Optimized CUBIC protocol for 3D imaging of chicken embryos at single-cell resolution

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#Equal contribution

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**Summary statement:** This work validates a CUBIC protocol for whole chicken embryo brain and *in-toto* chicken embryo 3D histological imaging.

**ABSTRACT**

The CUBIC tissue clearing protocol has been optimized to produce translucent immunostained whole chicken embryos and embryo brains. When combined with multispectral light sheet microscopy, the validated protocol presented here provides a rapid, inexpensive and reliable method for acquiring accurate histological images that preserve three-dimensional structural relationships with single-cell-level resolution in whole early-stage chicken embryos, and in the whole brains of late-stage embryos.
INTRODUCTION

The easy accessibility and physiological independence of chicken embryos have made them an important biological model system for over a century in the fields of developmental biology, neurobiology, genetics, immunology, cancer, virology, cardiovascular and cell biology (Stern, 2005). Recent chicken embryo work has revealed dynamic gene expression patterns underlying somite formation (Pourquié, 2004; Davey and Tickle, 2007), unexpectedly large variations in embryonic brain regional metabolic activity during the last 20% of in-ovo development (Balaban et al., 2012), and no coordinated patterns of brain gene expression resembling sleep or waking (Chan et al., 2016).

To better exploit the potential of chicken embryos for simultaneously examining electrophysiological, metabolic and molecular correlates of the brain-wide development of neural network activity, it is necessary to adapt methods that can rapidly and efficiently detail the structure and gene expression of developing networks in three dimensions with single-cell-level resolution. This was not achieved by previous 3D technologies such as Optical Coherence Tomography and PhotoAcoustic Tomography (Wong et al., 2013).

Optical imaging of tissue samples is highly limited by visible range light-scattering. While penetration depths of up to 1mm have been achieved with two-photon microscopy (Theer et al., 2003), conventional confocal microscopy remains limited to 100µm (Poguzhelskaya et al., 2014; Nehrhoff et al., 2016). This requires large tissue samples to be cut into thinner sections, resulting in both tissue geometry distortion and loss of precise 3D morphology. New tissue-clearing methods enable analysis of thicker samples (Susaki et al., 2014), that are well-suited for new volumetric imaging modalities such as light sheet microscopy (Ripoll et al., 2015; Arranz and Ripoll, 2015). The Clarity method clears mouse brains yet retains immunohistochemical compatibility (Chung et al., 2013), providing a way to acquire 3D images with single-cell resolution without having to cut specimens into thin sections. We present a validated protocol for whole chicken embryos and whole chicken embryonic brains using modifications of an alternative tissue clearing method (the Clear, Unobstructed Brain Imaging Cocktails and computational analysis [CUBIC] method of Susaki et al., 2014, 2015) combined with a light sheet microscope adapted to generate 3D quantitative images with single-cell resolution.
RESULTS AND DISCUSSION

We modified, optimized, and validated the CUBIC technique on early whole chicken embryos and late-stage chicken embryo brains, and assessed the impact of clearing on sample transparency, size, and cellular and subcellular tissue integrity using CT imaging and confocal, light-sheet and electron microscopy.

It is well known that embryonic tissue differs from that of adult organisms, both in the type and the number of cells, and in chemical composition. Although six days of incubation in the lipid-removing Reagent 1 were necessary to clear adult mouse brains, two days were sufficient to achieve a highly transparent sample for late-stage chicken embryo brains of similar size (Fig. 1A, B) and 4 hours for early-stage whole embryos (Fig. 1C, D, G). Transparency in embryo brains was compared to a widely-used clearing protocol for light-sheet microscopy imaging, Benzyl Alcohol Benzyl Benzoate (BABB) (Genina et al., 2010) (Fig. 2A). Both BABB and CUBIC achieved similar transparency.

