Label-free three-photon imaging of intact human cerebral organoids: tracking early events in brain development and deficits in Rett Syndrome

Murat Yildirim^{1,2,3,*}, Chloe Delepine^{1,3,*}, Danielle Feldman^{1,3}, Vincent Pham^{1,3}, Stephanie Chou^{1,3}, Jacque Pak Kan Ip^{1,3,5}, Alexi Nott^{1,3}, Li-Huei Tsai^{1,3}, Guo-li Ming⁴, Peter T. C. So^{2,6}, Mriganka Sur^{1,3}

¹*Picower Institute for Learning and Memory, Massachusetts Institute of Technology, Cambridge, MA, 02139, USA;*

²*Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA, 02139, USA;*

³*Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, MA, 02139, USA;*

⁴*Department of Neuroscience and Mahoney Institute for Neurosciences, Institute for Regenerative Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, 19104, USA;*

⁵*School of Biomedical Sciences, The Chinese University of Hong Kong, Hong Kong, China.*

⁶*Department of Mechanical Engineering, Massachusetts Institute of Technology, Cambridge, MA, 02139, USA;*

**These authors contribute equally.*

Corresponding authors: Mriganka Sur, Picower Institute for Learning and Memory, 43 Vassar Street Cambridge, MA, 02139; email: msur@mit.edu; Murat Yildirim, Picower Institute for Learning and Memory, 43 Vassar Street Cambridge, MA, 02139; email: muraty@mit.edu

ABSTRACT

Human cerebral organoids are unique in their development of progenitor-rich zones akin to ventricular zones from which neuronal progenitors differentiate and migrate radially. Analyses of cerebral organoids thus far have been performed in sectioned tissue or in superficial layers due to their high scattering properties. Here, we demonstrate label-free three-photon imaging of whole, uncleared intact organoids (~2 mm depth) to assess early events of early human brain development. Optimizing a custom-made three-photon microscope to image intact cerebral organoids generated from Rett Syndrome patients, we show defects in the ventricular zone volumetric structure of mutant organoids compared to isogenic control organoids. Long-term imaging live organoids reveals that shorter migration distances and slower migration speeds of mutant radially migrating neurons are associated with more tortuous trajectories. Our label-free imaging system constitutes a particularly useful platform for tracking normal and abnormal development in individual organoids, as well as for screening therapeutic molecules via intact organoid imaging.

INTRODUCTION

Human cerebral organoids derived from embryonic or induced pluripotent stem cells are unique in their ability to recapitulate early events of embryonic brain development[1]. These spheroid structures contain progenitor-rich zones around ventricle-like cavities akin to ventricular zones (VZ) from which neuronal progenitors migrate radially to generate the cortical plate (CP)[1-5]. Human cerebral organoids also recapitulate gene expression programs of the fetal cortex[2, 6-10] as well as the fetal brain epigenome[9]. Thus, they have been used to model neurogenesis-relevant human pathologies such as microcephaly[1, 11], licencephaly[12], heterotopia[13], Zika virus infection[2, 14, 15] and idiopathic autism[16]. Rett Syndrome (RTT) is an X-linked neurodevelopmental disorder caused by mutations in the gene encoding Methyl CpG binding protein 2 (MeCP2). MeCP2 is a pleiotropic regulator of gene expression and impacts multiple components of brain development and function[17]. 2D human stem cell models of RTT, generated by reprogramming of patient cells or genome editing, have revealed deficits in human RTT neurons including aberrant transcription[18-22], impaired neuronal maturation and electrophysiological function[19, 23-25], and up- or down-regulation of key signaling pathways and activity-related genes[19]. Using fixed tissue slices from 3D cerebral organoids, we have recently demonstrated impaired proliferation of the progenitor pool and delayed maturation and presumed migration of neurons[26]. However, the use of fixed tissues did not allow cell tracking and analysis of parameters such as speed and trajectory of neuronal

displacement; thus the dynamics and cellular mechanisms of the presumptive migration deficit remain to be characterized. Significantly, live cell time-lapse imaging of radial migration of MECP2-deficient neurons from the VZ to CP inside intact live 3D organoids has not been performed so far.

The vast majority of analyses of cerebral organoids have been performed in sectioned tissue as opposed to intact 3D structures[12, 13, 27-33]. Indeed, cerebral organoids appear opaque due to the optical density of neuronal tissues and the large number of apoptotic cells in the center[28]. Sectioning induces unwanted tissue damage and cell loss; furthermore, even though two-dimensional imaging provides valuable information, it does not provide a full picture of an organoid as a cohesive developmental unit. Even if desirable, however, live cell imaging requires phototoxic dyes and limited incubation conditions, which are not ideal for long-term imaging. Thus, label-free and high-resolution deep-tissue imaging are required to perform intact organoid imaging. Third-harmonic generation (THG) is an intrinsic signal which results from tripling of the frequency of the excitation wavelength. THG signal is either generated at structural interfaces such as local transitions of the refractive index or inside the materials whose third order nonlinear susceptibility χ^3 is higher. Thus, THG excitation of biological tissues occurs predominantly at interfaces that are formed between aqueous interstitial fluids and lipid-rich structures, such as cellular membrane[34] and lipid bodies[35]. Optimized designs using high power lasers and high sensitivity photomultipliers have enabled the application of THG microscopy to 3D tissue microscopy by improving the imaging depth in tissues with varying scattering coefficients [35-38]. THG microscopy has been applied recently to the non-invasive monitoring of human adipose tissue[39], cell nuclei and cytoplasm in liver tissue[40] and subcortical structures within an intact mouse brain [41-43]. THG imaging provides the advantages of micron scale resolution, label free imaging, deep tissue penetration depth, and non-destructiveness. Here, we describe a custom-made three-photon microscope with optimized laser and optics design to perform label-free THG imaging of intact organoids, and its use in imaging organoids generated from RTT and control patients to assess early events of early brain development. We demonstrate that high resolution label-free THG signal can be collected from intact cerebral organoids. Comparing organoids from RTT patients and isogenic controls, we show that the ventricular zone in mutant organoids has larger volume, larger surface area, and lower ventricular thickness compared to isogenic control organoids. Finally, label-free live cell imaging provides a unique way of observing neuronal migration inside the VZ/SVZ and CP of developing organoids without any phototoxicity or photobleaching, and reveals that the smaller ventricular thickness in mutant organoids is associated with shorter

migration distances, more tortuous trajectories, and slower migration speeds of radially migrating neurons.

RESULTS

Optimized system for label-free THG imaging of intact cerebral organoids

We developed a three-photon microscope[43] and modified it (Fig. 1A-C, see Methods) to perform label free THG imaging of intact, uncleared, fixed and live organoids. Since low average laser power is essential for live cell imaging, we focused on optimizing laser and microscopy parameters to reduce the average power requirement for intact organoid imaging. First, we utilized moderate repetition rate (<1 MHz) to maximize the energy per pulse and reduce the average laser power requirement for THG imaging. Second, we minimized the pulse width on the sample by building an external pulse compressor. The pulse width on the sample was reduced to 27 fs in the deepest part of the organoids (**see Methods**) enabling us to further reduce the average power requirement by 2-3 fold compared to other three-photon studies in the literature[41, 43, 44] (Supplementary Figure 1-2). Finally, we designed all intermediate optics in the excitation and emission path to maximize the generation and collection efficiency of THG signal (Supplementary Figure 3). This optical design procedure enabled us to reduce the spherical aberration in the system and increase the collected signal by 2-fold compared to off-the shelf optics[41] (Supplementary Figure 4).

THG signals from intact cerebral organoids reveal distinct zones

Cerebral organoids were derived from two pairs of isogenic iPSC clonal lines (see Methods). One pair (Line 1) was produced as previously described[26] from one individual with RTT harboring a heterozygous single nucleotide deletion (frameshift 705delG) in the transcriptional repression domain of MeCP2. Because of the monoallelic expression of X-chromosome genes and clonal selection of iPSCs, the “RTT-WT” line expressed exclusively the wild-type allele of *MECP2*, while the “RTT-MT” line expressed exclusively the mutated allele. The second pair of isogenic iPSC clonal lines (Line 2), produced as previously described[45], was from another individual with RTT harboring a heterozygous single nucleotide mutation *MECP2*R306C located in the transcriptional repression domain (TRD) or more specifically NCoR/SMRT interaction domain (NID) of MeCP2, which selectively blocks its interaction with the NCoR/SMRT complex[18, 46]. Because the *MECP2*R306C-derived iPSCs had skewed X-inactivation, and expressed only the *MECP2*R306C allele, the *MECP2*R306C RTT-WT line was

generated from the MECP2R306C iPSCs using CRISPR/Cas9-mediated gene editing to correct the R306C mutation[45].

