slices of 120  $\mu$ m thickness by using a vibrating blade microtome (vibratome, Leica) for SRS imaging. The experimental protocols for all mice studies (ACAAAQ0496) were approved by IACUC at Columbia University.

# Tumor xenografts in mice

To establish the colon tumor xenograft, we injected  $1 \times 10^7$  human colorectal LS174T cells subcutaneously into the lower flank of nude mice. Ten days later, tumor bearing mice were injected with dAA for 2.5 days.

#### **RESULTS AND DISCUSSION**

#### Injection of deuterated amino acids

Mice were anesthetized and kept warm on a heating pad. The neck skin of the mouse was cleaned with 2% chlorhexidine solution followed by 70% isopropyl alcohol, and the planned incision area was infiltrated subcutaneously with 1% lidocaine to relieve pain and numb the skin. A ~2 cm incision was made along the midline region of the throat to expose the common carotid artery, which was then tied with a suture thread (Prolene 86979 Ethicon) on the side closer to the heart to stop the blood flow. The artery was then cut with a precision stainless micro-scissor (63041-984, VWR), and a polyurethane based Micro-Renathane catheter tube (MRE033, Braintree Scientific, INC.) was carefully cannulated into the opening of the carotid artery. After implantation of the catheter, a syringe filled with 100 mg/ml D-labeled amino acids (dAA) dissolved in mammalian Ringer's solution was then connected with the cannulated tubing. The dAA solution was perfused at 0.001 ml/min by using a syringe pump (AL- 1000, WPI). In the short-term administration case, the injection was continuous for 8.5 h at 0.001 ml/min, resulting in a total amount of 0.51 ml (51 mg dAA) per mouse. The long-term administration consisted of 7 sessions of injection and a 2-h break between each session (total ~2.5 days). Each session lasted for 7 h with a perfusion rate of 0.001 ml/min, resulting in a total amount of 2.94 ml (294 mg dAA) per mouse.

#### SRS imaging of protein metabolism in the mouse brain

The increased dAA delivery efficiency allows us to visualize protein metabolism in the mouse brain, which could not be achieved previously.<sup>14</sup> We focus on key regions in the brain that involve extensive protein synthesis activity, including the cerebellar cortex, cerebral cortex, and hippocampal regions.

The cerebellum is the part of the brain that fine-tunes the motor activity. Purkinje cells are the largest neuronal cells and the only output cells of the cerebellar cortex. Previous studies have demonstrated that conditions such as ischemia, traumatic brain injury, chronic epilepsy, or mitochondria disorder can lead to Purkinje cell death, and neurological diseases such as Autism, Huntington's, or Alzheimer's can also cause Purkinje cell loss.<sup>16</sup> Hence, the protein metabolic activity in Purkinje cells can be an important indicator of the degenerative process in the brain. We imaged the

cerebellar cortical region in mice after intra-arterial dAA injection for 2.5 days, at frequencies targeting newly synthesized proteins (green image,  $2133 \text{ cm}^{-1}$ ), total proteins (red image,  $2940 \text{ cm}^{-1}$ ), and total lipids (blue image,  $2845 \text{ cm}^{-1}$ ). We observed active protein synthesis in Purkinje cells [white circles, Fig. 1(a)] in the C—D SRS image at  $2133 \text{ cm}^{-1}$ . The differential patterns in the C—D protein image and the total protein image suggest that Purkinje cells are more active than others, thereby demonstrating metabolic heterogeneity. To show protein synthesis activity with reference to tissue histology, we overlaid the C—D channel onto the total protein channel, which reveals accumulation of protein synthesis mainly inside the cell cytoplasm and nucleus but not in cell membranes [C—D and CH<sub>3</sub> merged, Fig. 1(a), last column].