CUBIC was initially reported to cause swelling after immersion of the sample in Reagent 1; this effect was reduced after sucrose dehydration steps (Susaki et al., 2015). Changes in the weight of isolated late-stage chicken embryo brains treated with both BABB and CUBIC were assessed before and after clearing. BABB significantly reduced chicken embryo brain weight by an average of ~25%, while CUBIC resulted in a non-significant increase of less than 10% in average brain weights (Fig. 2B). A subset of embryo brains had CT images taken before and after clearing. Data analysis revealed a highly significant correlation between changes in brain weight and changes in brain volume (r=0.94, p<0.0001, n = 13; Fig. 2C). BABB significantly decreased brain weight and brain volume, while CUBIC resulted in non-significant increases in both weight and volume (Fig. 2C). This indicates that brain weight changes during the clearing process are a reliable proxy for brain volumetric changes.

In CUBIC treated brains, general tissue morphology (assessed with Hematoxylin/Eosin [H/E] staining) was well-maintained at cellular and subcellular levels of resolution, even though much of the tissue lipid content was lost (Fig. 2D). Transmission Electron Microscopy (TEM) revealed that subcellular structures were largely preserved, even though the relative lack of lipids resulted in decreased image contrast (Fig. 2E, Fig. S1; previously described by Susaki et al., 2014; the osmium tetroxide used for TEM staining binds to lipids to enhance image contrast. R1 removes lipids, decreasing osmium tetroxide binding, so that the image contrast also decreases). In summary, the CUBIC
protocol employed here maintains the general integrity of cellular and tissue structures, increasing brain volume between ~3-10% on average, while BABB shrinks brain volumes by ~20-30%. These results agree with similar measurements using BABB- and CUBIC-cleared mouse embryos and embryonic heart tissue (Kolesova et al., 2016).

Entire brains from embryos incubated for 16 days (E16) were cleared with the optimized CUBIC methodology and triple-immunostained to show orexin and catecholaminergic (TH-positive) neurons and cFos-active nuclei with DAPI counterstaining to identify all cell nuclei. These staining combinations were previously used by Landry et al. (2014, 2016) and Chan et al. (2016) in chicken embryos and post-hatched chicks. Lightsheet microscopy of the CUBIC-cleared brains produced images with sufficient resolution to clearly recognize single labelled cells in the hypothalamus (Fig. 1E, F, Movies 1-8), as well as to follow their labelled neuronal processes (Fig. S3). A similar protocol was developed for E4 whole chicken embryos, with shorter incubation times (Fig. 1C).

Compatibility with confocal microscopy was assessed using 1-mm thick sections of chicken embryo brain. We investigated whether penetration depth could be increased by clearing. High transparency was achieved in thick tissue sections after immersion in Reagent 1 for four hours (Fig. 4A). This treatment enabled incubation times to be reduced to one day for each of the primary and secondary antibody solutions. A penetration depth of 500µm was achieved with a 10x objective and a depth of ~150µm was achieved with a 20x objective (Fig. 3A). In contrast, uncleared control sections produced more light scattering, which limited penetration to ~100µm with a 10x objective. To confirm that the penetration depth was limited by light scattering rather than insufficient antibody penetration, the 1-mm sections were sliced into ~150µm sections along their z-axis. Positive TH and DAPI signals were obtained from all five thin slices throughout the depth of the z-axis, confirming that the antibodies fully penetrated the 1-mm section (Fig. 3B). Taken together, these results demonstrate that an optimized CUBIC protocol can also be used for thick tissue sections with confocal microscopy.

To confirm that the optimized CUBIC-clearing and immunostaining protocol provides comparable results to standard histology and immunohistochemistry, we compared our results to those studied by Godden et al. (2014), Landry et al. (2014, 2016) and Chan et al. (2016). The stained cells obtained with our optimized protocol showed identical spatial distributions to those obtained in these previous studies at both E16 (orexinergic neurons are shown in Fig. S4, Movie 6) and at E20 (Movie 8).
In conclusion, the validated CUBIC method proposed here represents an important resource facilitating future chicken embryo imaging studies, and provides a powerful combination of clearing and immunostaining compatible with both 3D laser sheet microscopy and confocal imaging that can be used for studying DNA, RNA and protein expression patterns, neuronal connectivity, and subcellular-to-systems brain morphology at single-cell resolution.