Figure 1: Three-photon microscope and imaging system. **A.** Femtosecond laser pulses from a pump laser (1045 nm) were pumped through a noncollinear optical parametric amplifier (NOPA) to obtain 1300 nm excitation wavelength. Power control of these laser pulses was performed using a combination of a half-wave plate (HWP) and polarizing cube beam splitter (PCBS). A quarter-wave plate (QWP) was used to control the polarization state of the laser pulses to maximize the third harmonic generation (THG) signal. Laser beams were scanned by a pair of galvanometric scanning mirrors (SM), and passed through a scan lens (SL) and a tube lens (TL) on the back aperture of a 1.05 NA, 25x objective. The sample (S) was placed on a two-axis motorized stage, while the objective (OL) was placed on a one-axis motorized stage for nonlinear imaging. Emitted light was collected by a dichroic mirror (CM1), collection optics (CO), laser blocking filters (BF), and nonlinear imaging filters (F) and corresponding collection optics (COA, COB, and COC) for each photomultiplier tube (PMT A, PMT B, and PMT C). **B.** Fixed intact organoids were placed inside imaging chamber gaskets on top of the bottom glass. Gaskets were filled with Hprotos and covered with a cover glass. **C.** Live organoids were placed on top of the bottom plate of the incubator. Imaging gaskets were placed to secure organoids and cell medium was applied to fill the gaskets. Top plate was placed on top of the gaskets and the microincubator closed with 6 screws. Cell

medium mixed with 5% CO₂, 5% O₂, and balanced N₂ was pumped to the chamber; incubator temperature was set to 37°C.

First, we established that cortex-like substructures could be distinguished in the organoids by cell density differences. In accordance with previous studies[1, 2, 6], after 35 days of culture, the cerebral organoids demonstrated formation of well-organized structures composed of a ventricular zone (VZ)-like layer (KI67-positive proliferative cells) around a ventricle-like cavity and a surrounding cortical plate (CP)-like structure (Tuj1 or DCX-positive neurons) (Fig. 2A-B; see details of the generation of organoids in Methods). Notably, CP and VZ substructures demonstrated distinct cell density and morphology as shown by nuclear (7AAD) stain and plasma membrane specific dye (WGA) (Fig. 2C). The VZ region, densely populated by neural progenitor cells and few immature neurons, could be markedly distinguished from the CP substructure with a less dense population of neurons. Thus, we subsequently used the cell density to localize the CP structures in three-photon fluorescence/THG imaging experiments.

Next, we demonstrated that high resolution label-free THG signal could be collected from intact cerebral organoids. Nuclear stain was used as a control to localize the high cell density VZ from the low cell density CP (Fig. 2D). Strikingly, THG imaging provided a clean delineation between VZ and CP as THG signal intensity was lower in the former area than the latter (Fig. 2C-D). The intrinsic THG signal mostly arose distinctly from cell membranes. Consequently, the THG signal nicely revealed the typical radially elongated and bipolar radial glia cell morphology as well as the round-shaped somata of neurons (Fig. 2D-E). Additionally, neurons in the CP and newborn neurons inside the VZ showed high intensity somatic signals (Fig. 2D-E). Finally, we performed THG imaging of an intact organoid and then stained it with neuronal marker DCX. We were able to find the same field of view in our stained organoids and compare it with our THG imaging results (Supp. Fig. 5). Our results confirmed that strong THG signal is mostly detected in the CP (DCX-rich, low cell density), rather than the VZ (DCX-negative, high cell density) (Supp. Fig. 5).

To further validate that the intrinsic THG signal arose from neurons, we expressed green fluorescent protein (GFP) in control organoids by electroporation (see Methods). We previously demonstrated that >90% of the GFP-positive cells in electroporated organoids are neurons [47]. Three-dimensional imaging of cells in organoids (~2 mm thickness) revealed that both WT and MT organoids in fixed and live conditions had > 85% overlap between GFP and THG signals (Supp. Figs. 6-7, 19-20, see Methods).

Three-dimensional rendering of a ~450-image z-stack with 2- μ m increments revealed our system's ability to perform high-resolution imaging (Supp. Videos 1 and 2; Fig. 3 A-B, left). We performed depth-resolved imaging of a 250x250x900 μ m³ region selected to include a cortex-like structure, centered around the ventricle-like cavity, for both RTT-WT (Fig. 3A) and RTT-MT organoids (Fig. 3B). The strong THG signal produced at the interface between an organoid's surface and the cover glass helped us determine boundaries of the intact organoids. Since our field of view (FOV) was 250 μ m, serial imaging of multiple sites was necessary to delineate individual CP regions. Notably, we were able to collect high-resolution THG signal from the entire 900 μ m-deep regions, highlighting the performance of three-photon fluorescence and THG detection for deep-tissue imaging, and in particular, intact uncleared whole organoid imaging. The maximum imaging depth was only limited by the working distance of the objective, which was approximately 2 mm in our experiments (see Supp. Video 3).

Figure 2: Label-free Third Harmonic Generation (THG) imaging of intact cerebral organoids. A-B. Confocal imaging of immunolabeled 2D cerebral organoid slices where cerebral organoids present well-organized spherical structures located around ventricle-like cavities. Around these cavities the ventricular zone-like substructure (VZ), densely populated with progenitors (KI67+ cells) and young migrating neurons (TUJ1+ cells), can be distinguished from the cortical plate-like substructure (CP) with a less dense population of neurons (TUJ1, DCX+ cells). **C.** Cortical plate and ventricular zone substructures presented distinct cell density and morphology as shown by nuclear (7AAD dye) and plasma membrane labeling (WGA dye). **(D-E).** Label-free THG imaging from intact fixed 3D cerebral organoids show distinct signals from the cortical plate and ventricular zone, as confirmed by the nuclear labeling (7AAD dye) observed with the same setting but using three-photon epifluorescence (3PEF). THG signal seems to occur at the plasma membranes in the VZ and to be brighter in the CP substructure.

Figure 3: 3-D label-free THG imaging of fixed organoids and characterizing their extinction lengths. (A-B) 3D reconstruction of label-free THG signal from ventricular regions highlighted in Figure 2 D-E. THG allows imaging of deep structures (here, till 900 μm). Scale bars represent 100 μm . **(C-D)** Characterization of the extinction lengths of a WT and a MT fixed organoid at 1300 nm excitation wavelength. Semi-logarithmic plot for ratio of PMT signal and cube of laser power with respect to imaging depth for third harmonic generation (THG) imaging. Slope of these curves result in 162.5 μm and 148.2 μm attenuation lengths for the WT and MT fixed organoids, respectively. **(E)** Comparison of the extinction

lengths of four WT and MT fixed organoids at 1300 nm excitation wavelength. The average extinction length of wild-type (WT) organoids is significantly higher than that of mutant (MT) organoids ($p < 0.05$, t-test).

Label-free imaging of fixed cerebral organoids reveals differences between RTT and isogenic control organoids

First, we characterized the extinction (combined scattering and absorption) lengths of WT and MT organoids. Our label-free THG imaging characterization shows that the extinction lengths of a fixed WT and a MT organoid are $162.5 \mu\text{m}$ and $148.2 \mu\text{m}$, respectively which are approximately half of the attenuation length of a primary visual cortex of an awake mouse brain[42, 43] (Fig. 3C-D). In other words, fixed WT and MT organoids are at least two times more scattering than the mouse visual cortex at 1300 nm excitation wavelength. We also examined different WT and MT organoids for comparison (Fig. 3E). Our results show that average extinction length of WT organoids ($150.7 \pm 5.2 \mu\text{m}$) is significantly higher than that of MT organoids ($129.4 \pm 6.9 \mu\text{m}$, $p < 0.05$).

To study the 3D organization of VZ structures in RTT-WT and RTT-MT organoids, depth-resolved serial imaging of whole organoids was performed automatically with a custom algorithm to reduce the total imaging duration (see Methods). We acquired both 7AAD three-photon fluorescence and THG signal from RTT-WT (Fig. 4A) and RTT-MT (Fig. 4B) organoids. Then, we determined the boundaries of individual VZ structures using THG signal and rendered individual cortical regions in both RTT-WT (Fig. 4A) and RTT-MT (Fig. 4B) organoids (see **Methods**, see Supp. Video 4, see Supp. Fig. 8). First, we quantified the total volume of each region of interest and found slightly (but not significantly) higher volumes in mutant organoids than in control ones ($1.13 \pm 0.18 \times 10^7 \mu\text{m}^3$ for RTT-WT, and $1.39 \pm 0.15 \times 10^7 \mu\text{m}^3$ for RTT-MT organoids, $p = 0.3013$; $n = 10$ organoids) (Fig. 4C). However, the surface area of the VZ region in RTT-MT organoids was significantly higher than that in RTT-WT organoids ($5.70 \pm 0.75 \times 10^5 \mu\text{m}^2$ for RTT-WT, and $11.0 \pm 0.71 \times 10^5 \mu\text{m}^2$ for RTT-MT organoids, $p < 0.0001$; $n = 10$ organoids) (Fig. 4C). In addition, the ratio of volume to area was used as a relative measure of the VZ thickness. The progenitor-rich structures in the RTT-MT organoids had significantly lower unit thickness relative to RTT-WT organoids ($23.11 \pm 4.12 \mu\text{m}$ for RTT-WT, and $9.99 \pm 0.27 \mu\text{m}$ for RTT-MT organoids, $p < 0.0001$) (Fig. 4C). Finally, the number of ventricles was significantly higher in RTT-MT organoids (12.3 ± 0.7 for RTT-WT, and 22.0 ± 1.3 for RTT-MT organoids, $p < 0.0001$, $n = 10$ organoids) (Fig. 4C). In summary, using label-free intrinsic THG imaging we show that RTT-MT organoids contain an increased number of VZ structures with larger surface area and volume,

but lower VZ thickness, compared to RTT-WT organoids (values for each line separately are shown in Supp. Figs 9-12).