We next imaged the deep layers of the cerebral cortex from the same mouse. The cerebrum is the largest part of the brain and is responsible for the integration of complex sensory and neural functions. Inside the cerebral cortex, pyramidal neurons (pyramidal cells) are found to be the primary excitation units of the mammalian prefrontal cortex.<sup>17</sup> Interestingly, we found active protein synthesis in the cell bodies of pyramidal neurons [white arrowheads, Fig. 1(b)]. This spatial distribution in general matches with intense CH<sub>3</sub> signals from the total protein channel, yet newly synthesized proteins appear more restricted to cell bodies [Fig. 1(b), last merged image of C—D and CH<sub>3</sub>].

We then imaged the dentate gyrus (DG) region, which is involved in long-term memory formation and adult neurogenesis.<sup>18</sup> Our previous study<sup>14</sup> on organotypic brain tissue cultured ex vivo indicated that the DG is the most active region for new protein synthesis. Consistently, here we have observed more intense C—D signals in DG than other surrounding areas in the hippocampus [Fig. 1(c)]. In summary, our new method offers *in vivo* labeling for imaging new protein synthesis in the brain with subcellular resolution in addition to endogenous protein and lipid distribution, which is highly challenging for other existing methods.<sup>19</sup>



FIG. 1. Protein metabolism in various mouse brain regions with deuterium-labeled amino acid (dAA) incorporation via intra-arterial injection *in vivo* for 2.5 days. (a) Protein metabolism in the mouse cerebellum. Active amino acid incorporation and protein synthesis are imaged in the cortical regions of the cerebellum. The C–D images at 2133 cm<sup>-1</sup> show the newly synthesized protein from dAA. The CH<sub>3</sub> 2940 cm<sup>-1</sup> and CH<sub>2</sub> 2845 cm<sup>-1</sup> channels show the intrinsic distribution of total proteins and lipids in the same region. White dashed circled neurons are Purkinje cells. The last merged image of C–D and CH<sub>3</sub> shows the heterogeneity of newly synthesized proteins. (b) Protein metabolism in the mouse cerebral cortex. Active amino acid incorporation and protein synthesis are imaged in the deep layer of the cortical region of the cerebrum. The C–D signals mainly appear in neuronal cells, such as pyramidal cells. (c) Protein metabolism in the mouse hippocampus. Active protein synthesis occurs in the dentate gyrus region of the hippocampus. Scale bar: 20  $\mu$ m.

#### Protein metabolic dynamics in choroid plexus

As a secretory organ, the choroid plexus (CP) is located inside the brain ventricles and has been primarily investigated for its role in producing cerebrospinal fluid (CSF) and regulating CSF composition.<sup>20</sup> CP has also been shown to be responsible for protein synthesis<sup>21</sup> and neuroendocrine signaling.<sup>22</sup> However, the limitations of current protein labeling and contrast imaging techniques make it extremely challenging to visualize the spatial and temporal distributions of protein synthesis in CP. With our new *in vivo* dAA labeling and SRS imaging method, we successfully imaged the newly synthesized proteins [Fig. 2(a), green channel], the total proteins [Fig. 2(a), red channel], and the total lipids [Fig. 2(a), blue channel] in the same CP after 2.5 days of dAA labeling. The increased labeling efficiency allows for short-term labeling; thus, we also did an 8.5-h labeling to test the strength of new protein signals [Fig. 2(b)].

In both 2.5 days and 8.5 h labeling experiments, the newly formed C—D proteins in CP region presented overwhelmingly stronger signals at  $2133 \text{ cm}^{-1}$  than the surrounding brain tissues. In order to compare the C—D signals produced at two different labeling durations, we generated quantitative ratiometric images. We discovered that the intensity ratio of new proteins over the total endogenous proteins (CH<sub>3</sub>) in CP after 2.5 days of labeling is approximately twice as high as that after 8.5 h of labeling, indicating that 2.5 days labeling approaches steady state. Note that the signal in the CP region is much stronger than the tissues near the brain ventricles, suggesting a much higher protein synthesis activity in the CP region.