MATERIALS AND METHODS

Egg incubation and sample preparation
Fertilized chicken eggs were incubated in standard conditions. Immersion-fixed E4 whole embryos and perfusion-fixed E16, E18 and E20 chicken embryo brains were obtained. One-millimeter-thick coronal sections were obtained by cutting perfusion-fixed brains using a vibratome (Vibrating Microtome 7000smz-2, Campden Instruments Ltd., Loughborough, UK) and were collected in rostral-to-caudal order (Fig. 4A, B).

Optimized CUBIC clearing protocol
Brains were incubated in Reagent 1 in a shaker for 2 days at 37°C at 80rpm and then washed with PBS 3 times for 2 hours at room temperature. Thereafter, they were dehydrated for 30 minutes in 20% sucrose in PBS and then incubated in Reagent 2 for 1 day at 37°C and 80rpm. Incubation times in Reagent 1 and 2 were reduced for whole embryos (to 4 and 2 hours, respectively) and One-millimeter-thick sections (4 hours, each). Solutions are described in Supplementary Information.

Transparency measurements
The transparency in brains cleared with the optimized CUBIC and BABB (as in Genina et al., 2010) protocols were compared with a conventional light microscope. Detailed measurement information is provided in Supplementary Information.
CT imaging
The volumes of brains cleared with the optimized CUBIC and the BABB protocols were measured using an Argus PET/CT preclinical scanner (SEDECAL, Madrid, Spain). Details are provided in Supplementary Information.

TEM
Optimized CUBIC-cleared and uncleared brains were postfixed in osmium tetroxide and potassium ferricyanide, cut in ultrathin sections and examined by TEM using a JEOL 1230 microscope (IZASA Scientific SLU, Madrid, Spain) for ultrastructural analyses (further details in Supplementary Information).

Immunostaining
Fluorescent immunostaining and optimized CUBIC clearing protocols were integrated for chicken embryo brains (Fig. 1A). Brains were incubated in Reagent 1 (containing DAPI; Invitrogen; 1:5000) for 2 days, washed, then incubated in primary antibody solution (antiorexin, -TH, and -c-Fos antibodies; together, each at 1:250) in a shaker for 3 days at 37°C and 80rpm. After washes, the fluorescent secondary antibody solution (each at 1:300) was applied for 3 days at 37°C and 80rpm. Brains were then washed, dehydrated in sucrose solution and finally incubated in Reagent 2 for 1 day. Similar protocols were developed with reduced incubation times in the two antibody solutions for early-stage whole chicken embryos (12 hours, each; Fig. 1C) and thick sections (1 day, each). Antibodies are listed in Table S1.

Light sheet microscopy
Immunostained brains were analysed with a custom made light sheet microscope. Information about the set-up, image acquisition and processing are found in Supplemental Information, Fig. S2, and Table S2.

Confocal microscopy
An inverted confocal microscope (Leica TCS SPE Confocal Microscope, Leica Microsystems) was used. Confocal images from one-millimeter-thick coronal sections were acquired and processed as described in Supplementary Information.
Acknowledgments

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Competing financial interests

The authors declare no competing financial interests.

Author contributions

MVG, EB and MP designed and performed the experiments. DB, MVG, MTL and JJV imaged embryos and performed the image processing. EB, JJV and MD supervised the experiments. JR designed and assembled the light sheet microscope, and developed the control software. MVG, MP, JJV, EB and MD wrote the manuscript.

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Supplementary information

Supplementary information available online.
References


Figure 1. Chicken embryo clearing. A: Schedule for chicken embryo brain clearing and immunostaining. B: Appearance of an E16 brain after each protocol step. Brains become transparent after treatment with Reagent 1, opaque when washed and immunostained, and transparent again after incubation with Reagent 2. C: Schedule for whole chicken embryo development.
clearing and immunostaining. **D:** Untreated (left) and cleared (right) E4 embryos. Bar, 4 mm. 