Figure 4: Three-dimensional characterization of control (RTT-WT) and mutant (RTT-MT) VZ regions in intact organoids. A, B. THG signal acquired from the whole RTT-WT (A) and RTT-MT (B) organoids (left) was segmented into individual ventricular regions (right). C. (left; top) RTT-MT organoids showed slightly higher VZ volume than those in RTT-WT organoids ($1.13 \pm 0.18 \times 10^7 \mu\text{m}^3$ for RTT-WT, and $1.39 \pm 0.15 \times 10^7 \mu\text{m}^3$ for RTT-MT organoids, $p=0.3013$; $n=10$ organoids). (left; bottom) The surface area of the VZ region in RTT-MT organoids was significantly higher than that in RTT-WT organoids ($5.70 \pm 0.75 \times 10^5 \mu\text{m}^2$ for RTT-WT, and $11.0 \pm 0.71 \times 10^5 \mu\text{m}^2$ for RTT-MT organoids, $p<0.0001$; $n=10$ organoids). (right; top) The VZ thickness (V/A ratio) in RTT-MT organoids was significantly lower than that in RTT-WT organoids

($23.11 \pm 4.12 \mu\text{m}$ for RTT-WT, and $9.99 \pm 0.27 \mu\text{m}$ for RTT-MT organoids, $p < 0.0001$). (right; bottom) The number of ventricles was significantly higher in RTT-MT organoids than that in RTT-WT organoids (12.3 ± 0.7 for RTT-WT, and 22.0 ± 1.3 for RTT-MT organoids, $p < 0.0001$, $n = 10$ organoids).

Label-free imaging of live cerebral organoids captures lower migration speed and displacement in RTT organoids

Direct visualization and time-lapse characterization of neuronal migration dynamics have not been examined so far. Thus, we tracked migrating neurons in real time using THG microscopy. We performed live imaging of RTT-WT and RTT-MT organoids at 35 DIV for 12-96 hours, with an acquisition interval of 20 minutes (Supp. Fig. 13). Given the sparseness and high signal to noise ratio, we were able to track cells harboring a bright somatic THG signal in both RTT-WT and RTT-MT organoids (Fig. 5A, see Supp. Videos 5 and 6, see **Methods**). Although the tracked cells in both RTT-WT and RTT-MT organoids migrated radially from the inner VZ to the outer VZ, RTT-MT cells showed decreased displacement, more tortuous trajectories, and reduced linear speed than RTT-WT cells (Fig. 5B and 5C, also see Fig. 6). Specifically, we observed in RTT-WT organoids an average migration speed of $23.3 \pm 0.2 \mu\text{m}/\text{hour}$ ($n = 6$ organoids, $N = 208$ cells). The average speed for each line was $20.6 \pm 0.1 \mu\text{m}/\text{hour}$ for Line 1 and $25.7 \pm 0.2 \mu\text{m}/\text{hour}$ for Line 2 (Supp. Fig. 14). These average speed values are comparable to the speed recorded in neurons migrating off a human wild-type cerebral organoid onto a Matrigel-coated surface[12] and in ferret cortical explants[48]. In contrast, RTT-MT cells had a reduced average speed of $13.1 \pm 0.1 \mu\text{m}/\text{hour}$ ($n = 6$ organoids, $N = 217$ cells). The average speed for each line was $11.4 \pm 0.1 \mu\text{m}/\text{hour}$ for Line 1 and $14.6 \pm 0.1 \mu\text{m}/\text{hour}$ for Line 2 (Supp. Fig. 14). Collectively, the average speed in WT organoids was significantly higher than the average speed in MT organoids (Figure 5C). In addition, we compared migration speeds of individual cells with respect to their location in each organoid as well as between organoids (Supp. Fig. 15). In conclusion, we did not find any statistically significant difference in migration speeds of individual cells at different locations in the same organoid (Supp. Fig. 15 A-B) as well as between organoids of the same type (Supp Fig. 15 C-D). We also quantified the straightness of trajectory by taking the ratio of displacement and total trajectory length. With a straightness value close to 1, RTT-WT cells (straightness value of 0.877 ± 0.004) had significantly more straight trajectories than RTT-MT cells (0.654 ± 0.005) which showed much more nonlinear migrating patterns (Fig. 5A and 5C).

Since the average speed of RTT-MT was lower and these cells had a less straight trajectory, net displacement of MT cells ($132.1 \pm 34.4 \mu\text{m}$) was significantly lower than that of

WT cells ($241.8 \pm 51.2 \mu\text{m}$) (Fig. 5C). The straightness and net displacement values of RTT-
WT and RTT-MT in both lines showed similar trends (Supp. Figs. 16-17). To check whether
cells in the organoids were still viable after imaging session of ~18 hours, we performed
Caspase3 immunostaining for both imaged and not-imaged control organoids as well as a 90-
days old organoid with a necrotic core as a positive control (Supp. Fig. 18). Our results show
that imaging live organoids in the mini-incubator with three-photon microscopy did not induce
any significant cell death compared to that in organoids that were not kept in the mini-incubator
or imaged (Supp. Fig. 18).

Electroporation of organoids with GFP construct is a powerful method to label migrating
neurons with high efficiency and low toxicity [26]. We performed a comparative live-cell imaging
in GFP-electroporated wild-type and mutant organoids and confirmed that most cells expressing
GFP also emitted prominent THG signal (Supp. Fig. 19 A-B). Indeed, we found that 23/25 GFP-
positive cells in the WT organoids and 27/30 cells in the MT organoids emitted somatic THG
signals (Supp. Fig. 20). Cells expressing both GFP and THG signal demonstrated similar
migration deficits in their migration speeds (Supp. Fig. 19 C-D) as described above (Figs. 5-6).

Figure 5: Dynamics of neuronal migration in RTT-WT and RTT-MT organoids. (A) Representative time-lapse THG images of migrating cells in the ventricular zone in RTT-WT (top) and RTT-MT (bottom) organoids in every 2 hours. The migrating trajectory of each representative cells is shown on the right panel to the time-lapse images. Color bar represents the instantaneous speed of these cells. **(B)** Representative track speed over time for cells in RTT-WT (left) and RTT-MT (right) organoids. **(C)** Summary of displacement, average migration speed and straightness of the migration trajectory (n=6 RTT-WT and 6 RTT-MT organoids, 208 cells for WT and 217 cells for MT. Scale bar is 50 μm . **, p<0.01; ***, p<0.001; ****, p<0.0001).

Figure 6: Dynamics of neuronal migration in RTT-WT and RTT-MT organoids. Representative three-dimensional THG images of the live RTT-WT (A) and RTT-MT (C) organoids. Dashed red lines show the planes of imaging presented in (B) and (D). Representative time-lapse, depth-resolved THG images of migrating cells in the VZ region for RTT-WT (B) and RTT-MT (D) organoids in every 2 hours. The migrating trajectory of each representative cells is shown on the bottom right panel to the time-lapse images. Color bar represents the instantaneous speed of these cells. (E) Representative track speed over time for cells in RTT-WT and RTT-MT organoids. (F) Summary of displacement, average migration speed and straightness of the migration trajectory. Scale bar is 50 μm .

DISCUSSION

Since the demonstration that neural differentiation of human induced pluripotent stem cells could be induced in 3D and used to recapitulate the early stages of embryonic brain development, cerebral organoids have raised considerable interest as a model for understanding embryonic stages of cortical development and neurodevelopmental disorders [1-5, 9-17]. However, high-resolution 3D imaging of intact and uncleared organoids is challenging due to high light scattering. Thus, the vast majority of analyses of organoids has been limited to imaging tissue sections or slices, with one-photon microscopy [1, 3, 12, 13] or imaging superficial parts of intact organoids with two-photon microscopy [49]. Especially for live cell imaging, the one photon imaging depth is limited to 20 μm , with improvement to 40 μm with tunable refractive-index matching medium [50]. Two photon imaging depth is limited to couple of hundred of micrometers with live cerebral organoids [49]. To image intact and uncleared organoids, we thus developed custom three-photon microscopy to perform both fluorescence and label free (THG) three-photon imaging. Here, we demonstrate for the first time that 3-photon fluorescence and THG microscopy is capable of high spatial resolution imaging deep within intact human cerebral organoids, enabling 3D imaging of intact cerebral organoids as well as long-term live cell time-lapse imaging of radially migrating neurons in intact cerebral organoids. Since we used moderate repetition rate of the laser (800 kHz), uniquely reduced the pulse width < 30 fs in the deepest parts of the organoid for the first time (**see Methods**), and optimized the intermediate optics in the excitation and emission paths, the average power on the surface of the organoids did not exceed 5 mW, which induced no noticeable tissue damage such as cell death (Supp. Fig. 18). No specific processing of the fixed organoids, such as clearing techniques [51, 52] was necessary; however, incubation in index matching solution improved the imaging depth.