# Quantitative visualization of protein metabolism in the pancreas

We next applied this method to investigate protein metabolism in another important secretory organ, the pancreas. A previous study using radioautograph measured the activity of protein synthesis in various regions of the acinar cell<sup>23</sup> and found that most newly synthesized proteins migrate to and accumulate in the Golgi zone and then finally enter the excretory ducts. Their results indicated that two pools of proteins are synthesized in the ergastoplasm: "sedentary" with a slow turnover (62.5 h) and "exportable" with a rapid turnover (4.7 min).<sup>23</sup> The "exportable" protein takes minutes to synthesize but can take hours to accumulate into a significant amount in the duct. Hence, we studied protein synthesis in the pancreas with two different labeling durations: 8 h and 2.5 days, under C—D proteins at 2133 cm<sup>-1</sup>, CH<sub>3</sub> proteins at 2940 cm<sup>-1</sup>, and CH<sub>2</sub> lipids at 2845 cm<sup>-1</sup> [Figs. 3(a)–3(d)].

While the signals of 8.5-h dAA-labeled C—D protein mainly appeared in the duct region, signals of 2.5-day dAA-labeled C—D protein were obvious not only in the central duct region but also in



FIG. 2. Protein metabolism in the choroid plexus (CP) at the mouse brain ventricular region after dAA incorporation via intra-arterial injection *in vivo*. (a) Protein metabolism in CP after 2.5 days of dAA labeling. (b) Protein metabolism in CP after 8.5 h of dAA labeling. Active amino acid incorporation and protein synthesis are imaged in the CP region. The C–D image at 2133 cm<sup>-1</sup> shows the newly synthesized protein from dAA. The CH<sub>3</sub> 2940 cm<sup>-1</sup> and CH<sub>2</sub> 2845 cm<sup>-1</sup> channels show the intrinsic distribution of total proteins and lipids in the same region, respectively. The C–D/CH<sub>3</sub> quantitative ratiometric image shows the newly synthesized protein over the intrinsic total proteins. Scale bar: 20  $\mu$ m.



FIG. 3. C–D protein synthesis in the mouse pancreas at different time points *in vivo*. (a) Protein metabolism in the mouse pancreas with intra-arterial dAA injection for 2.5 days. Active amino acid incorporation and protein synthesis are imaged in the pancreas. The C–D image at 2133 cm<sup>-1</sup> shows the newly synthesized protein from dAA. The CH<sub>3</sub> 2940 cm<sup>-1</sup> and CH<sub>2</sub> 2845 cm<sup>-1</sup> channels show the intrinsic distribution of total proteins and lipids in the same region. The C–D/CH<sub>3</sub> ratiometric image shows the newly synthesized protein over the intrinsic total proteins in the pancreas. (b) Protein metabolism in the mouse pancreas with dAA injection for 8.5 h via carotid artery. The C–D/CH<sub>3</sub> quantitative ratiometric image shows the newly synthesized protein over the intrinsic total proteins. (c) and (d)] Enlarged areas after 2.5 days and 8.5 h dAA injection, respectively. (e) Intensity profiles along the line drawn in (c). The line profile of the C–D signal is more evenly distributed between beta cells in the pancreas after 2.5 days of dAA treatment. (f) Intensity profiles along the line drawn in (d). The line profile of C–D signals is disconnected between beta cells in the pancreas after 8.5 h of dAA treatment. Scale bar: 20  $\mu$ m.

the surrounding acinar cell bodies. The ratiometric images quantitatively evaluated the ratio of newly synthesized C—D proteins over total CH<sub>3</sub> proteins. The C—D and CH<sub>2</sub> merged images [Figs. 3(c) and 3(d), last column, C—D and CH<sub>2</sub> merged images] clearly display the different distributions of new proteins and old lipids, especially in the 8.5-h labeling case. We also present the normalized intensity profiles along the white straight lines [in Figs. 3(e) and 3(f)] in the same region of interest. As expected, the intensity profile of C—D proteins is continuous in the 2.5-day labeled tissues but appears discontinuous between two pancreatic islets in the 8.5-h labeled tissues. Our observation suggests that only "exportable" proteins were transported and accumulated in the duct at 8.5-h labeling,

while "sedentary" proteins were slowly formed in the ergastoplasm region after 2.5 days of dAA labeling.