**E, F:** Light-sheet microscope fluorescence images (inset with rectangle shows brain location of 3D image) of an E16 whole brain (red: orexinergic neuron bodies; grey: DAPI). **G:** Midsagittal view of a whole E4 chicken embryo. Axes: (L) lateral; (D) dorsal; (R) rostral. Anatomical markers: (3v) third ventricle; (b) brain; (t) tail.
Figure 2. CUBIC and BABB clearing of chicken embryo brains. Symbols above all graphs denote statistically-significant differences; groups with different symbols are significantly different from each other. A, Light attenuation in CUBIC brains (n=8; measured before and after clearing) and BABB brains (n=8; only measured after clearing). Left graph: CUBIC brains showed a highly significant reduction in light attenuation after clearing [before (open black circles, bar indicates mean): 0.77±0.01 (s.e.m.) µ/mm, after (open grey circles, bar indicates mean): 0.017±0.001; Wilcoxon matched-pairs sign-rank test, 8/8 differences<0, z=-2.521, p=0.012]. After clearing, the attenuation coefficients of BABB–cleared (filled grey circles; bar indicates mean) and CUBIC-cleared brains were not significantly different from each other [Mann-Whitney U-test, U=31, U'=33, z=-0.105, p=0.92]. Right: Percentage change in light attenuation of CUBIC-cleared brains (bar indicates mean = -97.79±0.17%). B,
Weight measurements of E16 chicken embryo brains before and after clearing. Left graph: A Scheirer-Ray-Hare 2-Way ANOVA with Method (BABB [filled circles] vs. CUBIC [open circles]) and Time (Before Clearing [black circles] vs. After Clearing [grey circles]) as factors indicated a significant overall difference due to Method (H=10.75, df=1, p=0.001), no significant difference due to Time (H=3.48, df=1, p=0.062) and a significant Method*Time interaction (H=12.69, df=1, p=0.0003). Post-hoc Wilcoxon Matched-pair Post-hoc tests corrected for multiple comparisons indicated that BABB samples significantly decreased their weight (mean±s.e.m. before=0.75±0.03g, after=0.55±0.03g, 15/15 differences< 0, z=-3.41, p=0.0014) while CUBIC samples did not (mean±s.e.m. before=0.75±0.01g, after=0.81±0.03g, 8/14 differences<0, z=-1.73, p=0.17). There was no significant difference between BABB and CUBIC weights prior to clearing (Mann-Whitney U-test corrected for multiple comparisons, U=96, U’=114, z=-0.39, p>0.95), while there was a significant difference after clearing (U=6, U’=204, z=-4.3, p<0.0001). Right graph: Percentage change in the weight of BABB-cleared brains (filled circles, bar indicates mean=-26.49±2.25%) and CUBIC-cleared brains (open circles, bar indicates mean=8.96±4.23%); the two methods had significantly different percentage weight changes (Mann-Whitney U-test, U=2, U’=208, z=-4.49, p<0.0001). C, The relationship between weight change and volume change. The final 13 brains (7 BABB, 6 CUBIC) processed for measurement in (B) above were subjected to CT imaging before and after clearing (see Supplementary Information), and their 3-dimensional volumes were calculated from the CT images. There was a highly-significant correlation between % Weight Change (x-axis) and % Volume change (y-axis) (r=0.94, n=13, p<0.0001); BABB, solid (black) circles, CUBIC, open (white) circles; the black plus indicates mean BABB values (weight change: -33.93±2.22%; volume change: -26.62±3.99%); the white plus indicates mean CUBIC values (weight change: 18.79±2.22%; volume change: 10.83±4.10%). BABB and CUBIC samples had significantly different weight changes (Mann-Whitney U-test, U=0, U’=42, z=-3.00, p=0.0027) and volume changes (Mann-Whitney U-test, U=0, U’=42, z=-3.00, p=0.0027). The BABB brains showed significant pre-to-post-clearing changes in both weight and volume (Wilcoxon matched-pairs sign rank test corrected for multiple comparisons, both variables 7/7 differences <0, z=-2.37, p=0.036), while the CUBIC brains did not show significant changes in either weight or volume (Wilcoxon matched-pairs sign rank test corrected for multiple comparisons, both variables 1/6 differences <0, z=-1.99 p=0.093). D, Hematoxylin/Eosin (H/E) staining of CUBIC-cleared (right) and uncleared (left) chicken embryo brains. Images were acquired with 20x (top) and 40x (bottom) objectives. Cytoplasm and nuclear staining appear generally
preserved after clearing. E, Subcellular resolution observed with transmission electron microscopy in cleared (right) and uncleared (left) chicken embryo brain. Membrane integrity was preserved in uncleared fixed brains and less so in the cleared fixed ones. Arrows indicate membranes (top) that are shown at higher magnification (bottom). Bars, 4µm (top) and 2µm (bottom).
Figure 3. The optimized clearing protocol improves penetration of laser light and antibodies into thick brain sections for confocal microscopy. A: Confocal images of thick hypothalamic sections (inset with rectangle shows brain location of 3D image) in an E16 chicken embryo brain. Images were obtained from untreated (left) and cleared (right) sections at different depths, as indicated. B: Antibody penetration in cleared sections. Cleared thick sections were sliced (vibratome) and visualized in the confocal microscope to study the depth of antibody penetration (z-axis). Blue, DAPI stained nuclei; red, TH-positive neuron bodies. Scale bar, 100µm for all images. 10x objective. Z-stack step size: 23µm.
Figure 4. CUBIC clearing and immunostaining imaging of thick sections from E16 chicken embryo brains. A: Four consecutive rostral (R) to caudal (C) sections (red rectangles [1-4] in panel B; D, dorsal, V, ventral) from a brain before (left) and after (right)
CUBIC clearing. **B:** Schematic sagittal and coronal brain sections showing the hypothalamic region imaged in panel D (marked in light blue); inset with rectangle shows the location of the 3D image in panel C. **C:** 3D volumetric rendering of neurons in the hypothalamus. Red, TH-positive neurons; green, orexinergic neurons. Scale bar, 100μm. 10x objective. **D:** Confocal images of the same hypothalamic region obtained with different filters. Grey, c-Fos positive nuclei (upper left panel); green, orexinergic neurons (upper right panel); red, TH-positive neurons (lower left panel); blue, DAPI stained nuclei (lower right panel, with all images superimposed). Scale bars, 100μm. 10x objective. Z-stack step size: 23μm.
SUPPLEMENTARY METHODS