Most of the THG signal we observed in RTT-WT and RTT-MT organoids arose from cellular membranes or showed a high intensity somatic profile observed mostly in the CP region. It was shown previously that the cell nucleus [40, 53] and several organelles in the cell cytoplasm could generate a THG signal [37, 54]. Thus, we propose that the bright somatic signal is the result of the high concentration of organelles in the neuronal soma. THG imaging of intact fixed organoids provided clear demarcation between VZ and CP structures, likely due to differences in cell composition, morphology and density in these regions. This clear label-free demarcation of VZ and CP further allowed us to characterize VZ 3D structures in RTT-WT and RTT-MT organoids. We characterized the extinction lengths of RTT-WT and RTT-MT organoids (Fig. 3C-E) and found that average extinction length of WT organoids is significantly higher than

that of MT organoids. This result can be explained by structural differences between WT and MT organoids including higher surface area, lower radial thickness and most importantly higher density of ventricular regions in MT organoids. Moreover, a bright THG signal arose from neuronal soma, allowing live cell tracking of migrating neurons in the VZ. To check the source of THG signal in the VZ, we labeled migrating neurons with GFP through electroporation. We showed that more than 85% of the GFP positive cells had also significant THG signal, suggesting that label-free THG imaging of live cerebral organoids can reveal the migration trajectories and deficits at the single cell level (Supp Fig. 6-7, 19-20). In addition, we also performed DCX staining to the same field of view that we imaged with our system. This staining also shows that THG signal is mostly overlapped with DCX signal which indicates that THG signal is mostly populated in the cortical plate but not in the ventricular zone (Supp. Fig. 5).

Our findings of 3D morphological alterations in RTT-MT organoids including an increase in total ventricular zone volume, a significant increase in ventricular zone surface area, and a significant decrease in mean ventricle thickness, enable noninvasive and comprehensive quantification of organoids consistent with enhanced proliferation of the progenitor pool[26]. As VZ expansion is uniquely different in human versus mouse neurogenesis[55], it is likely that this phenotype would go unobserved in mouse (or other lissencephalic) models of RTT. Moreover, we and others previously suggested with endpoint experiments that the putative migration of young neurons through the VZ is impaired in mice after *in utero* electroporation of *Mecp2*-sh RNA as well as in human RTT cerebral organoids [26, 56]. However, the dynamics of the migration deficit were never previously characterized. Thus, here we performed label-free live cell tracking in intact and uncleared human cerebral organoids at different depths and locations to explore the dynamics of neuronal radial migration in RTT-WT and RTT-MT organoids. RTT-MT neurons demonstrated reduced migration speed and less straight trajectories and consequently a lowered displacement than RTT-WT cells in cerebral organoids at 5 weeks. Furthermore, these trends in migration speed were independent of the imaging depth or the imaging location in both MT and WT organoids. Although there was a range of radial thickness in MT organoids, lower migration speed and tortuous migration patterns were common. Altered cytoskeleton dynamics have been previously suggested in rat, mouse and human models of Rett syndrome and could contribute to migration defects [57-61]. Non-cell autonomous effects could also contribute to the neuronal radial migration deficit, such as impaired cell-to-cell communication, adhesion and radial glia dysfunction. Further studies are required to study the mechanisms responsible for the migration deficit as well as to rescue the phenotype. Taken together, these results suggest that changes in ventricular wall thickness result not only from the

decreased neurogenesis and increased proliferation of the progenitor pool but also from the abnormally slow displacement and tortuous trajectory of the neurons.

Our findings demonstrate the promise of three-photon and label-free THG imaging for monitoring spatiotemporal dynamics in intact and uncured human cerebral organoids. We believe that our optimized three-photon microscopy system is straightforward to use, and can provide a new platform for monitoring structural and functional abnormalities in fixed and live organoids derived from patients with neurogenetic disorders as well as for therapeutic drug screening.

METHODS

Custom microscope for three-photon and THG imaging

To obtain an excitation wavelength of 1300 nm required for label-free THG imaging deep within intact organoids, ultrashort laser pulses (300 fs, 400 kHz, 16 W) at 1045 nm from a pump laser (Spirit, Spectra Physics) were sent to a noncollinear optical parametric amplifier (NOPA, Spectra Physics). In order to shorten the pulse width on the sample to maximize the generated THG signal, we built a two-prism based external compressor to prechirp the pulse before sending it to the microscope. The internal compressor in NOPA could compress the Gaussian pulse width to 22 fs (which was the transform limited pulse width for 110 nm spectral full width half maximum bandwidth). Due to the dispersion in the microscope, the Gaussian pulse width on the sample was ~250 fs. After dispersing the pulse with 65 cm long prism-pair, and silicon broadband window (Thorlabs, WG81050) the pulse width on the sample was reduced to 27 fs in the deepest part of the organoids (pulse widths < 30 fs obtained, to our knowledge, for the first time). To measure the pulse width inside the organoids, we placed a live organoid (< 1 mm thickness) on the external sensor of the autocorrelator (CARPE). Then, we measured the pulse width of the laser after it exists from the thick organoid. In addition, we built a delay line to double the repetition rate thereby increasing the frame rate of THG imaging. Subsequently, the frame rate at 512x512 pixel resolution was increased up to 2 Hz. Power control was performed with a combination of half-wave plate (AHWP05M-1600, Thorlabs) and low-GDD ultrafast beamsplitter (UFBS2080, Thorlabs) with 100:1 extinction ratio. The laser beams were scanned by a pair of galvanometric mirrors (6215H, Cambridge Technologies) to image the laser spot on the back aperture of the objective using a pair of custom-designed scan and tube lenses. A pair of collection lenses for three photon-multiplying tubes (PMT) collected the emitted signal from the cerebral organoids. To maximize the signal detected in PMTs, optics in both excitation and emission paths were optimized considering the objective lens using Zemax software.

Fluorescent signals from immunostaining labels (7AAD) were detected using GaAsP photomultiplier tubes (H7422A-40, Hamamatsu, Japan) and a bandpass filter (Semrock, 650/70 nm), and THG signal was detected using bialkali (BA) photomultiplier tube (R7600U-200) and a narrow-bandwidth bandpass filter (430/20 nm). A custom designed objective lens (25x, 1.05-N.A., Olympus) was used. Automated image acquisition, control of scanners, and sample stage were carried out using ScanImage (Vidrio)[62]. Imaged cells were located at a depth of 0–2000 μ m below the surface of cerebral organoids. The images were taken with 2 μ m and 5 μ m axial increments in fixed and live organoids, respectively. Laser power ranged from 0.5 – 5 mW at the sample depending on depth and fluorescence expression levels. Both fixed and live organoids were placed on a two-axis motorized stage (MMBP, Scientifica) and the objective lens was placed on a single-axis motorized stage (MMBP, Scientifica) to move it in the axial direction.

Methods for fixed and live organoid imaging

For fixed organoid imaging, five-week-old cerebral organoids were placed into an imaging chamber gasket (C18161, ThermoFisher) filled with index-matching medium Hprotos[51]. Top and bottom parts of the imaging chamber gasket were sandwiched between a glass slide (48311-703, VWR) and a cover glass (48366-227, VWR) (Figure 1B). To perform whole fixed organoid imaging, automatic serial imaging of multiple sites was performed via determining the coordinates of four corners of the entire field of view. A Matlab-based algorithm moved the stage in the required x and y axis locations to image the full field of view and then moved the focal plane in the z direction.