# Imaging protein metabolic heterogeneity in the liver

The significance of liver metabolic zonation is increasingly recognized. It describes interlobular functional divisions due to the oxygen/nutrient gradient from the portal vein (PV) to the central vein (CV).<sup>24</sup> Our previous study imaged protein synthesis in the liver tissue with deuterium-labeling via oral administration of dAA in water but did not examine protein metabolic heterogeneity in the liver.<sup>14</sup> Motivated by this, we imaged protein synthesis in regions near the PV and CV and compared the differences in protein metabolic activities between these two regions.

We labeled the liver with dAA injection for 2.5 days and imaged C—D protein synthesis (green channels, Fig. 4), as well as CH<sub>3</sub> protein and CH<sub>2</sub> lipids (red and blue channels, respectively, Fig. 4). Although the spatial distributions of C—D proteins near PV and CV are not very well pronounced, the quantitative ratiometric images (C—D/CH<sub>3</sub>, Fig. 4) immediately differentiate these two regions. The region near PV displayed a higher C—D/CH<sub>3</sub> ratio compared to the region near CV, indicating that the PV region has higher protein synthetic activities. Our observations revealed metabolic heterogeneity of new protein distribution near PV and CV regions in the liver.

# Colon tumor metabolic heterogeneity of protein synthesis

Label-free SRS imaging has been proven to separate the protein-rich glioblastoma tumor from normal brain tissue that is enriched with lipids.<sup>25</sup> However, it highly depends on the host organ's environment and has not been applied to other types of tumors. The colon tumor, for example, is not as biased in the protein/lipid content as the brain tumor. We demonstrate here that *in vivo* dAA labeling can help reveal colon tumor boundaries [dashed lines, Figs. 5(a) and 5(b)] and intratumoral metabolic heterogeneity [Fig. 5(c)] by examining spatial differences in protein synthesis.

We applied our new labeling strategy in a colon tumor xenograft mouse model. We found a prominent difference in C—D signal intensities between the tumor region and the supporting tissues [C-D channel, Figs. 5(a) and 5(b)]. Interestingly, the tumor boundary immediately next to supporting tissues had stronger protein synthesis than both supporting tissues and tumor interior, as evidenced by the line profiles across the tumor edge (dashed line box) [Fig. 5(b)]. This edge is likely the invasion



FIG. 4. Metabolic heterogeneity of proteins in the mouse liver with quantitative ratiometric images after intra-arterial dAA injection for 2.5 days. The C–D image at 2133 cm<sup>-1</sup> shows the newly synthesized protein from dAA. The CH<sub>3</sub> 2940 cm<sup>-1</sup> and CH<sub>2</sub> 2845 cm<sup>-1</sup> channels show the intrinsic distribution of the total proteins and lipids in the same region. The quantitative C–D/CH<sub>3</sub> ratiometric image shows the newly synthesized protein over the intrinsic total proteins in the liver. (a) Protein metabolism in mouse liver regions close to the central vein (CV). (b) Protein metabolism with dAA in regions close to the portal vein (PV), hepatic artery (HA), and bile duct (BD) of the mouse liver. The region close to the portal vein has a higher C–D/CH<sub>3</sub> ratio compared to the region close to the central vein. H: hepatocyte. Scale bar: 20  $\mu$ m.