Egg incubation
Fertilized chicken eggs (COBB500 white broiler; Gallus gallus) were generously provided by COBB Española S.A. They were incubated under constant conditions (37.5°C and 60% relative humidity) in a commercial incubator (Ova-Easy 380 Advance Series II Cabinet Incubator, Brinsea, Weston-Super-Mare, UK) until the desired developmental stage was reached.

Sample preparation
Chicken embryos were anesthetized with isoflurane in ovo. Young embryos at day 4 were fixed by immersion in 4% Paraformaldehyde (PFA) in chick embryology phosphate buffered saline (cPBS, 150 mM NaCl, 2.8 mM KH₂PO₄, 7 mM K₂HPO₄; pH 7.4), at 4°C overnight. Embryos at day 16, 18, and 20 were fixed with 4% PFA in cPBS by transcardial perfusion. The heads were then postfixed in 4% PFA in cPBS, at 4°C overnight. Brains were extracted, washed and kept in cPBS at 4°C until used.

Solutions for the optimized CUBIC clearing and immunostaining protocols
The whole-organ clearing method described by Susaki et al. (2014, 2015) for mouse brain and other organs was optimized in the present study for early whole embryos and late-stage chicken embryo brains.

Reagent 1 was prepared by mixing 3.5 mL of distilled water (35% by wt), 2.5 g of urea (25% by wt), 2.5 g of N, N, N’, N’-tetakis (2-hydroxy-propyl) ethylenediamine (25% by wt) and 1.5 g of Triton X-100 (15% by wt).

Reagent 2 was prepared by mixing 1.5 mL of distilled water (15% by wt), 1 g of triethanolamine (1% by wt), 5 g of sucrose (50% by wt) and 2.5 g of urea (25% by wt).