For live organoid imaging, we increased the imaging field of view from 250 μ m to 320 μ m. The five-week-old live organoids were placed in a microincubator (RC-30, Warner Instruments). The microincubator temperature was controlled and maintained at 37°C with a pair of resistive heater elements. A peristaltic pump was connected to the microincubator to provide continuous flow of cell medium supplemented with 5% CO₂, 5%O₂, and balanced N₂ (Airgas) (Fig. 1C). We performed Caspase3 immunostaining to check whether our imaging system induced any significant cell death. Compared with healthy and not-imaged organoids, the organoids which were imaged up to 18 hrs in our imaging system did not show any increased cell death (Supp. Fig. 18).

iPSC generation and colony maintenance

Induced pluripotent stem cells (iPSC) were reprogrammed from Rett Syndrome patient samples obtained from the Coriell cell line repository[26, 45]. These lines were derived from: (1) a patient carrying a MECP2 V247X mutation (Coriell Institute biobank GM07982); (2) a patient with MECP2 R306C mutation (Coriell Institute biobank GM23298). All iPSC lines were primarily and routinely quality-controlled by karyotyping (G-band), mycoplasma testing and pluripotency testing (initially by teratoma test then routinely by immunostaining for pluripotency markers such as Sox2 and Oct4). We examined allele-specific transcription of the MECP2 gene in all the iPSC lines by performing RNA extraction, reverse transcription, PCR and Sanger sequencing of the amplified fragments. In all lines we were able to detect transcription of either the wildtype or the mutant alleles of the MECP2 gene, but not both, confirming the clonality and X inactivation status. We confirmed that the X inactivation profile is maintained at all stages of the organoid differentiation. Cell culture quality check and authentication were performed every ten iPSC passages. iPSC colonies were grown in iPSC media, consisting of 20% Knock-out Serum Replacement (KOSR) (Invitrogen), 1% penicillin/streptomycin (Invitrogen), 1% non-essential amino acids (Invitrogen), 0.5% L-glutamine (Invitrogen), 100 μ M 2-mercaptoethanol (Bio-Rad), DMEM/F-12 (Invitrogen), supplemented with 10 ng/mL bFGF (Stemgent). Culture media was changed daily. iPSC colonies were passaged weekly onto 6-well plates coated with 0.1% gelatin (EMD Millipore) and pre-seeded with a feeder layer of irradiated mouse embryonic fibroblasts (MEFs) (GlobalStem), which were plated at a density of 200,000 cells/well. Passaging entailed lifting colonies with 2.5 mg/ml Collagenase, Type IV (ThermoFisher) for 1 hr and dissociation into smaller pieces through manual trituration before seeding onto feeder layer of MEFs.

Cerebral organoid generation

iPSCs were detached from irradiated MEFs and plated at 9×10^4 cells per well of an ultralow attachment 96-well plate (Corning) in iPSC media supplemented with bFGF (10ng/mL) and ROCK inhibitor (50 μ M; Y-27632, Tocris) (Day 0). Embryoid bodies (EBs) were subsequently transferred (Day 6) in an ultra-low attachment 24-well plate (Corning) to neural induction media : 1% N2 supplement (Invitrogen), 1% Glutamax (Invitrogen), 1% non-essential amino acids (Invitrogen), 5ug/mL heparin (Sigma), DMEM/F12 (Invitrogen), supplemented with 10 μ M SB431542 (Tocris Bioscience) and 1 μ M dorsomorphin (Stemgent). EBs were embedded in Matrigel (Corning) droplets on Day 11 and transferred to neural differentiation media: DMEM/F12 : Neurobasal (Invitrogen), 0.5% N2 supplement, 1% Glutamax, 0.5% non-essential amino acids, 100 μ M 2-mercaptoethanol, insulin, 1% Pen/Strep (Invitrogen) supplemented with

1% B27 without vitamin A (Gibco, Life Technologies). On Day 15, embedded EBs were transferred to a shaker and grown in neural differentiation media supplemented with B27 with vitamin A (Gibco, Life Technologies). Organoids were either fixed on Day 35 by a 3h-incubation in 4% paraformaldehyde solution or imaged under the microscope for live cell characterization.

Cryosectioning and immunostaining

For cryosectioning, fixed organoids were incubated in sucrose solution overnight at 4°C before embedding and freezing in O.C.T medium. Frozen organoid tissue was sliced into 20 µm sections using a cryostat. Permeabilization/blocking was performed using 3% BSA/0.1% TX100 in TBS. Incubation of sections from cerebral organoids in primary antibodies solution was performed overnight at 4°C and in secondary antibodies solution at room temperature for 1 hour (Alexa Fluor, Molecular Probes). The following primary antibodies were used: DCX (Aves Labs, 1:200); PAX6 (Millipore; 1:200); TBR1 (Abcam 1:400); Cleaved caspase-3 (Cell Signaling, 1:500). Coverslips were affixed with ProLong Gold antifade reagent with DAPI (Life Technologies) and z-stacks were acquired using either a Leica TCS SP8 confocal microscope.

Intact organoid staining

For 3D imaging of whole organoids, intact fixed organoids were incubated with a 1:50 solution of the nuclear dye 7-Aminoactinomycin D (7AAD, MolecularProbes) in PBS for 1h at room temperature. Before imaging, organoids were transferred to a shaker and immersed twice for 3h in index matching solution [51]. Z-stacks were acquired with the three-photon microscope described above.

Electroporation

Organoids (specifically, the "ventricles" or progenitor zones) were electroporated with a GFP construct (pCAG-GFP) at 7 weeks post-EB formation. Plasmids were maxi-prepared using Qiagen Maxi kit. DNA solution (0.5 µg/µl) was mixed with Fast Green dye (0.1%) and injected into the organoids using a pulled-glass capillary microelectrode. Successful injection was confirmed by the visualization of Fast Green dye inside the injected organoid. Immediately after DNA injection, four 50-ms electrical pulses (40V) were applied at 1-s intervals using a 5-mm electrode and an electroporator (EM830, BTX). The organoids were returned to the incubator after electroporation. After 3-4 days, the electroporated organoids were subjected to the three-photon microscope for imaging.

Statistical analysis

All the statistical comparisons presented in the main and supplementary figures (Fig. 3E, Fig. 4C, Fig. 5C, Fig. 6F, Supp. Figs. 9-12, and Supp. Figs. 14,16-17) are based on Tukey method where the whiskers are drawn down to the 10th percentile and up to the 90th. Points below and above the whiskers are drawn as individual dots.

Area segmentation and rendering

The area segmentation was performed manually for individual 2-D THG images and confirmed with 7AAD images which are taken as ground truth (Fig. 4A-B). Then, Surface function of Imaris software was used to render these 2-D segmented areas into a 3-D volume. We used both opaque (Fig. 4A-B) and transparent modes (Supplementary Figure 8 C-D) of this function in order to represent 3-D structure of ventricular zones.

Live cell tracking

The three-dimensional THG images were used to characterize the speed, straightness, and displacement of cells in control and mutant organoids. To quantify these features of control and mutant cells, Spots function of Imaris software was used. This function first identifies the location of the individual cell, and tracks them in time-lapse images.

Colocalization of GFP, THG, and 7AAD Signals

The colocalization of GFP, THG, and 7AAD signals were performed through Coloc module of Imaris software. We used our z-stack 2-D images (~1000 images) to form three-channel three-dimensional data sets (Supp. Fig. 6). Then, Coloc module quantified the percentage of overlapping between each channel.

ACKNOWLEDGEMENTS

This work was supported by US National Institute of Health (NIH) grants MH085802 and NS090473 (MS), 4-P41-EB015871 (PTCS), K99EB027706 (MY), US National Science Foundation (NSF) grant EF1451125 (MS), a Picower Institute Engineering Collaboration Grant (MS, PTCS and MY), and an equipment grant from the Massachusetts Life Sciences Initiative. We would like to thank Dr. Alex Albenese and Dr. Kwunghun Chung for their assistance on Hprotos generation. We are grateful to members of the Sur and So labs for their help.

AUTHOR CONTRIBUTIONS

M.Y. conceived the project and designed the microscope with input from P.T.C.S. and M.S. M.S. supervised the work. M.Y. performed imaging experiments and C.D., D.F., S.C. and V.P. generated cerebral organoids. C.D. and V.P. performed cryosectioning, staining, immunostaining and confocal imaging. A.N., L-H.S. and G-L.M. provided cell lines. J.I. carried out organoid electroporation. M.Y. analyzed the data. M.Y. and C.D. wrote the paper and were assisted by P.T.C.S. and M.S.