FIG. 5. Tumor metabolic heterogeneity of the newly synthesized protein in mice in vivo. The C-D image at 2133 cm<sup>-1</sup> shows the newly synthesized protein from dAA. The CH<sub>3</sub> 2940 cm<sup>-1</sup> and CH<sub>2</sub> 2845 cm<sup>-1</sup> channels show the intrinsic distribution of total proteins and lipids in the same region. The C-D/CH<sub>3</sub> quantitative ratiometric image shows the newly synthesized protein over the intrinsic total proteins in the xenografted colon tumor. (a) Protein metabolism images of the xenografted colon tumor in the nude mouse after 2.5 days of dAA injection via carotid artery in vivo. Active amino acid incorporation and protein synthesis are imaged in the tumor. Second row images are enlarged regions of the xenografted colon tumor, and the last image of the C-D and CH<sub>3</sub> merged image shows that the newly synthesized C-D protein signal (green) and the intrinsic total protein CH<sub>3</sub> (red) in the xenografted colon tumor have different intensity distribution; the tumor side has more intense new C-D protein synthesized than the stroma side. The tumor-normal tissue boundary is drawn in white dashed line to separate the stroma and the tumor. (b) Protein metabolism at the tumor boundary. After dAA injection for 2.5 days in vivo, the C-D image shows the newly synthesized C-D protein signal, which shows metabolic heterogeneity at the tumor edge. The boundary between the tumor and the supporting tissue is drawn with a white dashed line. The second row images are intensity profiles as a function of distance enclosed by dashed rectangular boxes across the intra-tumor region, the tumor front, and the supporting tissues. (c) Intra-tumor metabolic heterogeneity in a large field of view. The C-D image shows that some regions (circled with white dashed lines) inside the colon tumor have intense C-D protein signals, but regions other than these have much lower C-D signals. The second row images are intensity profiles as a function of distance enclosed by dashed rectangular boxes in the intra-tumor region. Scale bar: 20  $\mu$ m in (a) and (b), 500  $\mu$ m in (c).

front of the colon tumor, which is more aggressive in occupying space and nutrients than other regions of the tumor. By contrast, the intensity profile of total proteins and total lipids [Fig. 5(b), intensity profiles of CH<sub>3</sub> and CH<sub>2</sub>] did not show such differences.

We also imaged total protein and lipid channels on the same region and generated quantitative ratiometric images [Figs. 5(a) row 1, 5(b) and 5(c), last column] to investigate spatial information on the percentage of protein replaced by the newly formed protein. The merged image [Fig. 5(a), row 2, last column] of C—D and CH<sub>3</sub> identified the morphological pattern of proteins and lipids in the colon tumor, tumor tissue with glandular lumens,<sup>26</sup> and stromal with relatively loosely packed cells but lacking glandular lumens.<sup>27</sup> The blood vessels with red blood cells in the lumen cross-sectional area are also clearly shown (circled with white dashed lines); they were formed via tumor induced angiogenesis,<sup>28</sup> which potentially can be used as evidence for tumor identification.<sup>28</sup>

Intratumoral metabolic heterogeneity is known to be highly correlated to aggressiveness and has been studied due to its fundamental importance as well as prognostic significance.<sup>29</sup> We imaged a large field of view in the colon tumor xenograft after 2.5 days of dAA labeling to visualize intertumoral protein synthesis [Figs. 5(c), C—D channel]. The spatial distribution of the newly synthesized protein inside the colon tumor exhibited a substantial amount of heterogeneity. We generated intensity profiles [Fig. 5(c), second row, line profiles] across the solid tumor [Fig. 5(c), dashed line box], which showed about 10 times higher C—D intensity on the cells that are circled with dashed lines than other regions in the tumor. This huge difference occurred only in the C—D new protein channel and the ratiometric image but not in the CH<sub>3</sub> protein or CH<sub>2</sub> lipid channels. The higher C—D intensity regions are mostly recruited mouse stromal cells.

# CONCLUSIONS

In this study, we significantly improved and optimized an *in vivo* labeling method for imaging protein metabolism in mice by intra-carotid arterial injection of dAA. Coupled with SRS imaging, we visualized protein synthesis activity in various organs, including the brain, pancreas, liver, and choroid plexus, which was not possible with previous methods. In addition, we visualized the metabolic heterogeneity of protein synthesis within the xenograft colon tumor, as well as between the tumor and the normal tissues. This new method is an efficient tool for the *in vivo* study of protein metabolism in mammals, in both physiological and pathological tissues.

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