For immunostaining, the following solutions were used: a) primary antibody solution containing the primary antibodies (see Table S1) in PBS (137 mM NaCl, 1.5 mM KCl, 8 mM Na₂HPO₄, 2.7 mM KH₂PO₄, pH 7.4), with the addition of bovine serum albumin (1% by wt), sodium azide (0.02% by wt), donkey serum (10% by vol), and Triton (0.1% by wt); b) secondary antibody solution with the secondary antibodies (see Table S1) in PBS
containing also bovine serum albumin (0.2% by wt), sodium azide (0.02% by wt), donkey serum (10% by vol). Both solutions were adjusted to pH 7.4. The three antibodies have been previously validated for use in birds by Western blot (anti-orexin: Godden et al., 2014; anti-TH: Chan et al., 2016; anti-c-Fos: Mouritsen et al., 2004).

Sucrose solution (20% by wt in PBS) was used for sample dehydration.

**Transparency measurements**

The transparency of the samples was measured by taking bright-field pictures with a conventional light microscope. Transparency of the CUBIC protocol cleared chicken embryo brains was compared to that obtained with the BABB protocol (Genina et al., 2010).

The sample was trans-illuminated with a light source and the light transmitted through it was captured by a camera on the opposite side through a 10x objective. Two pictures were taken: one without the sample, where the light intensity emitted by the source reached the detector since there was no object in the way ($I_0$) and a second one with the cleared brain between the light source and the detector, where only light that passed though the sample reached the camera ($I$). The transparency was calculated in terms of the attenuation coefficient, following the Beer-Lambert law:

$$I = I_0 \cdot e^{-\mu \cdot L}$$

where $I$ is the intensity of the light transmitted through the sample, $I_0$ is incident light intensity, $\mu$ is the attenuation coefficient and $L$ is the sample thickness that the light crosses in its pathway to the detector. $I$ and $I_0$ intensities were measured by taking the average pixel value in the exact region of interest both in the background and in the sample picture. The thickness of the sample was established from the 3D SPIM images. The attenuation coefficient $\mu$ was calculated as:

$$\mu = \frac{-\ln (I/I_0)}{L}$$
CT image acquisition and analysis
CT images of perfused and fixed whole chicken brains isolated from the skull and surrounding tissue were obtained using an Argus PET/CT preclinical scanner (SEDECAL, Madrid, Spain), with cone-beam geometry based on a tungsten anode x-ray tube and a digital CMOS X-ray detector. Imaging parameters were: Current: 340 µA; Voltage: 40 kV; Number of exposures per angle: 8; Angular sampling: 1 projection per degree; the images were acquired using MMWKS VISTA_PET/CT software version conducted using the FBK algorithm contained in Mangoose (GPU) software version 1.5, (SEDECAL, Madrid, Spain). The resulting 3D images had a spatial resolution of 120x120x120 µm³, and the brain volumes were calculated by thresholding the 3D brain image from the surrounding air using MMWKS software analysis tools. The same brains were imaged both before and after clearing.

Hematoxylin-eosin staining and ultrastructure analysis by TEM
Chicken embryos were perfused as described, and their brains were dissected out. For Hematoxylin/Eosin staining, control non-cleared brains and optimized CUBIC-cleared brains were cut and stained following the standard protocol, and images were acquired with a conventional visible light microscope using both 20x and 40x objectives.

For TEM, optimized CUBIC-cleared brains and brains without clearing were rinsed several times in PBS. Small pieces of tissue (1 mm³) were immersed in 4% Paraformaldehyde and kept at room temperature for one hour. The samples were then rinsed several times in PBS. The samples were postfixed in a 1% osmium and 0.8% potassium ferricyanide water solution and kept at 4ºC for 1 hour. Fixed samples were dehydrated in a series of graded ethanol solutions and embedded in LX 112 resin (Ladd Research Industries, Williston, VT, USA). Ultrathin sections (70 nm) were stained with uranyl acetate and lead citrate and examined by TEM using a JEOL 1230 microscope (IZASA Scientific SLU, Madrid, Spain) at 80 kV. (Fig. S1).