REFERENCES

1. Lancaster, M.A., et al., *Cerebral organoids model human brain development and microcephaly*. Nature, 2013. **501**(7467): p. 373-+.
2. Qian, X.Y., et al., *Brain-Region-Specific Organoids Using Mini-bioreactors for Modeling ZIKV Exposure*. Cell, 2016. **165**(5): p. 1238-1254.
3. Pasca, A.M., et al., *Functional cortical neurons and astrocytes from human pluripotent stem cells in 3D culture*. Nature Methods, 2015. **12**(7): p. 671-+.
4. Mariani, J., et al., *Modeling human cortical development in vitro using induced pluripotent stem cells*. Proceedings of the National Academy of Sciences of the United States of America, 2012. **109**(31): p. 12770-12775.
5. Kadoshima, T., et al., *Self-organization of axial polarity, inside-out layer pattern, and species-specific progenitor dynamics in human ES cell-derived neocortex*. Proceedings of the National Academy of Sciences of the United States of America, 2013. **110**(50): p. 20284-20289.
6. Velasco, S., et al., *Individual brain organoids reproducibly form cell diversity of the human cerebral cortex*. Nature, 2019. **570**(7762): p. 523-+.
7. Quadrato, G., et al., *Cell diversity and network dynamics in photosensitive human brain organoids*. Nature, 2017. **545**(7652): p. 48-+.
8. Pollen, A.A., et al., *Establishing Cerebral Organoids as Models of Human-Specific Brain Evolution*. Cell, 2019. **176**(4): p. 743-+.
9. Luo, C.Y., et al., *Cerebral Organoids Recapitulate Epigenomic Signatures of the Human Fetal Brain*. Cell Reports, 2016. **17**(12): p. 3369-3384.
10. Camp, J.G., et al., *Human cerebral organoids recapitulate gene expression programs of fetal neocortex development*. Proceedings of the National Academy of Sciences of the United States of America, 2015. **112**(51): p. 15672-15677.
11. Zhang, W., et al., *Modeling microcephaly with cerebral organoids reveals a WDR62-CEP170-KIF2A pathway promoting cilium disassembly in neural progenitors*. Nature Communications, 2019. **10**.
12. Bershteyn, M., et al., *Human iPSC-Derived Cerebral Organoids Model Cellular Features of Lissencephaly and Reveal Prolonged Mitosis of Outer Radial Glia*. Cell Stem Cell, 2017. **20**(4): p. 435-+.
13. Klaus, J., et al., *Altered neuronal migratory trajectories in human cerebral organoids derived from individuals with neuronal heterotopia*. Nature Medicine, 2019. **25**(4): p. 561-+.

- 599 14. Garcez, P.P., et al., *Zika virus impairs growth in human neurospheres and brain organoids*.
600 Science, 2016. **352**(6287): p. 816-818.
- 601 15. Dang, J., et al., *Zika Virus Depletes Neural Progenitors in Human Cerebral Organoids through*
602 *Activation of the Innate Immune Receptor TLR3*. Cell Stem Cell, 2016. **19**(2): p. 258-265.
- 603 16. Mariani, J., et al., *FOXG1-Dependent Dysregulation of GABA/Glutamate Neuron Differentiation in*
604 *Autism Spectrum Disorders*. Cell, 2015. **162**(2): p. 375-390.
- 605 17. Ip, J.P.K., N. Mellios, and M. Sur, *Rett syndrome: insights into genetic, molecular and circuit*
606 *mechanisms*. Nature Reviews Neuroscience, 2018. **19**(6): p. 368-382.
- 607 18. Lyst, M.J. and A. Bird, *Rett syndrome: a complex disorder with simple roots*. Nature Reviews
608 Genetics, 2015. **16**(5): p. 261-274.
- 609 19. Li, Y., et al., *Global Transcriptional and Translational Repression in Human-Embryonic-Stem-Cell-*
610 *Derived Rett Syndrome Neurons*. Cell Stem Cell, 2013. **13**(4): p. 446-458.
- 611 20. Chen, J.L., et al., *Behaviour-dependent recruitment of long-range projection neurons in*
612 *somatosensory cortex*. Nature, 2013. **499**(7458): p. 336-40.
- 613 21. Gomes, A.R., et al., *Modeling Rett Syndrome With Human Patient-Specific Forebrain Organoids*.
614 Frontiers in Cell and Developmental Biology, 2020. **8**.
- 615 22. Trujillo, C.A., et al., *Pharmacological reversal of synaptic and network pathology in human*
616 *MECP2-KO neurons and cortical organoids*. Embo Molecular Medicine, 2021. **13**(1).
- 617 23. Tang, X., et al., *KCC2 rescues functional deficits in human neurons derived from patients with*
618 *Rett syndrome*. Proceedings of the National Academy of Sciences of the United States of
619 America, 2016. **113**(3): p. 751-756.
- 620 24. Kim, J.E., et al., *Investigating synapse formation and function using human pluripotent stem cell-*
621 *derived neurons*. Proceedings of the National Academy of Sciences of the United States of
622 America, 2011. **108**(7): p. 3005-3010.
- 623 25. Farra, N., et al., *Rett syndrome induced pluripotent stem cell-derived neurons reveal novel*
624 *neurophysiological alterations*. Molecular Psychiatry, 2012. **17**(12): p. 1261-1271.
- 625 26. Mellios, N., et al., *MeCP2-regulated miRNAs control early human neurogenesis through*
626 *differential effects on ERK and AKT signaling*. Molecular Psychiatry, 2018. **23**(4): p. 1051-1065.
- 627 27. Li, Y., et al., *Induction of Expansion and Folding in Human Cerebral Organoids*. Cell Stem Cell,
628 2017. **20**(3): p. 385-+.
- 629 28. Lancaster, M.A. and J.A. Knoblich, *Generation of cerebral organoids from human pluripotent*
630 *stem cells*. Nat Protoc, 2014. **9**(10): p. 2329-40.
- 631 29. Andersen, J., et al., *Generation of Functional Human 3D Cortico-Motor Assembloids*. Cell, 2020.
632 **183**(7): p. 1913-+.
- 633 30. Bagley, J.A., et al., *Fused cerebral organoids model interactions between brain regions*. Nature
634 Methods, 2017. **14**(7): p. 743-+.
- 635 31. Karzbrun, E., et al., *Human brain organoids on a chip reveal the physics of folding*. Nature
636 Physics, 2018. **14**(5): p. 515-+.
- 637 32. Lancaster, M.A., et al., *Guided self-organization and cortical plate formation in human brain*
638 *organoids*. Nature Biotechnology, 2017. **35**(7): p. 659-+.
- 639 33. Miura, Y., et al., *Generation of human striatal organoids and cortico-striatal assembloids from*
640 *human pluripotent stem cells*. Nature Biotechnology, 2020. **38**(12).
- 641 34. Rehberg, M., et al., *Label-free 3D visualization of cellular and tissue structures in intact muscle*
642 *with second and third harmonic generation microscopy*. PLoS One, 2011. **6**(11): p. e28237.
- 643 35. Debarre, D., et al., *Imaging lipid bodies in cells and tissues using third-harmonic generation*
644 *microscopy*. Nature Methods, 2006. **3**(1): p. 47-53.
- 645 36. Yildirim, M., N. Durr, and A. Ben-Yakar, *Tripling the maximum imaging depth with third-*
646 *harmonic generation microscopy*. Journal of Biomedical Optics, 2015. **20**(9).

- 647 37. Yelin, D. and Y. Silberberg, *Laser scanning third-harmonic-generation microscopy in biology*.
648 Optics Express, 1999. **5**(8): p. 169-175.
- 649 38. Gualda, E.J., et al., *In vivo imaging of neurodegeneration in Caenorhabditis elegans by third*
650 *harmonic generation microscopy*. Journal of Microscopy, 2008. **232**(2): p. 270-275.
- 651 39. Chang, T., et al., *Non-invasive monitoring of cell metabolism and lipid production in 3D*
652 *engineered human adipose tissues using label-free multiphoton microscopy*. Biomaterials, 2013.
653 **34**(34): p. 8607-8616.
- 654 40. Lin, J., et al., *Label-free three-dimensional imaging of cell nucleus using third-harmonic*
655 *generation microscopy*. Applied Physics Letters, 2014. **105**(10).
- 656 41. Ouzounov, D.G., et al., *In vivo three-photon imaging of activity of GCaMP6-labeled neurons deep*
657 *in intact mouse brain*. Nature Methods, 2017. **14**(4): p. 388-+.
- 658 42. Yildirim, M., et al., *Quantitative third-harmonic generation imaging of mouse visual cortex areas*
659 *reveals correlations between functional maps and structural substrates*. Biomedical Optics
660 Express, 2020. **11**(10): p. 5650-5673.
- 661 43. Yildirim, M., et al., *Functional imaging of visual cortical layers and subplate in awake mice with*
662 *optimized three-photon microscopy*. Nat Commun, 2019. **10**(1): p. 177.
- 663 44. Weisenburger, S., et al., *Volumetric Ca(2+) Imaging in the Mouse Brain Using Hybrid Multiplexed*
664 *Sculpted Light Microscopy*. Cell, 2019. **177**(4): p. 1050-1066 e14.
- 665 45. Nott, A., et al., *Histone deacetylase 3 associates with MeCP2 to regulate FOXO and social*
666 *behavior*. Nat Neurosci, 2016. **19**(11): p. 1497-1505.
- 667 46. Lyst, M.J., et al., *Rett syndrome mutations abolish the interaction of MeCP2 with the NCoR/SMRT*
668 *co-repressor*. Nat Neurosci, 2013. **16**(7): p. 898-902.
- 669 47. Delepine, C., et al., *GSK3ss inhibitor CHIR 99021 modulates cerebral organoid development*
670 *through dose-dependent regulation of apoptosis, proliferation, differentiation and migration*.
671 PLoS One, 2021. **16**(5): p. e0251173.
- 672 48. Gertz, C.C. and A.R. Kriegstein, *Neuronal Migration Dynamics in the Developing Ferret Cortex*.
673 Journal of Neuroscience, 2015. **35**(42): p. 14307-14315.
- 674 49. Mansour, A.A., et al., *An in vivo model of functional and vascularized human brain organoids*.
675 Nature Biotechnology, 2018. **36**(5): p. 432-+.
- 676 50. Boothe, T., et al., *A tunable refractive index matching medium for live imaging cells, tissues and*
677 *model organisms*. Elife, 2017. **6**.
- 678 51. Murray, E., et al., *Simple, Scalable Proteomic Imaging for High-Dimensional Profiling of Intact*
679 *Systems*. Cell, 2015. **163**(6): p. 1500-1514.
- 680 52. Chung, K. and K. Deisseroth, *CLARITY for mapping the nervous system*. Nature Methods, 2013.
681 **10**(6): p. 508-513.
- 682 53. Tsai, M.R., et al., *In vivo optical virtual biopsy of human oral mucosa with harmonic generation*
683 *microscopy*. Biomedical Optics Express, 2011. **2**(8): p. 2317-2328.
- 684 54. Barzda, V., *Visualization of mitochondria in cardiomyocytes by simultaneous harmonic*
685 *generation and fluorescence microscopy*. Optics Express, 2005. **13**(20): p. 8263-8276.
- 686 55. Florio, M. and W.B. Huttner, *Neural progenitors, neurogenesis and the evolution of the*
687 *neocortex*. Development, 2014. **141**(11): p. 2182-2194.
- 688 56. Bedogni, F., et al., *Defects During Mecp2 Null Embryonic Cortex Development Precede the Onset*
689 *of Overt Neurological Symptoms*. Cerebral Cortex, 2016. **26**(6): p. 2517-2529.
- 690 57. Kaufmann, W.E., S. Naidu, and S. Budden, *Abnormal Expression of Microtubule-Associated*
691 *Protein-2 (Map-2) in Neocortex in Rett-Syndrome*. Neuropediatrics, 1995. **26**(2): p. 109-113.
- 692 58. Delepine, C., et al., *MeCP2 deficiency is associated with impaired microtubule stability*. Febs
693 Letters, 2013. **587**(2): p. 245-253.

59. Delepine, C., et al., *Altered microtubule dynamics and vesicular transport in mouse and human MeCP2-deficient astrocytes*. Human Molecular Genetics, 2016. **25**(1): p. 146-157.
60. Cortelazzo, A., et al., *Beta-Actin Deficiency with Oxidative Posttranslational Modifications in Rett Syndrome Erythrocytes: Insights into an Altered Cytoskeletal Organization*. Plos One, 2014. **9**(3).
61. Bhattacharjee, A., et al., *Neuronal cytoskeletal gene dysregulation and mechanical hypersensitivity in a rat model of Rett syndrome*. Proceedings of the National Academy of Sciences of the United States of America, 2017. **114**(33): p. E6952-E6961.
62. Pologruto, T.A., B.L. Sabatini, and K. Svoboda, *ScanImage: flexible software for operating laser scanning microscopes*. Biomed Eng Online, 2003. **2**: p. 13.

SUPPLEMENTARY FIGURES

Supplementary Figure 1

Supplementary Figure 1. Measurement of the pulse width of the laser at 1300 nm wavelength after it exits from the live cerebral organoid. (A) The gaussian pulse width becomes 58 fs after travelling the whole organoid without chirping the pulse width further. (B) After chirping the pulse, we can bring back the pulse width to 27 fs after the laser exits from the whole organoid.

Supplementary Figure 2

Supplementary Figure 2. Comparison of the effect of different pulse widths on the THG signal acquired in a cerebral organoid. (A) A representative three-dimensional reconstruction of THG images of a fixed cerebral organoid. (B) Comparison of THG images with three different pulse widths corresponding to our current study (left column), our previous study (middle column), and ref. 36 (right column) at two different depths (250 μm depth (top), 500 μm depth (bottom)). (C) Comparison of THG signals in the five different regions of interest in the same field of view in these three different studies at two different depths (250 μm (left), and 500 μm (right)). (D) Ratio of THG signal acquired between the current study and previous study or between the current study and ref. 36 at two different depths (250 μm (left), and 500 μm (right)).

Supplementary Figure 3

Supplementary Figure 3. Comparison of one-inch and two-inch size collection optics by placing an iris (I) in front of the collection side of the microscope. (A) Schematic of the experimental setup modification. (B) Imaging of axonal tracts in the white matter via THG microscopy with one-inch size iris (left), and with two-inch size iris (right). (C) Ratio of THG signal acquired with two-inch size iris and with one-inch iris. Scale bar is 50 μm.

Supplementary Figure 4

Supplementary Figure 4. In vivo point spread function (PSF) utilizing green retrobeads in anesthetized mice. (A) 3D rendering of 3p imaging of retrobeads in a 200 μm column within deep cortex, spanning L6 and extending into L5 and the top of WM. Lateral and axial PSF profile for 200 μm above WM (B), 100 μm above WM (C) and at the WM (D).

boundary of WM and L6 (D). Lateral and axial point spread function values are given in the inset of panels B, C, and D.

Supplementary Figure 5

Supplementary Figure 5. Identifying the identity of THG signals in an intact organoid. (Left) Virtual section of an intact organoid where THG signal (white) is mostly populated in the cortical plate (CP)-like zone but not populated in the ventricular zone (VZ). (Right) Staining of the same field of view on the left shows that cells with high THG signal also possess DCX (yellow) signal. The dashed blue line represents the boundary between CP-like region and VZ region.

762 **Supplementary**
 763 **Figure 6**

Supplementary Figure 6. Three-dimensional characterization of electroporated cells in a fixed control organoid. A. 3D reconstruction of label-free THG signal (blue) from an intact fixed organoid show clear overlap with electroporated cells with GFP (green) as well as nuclear labeling (red). THG allows imaging of deep structures (here, till 2 mm). Representative images at depths of 776 μm (B) and 1670 μm (C) show that most of the fluorescent signal (green) from the electroporated cells overlapped with the label free intrinsic THG signal (blue) as well as with the nuclear labeling in both overlapped and inset views (D and E). Scale bars are 50 μm .

Supplementary Figure 7

Supplementary Figure 7: The quantitative analysis of GFP, THG, and 7AAD signal overlap in fixed organoids with respect to depth of imaging. (A) GFP signal overlap with THG and 7AAD signals with respect to depth of imaging in a wild-type organoid. (B) Overall overlap between GFP, THG, and 7AAD signals in a wild-type organoid. (C) GFP signal overlap with THG and 7AAD signals with respect to depth of imaging in a mutant organoid. (D) Overall overlap between GFP, THG, and 7AAD signals in a mutant organoid.

788

Supplementary Figure 8

789

790

791 **Supplementary Figure 8. Three-dimensional characterization of control and mutant VZ regions in intact**
 792 **organoids.** VZ area segmentation in opaque (A) and transparent (C) modes in control organoids. VZ area
 793 segmentation in opaque (B) and transparent (D) modes in mutant organoids

794

Supplementary Figure 9

795

796 **Supplementary Figure 9. Comparison of average volume of ventricles per organoid in RTT-WT and RTT-MT**
 797 **organoids for Line 1 and Line 2.** (A) The average volume of ventricles per organoid of WT and MT organoids in
 798 Line 1 are $1.07 \pm 0.25 \times 10^7 \mu\text{m}^3$ and $1.43 \pm 0.20 \times 10^7 \mu\text{m}^3$, respectively (n=5 organoids, p=0.3056). (B) The average
 799 volume of ventricles per organoid of WT and MT organoids in Line 2 are $1.19 \pm 0.28 \times 10^7 \mu\text{m}^3$ and $1.36 \pm 0.22 \times 10^7$,
 800 respectively (n=5 organoids, p=0.6506).

801

Supplementary Figure 10

802

803 **Supplementary Figure 10. Comparison of average surface area of ventricles per organoid in RTT-WT and**
 804 **RTT-MT organoids for Line 1 and Line 2. (A)** The average surface area of ventricles per organoid of WT and MT
 805 organoids in Line 1 are $6.01 \pm 1.13 \times 10^5 \mu\text{m}^2$ and $11.5 \pm 1.02 \times 10^5 \mu\text{m}^2$, respectively ($n=5$ organoids, $p=0.0017$). (B)
 806 The average volume of ventricles per organoid of WT and MT organoids in Line 2 are $5.39 \pm 0.98 \times 10^5 \mu\text{m}^2$ and $10.6 \pm$
 807 $1.0 \times 10^5 \mu\text{m}^2$, respectively ($n=5$ organoids, $p=0.0021$).

808

Supplementary Figure 11

809

810

811 **Supplementary Figure 11. Comparison of average volume per unit area of ventricles per organoid in RTT-WT**
 812 **and RTT-MT organoids for Line 1 and Line 2. (A)** The average volume per unit area of ventricles per organoid of
 813 WT and MT organoids in Line 1 are $24.45 \pm 6.01 \mu\text{m}$ and $10.53 \pm 0.40 \mu\text{m}$, respectively ($n=5$ organoids, $p=0.0006$).
 814 (B) The average volume of ventricles per organoid of WT and MT organoids in Line 2 are $21.76 \pm 5.68 \mu\text{m}$ and $9.45 \pm$
 815 $0.37 \mu\text{m}$, respectively ($n=5$ organoids, $p=0.0013$).

816

817

Supplementary Figure 12

818

819 **Supplementary Figure 12. Comparison of average number of ventricles per organoid in RTT-WT and RTT-MT**
820 **organoids for Line 1 and Line 2. (A)** The average number of ventricles per organoid of WT and MT organoids in
821 Line 1 are 9.9 ± 0.4 and 19.1 ± 0.8 , respectively ($n=5$ organoids, $N=50$ ventricles for WT and $N=100$ for MT
822 organoids, $p<0.0001$). **(B)** The average number of ventricles per organoid of WT and MT organoids in Line 2 are
823 15.2 ± 0.8 and 24.2 ± 1.5 , respectively ($n=5$ organoids, $N=75$ ventricles for WT and $N=120$ for MT organoids, $p<0.0001$).

824

Supplementary Figure 13

825

826 **Supplementary Figure 13: Representative long-term imaging of live mutant organoid at 1 mm depth. A.**(Top)
827 Long-term THG imaging of RTT-MT organoids for 4 days. THG signal is acquired homogeneously in an intact
828 organoid. (Bottom) Long-term TPEF imaging of RTT-MT organoid for 4 days. TPEF signal is mostly confined to the
829 periphery of the organoid. This signal is generated by labeling neurons through GFP electroporation. Scale bar is 1
830 mm. **B.** Long term imaging of RTT-MT organoids in the field of view highlighted in blue square in part A. Four cells
831 are highlighted with arrows in different colors. Dashed green lines represent the apical surfaces of the ventricular
832 regions. Scale bar is 200 μ m.

833

834

Supplementary Figure 14

835

836 **Supplementary Figure 14. Comparison of average speed of migrating cells in RTT-WT and RTT-MT organoids**
 837 **for Line 1 and Line 2.** (A) The average speeds of WT and MT organoids in Line 1 are $20.6 \pm 0.1 \mu\text{m/hr}$ and $11.4 \pm$
 838 $0.1 \mu\text{m/hr}$, respectively ($n=3$ organoids, $N=100$ cells for WT and $N=105$ for MT organoids, $p<0.0001$). (B) The
 839 average speeds of WT and MT organoids in Line 2 are $25.7 \pm 0.2 \mu\text{m/hr}$ and $14.6 \pm 0.1 \mu\text{m/hr}$, respectively ($n=3$
 840 organoids, $N=108$ cells for WT and $N=112$ for MT organoids, $p<0.0001$).

841

Supplementary Figure 15

842

843 **Supplementary Figure 15: The distribution of migration speeds of the cells with respect to the depth of**
 844 **imaging in WT and MT organoids** (A) Collective representation of migration speeds with respect to depth of
 845 imaging in WT organoids. (B) Collective representation of migration speeds with respect to depth of imaging in MT
 846 organoids. (C) Representation of migration speeds of each organoid with respect to depth of imaging in WT
 847 organoids. (D) Representation of migration speeds of each organoid with respect to depth of imaging in MT organoids

848

Supplementary Figure 16

849

850 **Supplementary Figure 16. Comparison of the straightness of the migration trajectory of cells in RTT-WT and**
 851 **RTT-MT organoids for Line 1 and Line 2. (A)** The straightness of the migration trajectory of WT and MT organoids
 852 in Line 1 are 0.828 ± 0.004 and 0.581 ± 0.005 , respectively ($n=3$ organoids, $N=100$ cells for WT and $N=105$ for MT
 853 organoids, $p<0.0001$). (B) The straightness of the migration trajectory of WT and MT organoids in Line 2 are $0.924 \pm$
 854 0.004 and 0.717 ± 0.005 , respectively ($n=3$ organoids, $N=108$ cells for WT and $N=112$ for MT organoids, $p<0.0001$).

855

Supplementary Figure 17

856

857 **Supplementary Figure 17. Comparison of the displacement of migrating cells in RTT-WT and RTT-MT**
 858 **organoids for Line 1 and Line 2. (A)** The displacement of WT and MT organoids in Line 1 are $199.0 \pm 2.2 \mu\text{m}$ and
 859 $102.0 \pm 1.6 \mu\text{m}$, respectively ($n=3$ organoids, $N=100$ cells for WT and $N=105$ for MT organoids, $p<0.0001$). (B) The
 860 displacement of WT and MT organoids in Line 2 are $281.4 \pm 3.4 \mu\text{m}$ and $157.9 \pm 2.1 \mu\text{m}$, respectively ($n=3$ organoids,
 861 $N=108$ cells for WT and $N=112$ for MT organoids, $p<0.0001$).

862

863

864

Supplementary Figure 18

865

866 **Supplementary Figure 18: Characterization of cell viability in wild type (WT) and mutant (MT) organoids via**
867 **Caspase3 immunostaining.** (A) Representative images of a control (left) and an imaged (right) wild type organoids.
868 (B) Representative images of a control (left) and an imaged (right) mutant type organoids. (C) Representative image
869 of a 90-days old organoid as a positive control which has significant Caspase3 positive cells (left) and quantification
870 of percentage of Caspase3 positive cells in each condition (right). The 90-days old organoid has high percentage of
871 Caspase3 positive cells (>10%) whereas both control and imaged WT and MT organoids (0hr and 18 hr respectively)
872 have low percentages of Caspase3 positive cells (<2%). Graphs represent mean +/- SEM.

873

Supplementary Figure 19

874
875

Supplementary Figure 19: Validation of THG signal from identified neurons in organoids. A. Time-lapse THG and GFP-positive images of representative migrating cells in the ventricular zone in a RTT-WT organoid. B. Comparison between THG (left) and GFP-positive (middle) signals identified with three-photon epifluorescence (TPEF). THG and GFP-positive cells overlap (right, arrows). C. Tracking speed over time of GFP-labeled neurons in mutant (RTT-MT) and control (RTT-WT) organoids. D. Average speed of cells in control and mutant organoids. N=3 for both WT and MT organoids. n=25 cells for WT, 30 for MT.

881

882

883

884

885

886

887

Supplementary Figure 20

888

889 **Supplementary Figure 20: The quantitative analysis of the overlap between GFP and THG signals in live WT**
 890 **and MT organoids.** (A) 23/25 GFP-positive cells have also THG signals in WT organoids. (B) 27/30 GFP-positive
 891 cells have also THG signals in MT organoids.

892

SUPPLEMENTARY VIDEOS

893 **Supplementary Video 1.** Depth-resolved z-stack images of an intact, fixed RTT-MT organoid.
 894 Red color represents the 7AAD nuclear stain, and green color represents the label-free THG
 895 signal. Increments in the z direction are 2 μ m and absolute values of the z-position are
 896 presented on the top-left part of each image.

897 **Supplementary Video 2.** Depth-resolved z-stack images of an intact, fixed RTT-WT organoid.
 898 Red color represents the 7AAD nuclear stain, and green color represents the label-free THG
 899 signal. Increments in the z direction are 2 μ m and absolute values of the z-position are
 900 presented on the top-left part of each image.

901 **Supplementary Video 3.** Depth-resolved z-stack THG images up to 2 mm depth of an intact,
 902 fixed RTT-WT organoid. This video exemplifies that the maximum imaging depth of an intact,
 903 fixed organoid is limited by either the thickness of the organoid or the working distance of the
 904 objective lens. Increments in the z direction are 2 μ m and absolute values of the z-position are
 905 presented on the top-left part of each image.

906 **Supplementary Video 4.** THG signal acquired from an intact RTT-MT organoid was segmented
 907 into individual ventricular regions. Each color represents a three-dimensional structure of an
 908 individual ventricular region.

909 **Supplementary Video 5.** Neuronal migration in isogenic control (RTT-WT) intact live organoid
 910 imaged over the course of 12 hours. Neurons, identified with third harmonic generation (THG)
 911 imaging, are observed migrating in the ventricular zone from the ventricular cavity (at top)

912 towards the cortical plate (at bottom). Colored line for each neuron represents its migration
913 trajectory. Color bar at bottom right represents instantaneous velocity ($\mu\text{m}/\text{h}$) of neurons.

914 **Supplementary Video 6.** Neuronal migration in mutant (RTT-MT) intact live organoid imaged
915 over the course of 12 hours. Neurons, identified with THG imaging, are observed migrating from
916 the ventricular cavity (at top) through the ventricular zone towards the cortical plate (at bottom).
917 Colored line for each neuron represents its migration trajectory. Color bar at bottom right
918 represents instantaneous velocity ($\mu\text{m}/\text{h}$) of neurons.