Light Sheet microscope setup
A custom-made multispectral light sheet microscope constructed by J. Ripoll was used.
This setup had five different excitation laser lines with powers ranging from 50 mW to 200 mW (405 nm, 473 nm, 532 nm, 589 nm, and 635 nm; CNI Lasers), and emission filters matching the laser lines (475 nm, 531 nm, 607 nm, 624 nm, and 670 nm) (Fig. S2, Table S2).

The excitation laser beam was directed through a set of mirrors towards a cylindrical lens (f = 50mm; Thorlabs, Newton, NJ, USA), which, when combined with a 5x long working distance objective (Mitutoyo, Kawasaki, Japan), created a light sheet with a ~2µm thickness. The imaging system consisted of a long working distance objective that could automatically select between 2x and 5x (Mitutoyo), in the optical axis of a tube lens placed after the emission filter wheel (Thorlabs), and a Neo 5.5 sCMOS camera (Andor, Belfast, UK) with 2560x2160 active pixels and a physical detector size of 6.5 µmx6.5 µm. Samples were positioned by means of three motorized stages that allowed translation (X, Y, and Z axes) and one rotation motor (Zaber Technologies, Vancouver, BC, Canada).

Images were acquired with the light sheet microscope using custom software developed specifically for this setup using Labview (National Instruments, Austin, TX, USA). The user fits the sample in the field-of-view (FOV) with real-time visualization, and then selects the excitation laser line sequence with its respective emission filters.

**Light sheet image acquisition**

Detection was performed using the 2x and 5x objectives. Grey scale images of 16 bit depth were acquired with 1x1 binning to achieve a higher spatial resolution. The imaged FOV was 8.3x7 mm² when the 2x objective was used, and 3.2x2.8 mm² when the 5x objective was used.

**Light sheet microscopy image processing**

A common artefact in light sheet microscopy are "stripes" caused by the presence of highly-absorbent structures such as the impurities present in the medium or sample, including highly-absorbing fluorophores. Image stacks presenting stripe artefacts were processed with the stripe removal algorithm (Leischner et al., 2010). The algorithm, known as the "rolling ball" algorithm, is implemented in Matlab and involves application of a
series of morphological operations to smooth out the image (Available at https://drive.google.com/open? id=0BwxxJ4jzIZrbTFpNVFNMTBWVVU).

The images are first closed and then opened with a line structural element in the direction of the stripes. Stripes are removed from the original image through pixel-wise division by the smoothed background image (Fig. S3). Visualization and image contrast adjustments were performed using open source Fiji software based on ImageJ (NIH). Volumetric reconstruction of the image stack was visualized with the Fiji integrated plugin ImageJ 3D Viewer (Schmid et al., 2010).

Light sheet microscopy comparable results to standard immunochemistry procedures
Immunostainings performed on tissue cleared with the optimized CUBIC protocol provide results that perfectly reproduce those obtained with standard immunochemistry procedures applied to sections from uncleared tissue (Fig. S4).

Confocal image acquisition and processing
Immunostained and optimized CUBIC-cleared and noncleared one-millimeter-thick coronal sections from chick embryo brains were used. High-resolution images of the hypothalamus were acquired with an inverted confocal microscope (Leica TCS SPE Confocal Microscope, Leica Microsystems). Images were processed using ImageJ Open Source software and its extension Fiji (Schindelin et al., 2012). Image stacks were filtered with a 3x3 median filter to remove salt and pepper noise when needed. The task was performed with the ‘Despeckle’ operation in Fiji (Process > Noise > Despeckle) on the image stacks that presented this type of noise. Brightness and contrast were linearly adjusted.

References


Figure S1. Transmission electronic microscopy (TEM) images obtained from uncleared and cleared tissue. Different magnifications were acquired from different regions of chicken embryos in uncleared (top raw) and cleared (bottom raw) brains. The ultracellular resolution of TEM allows the detection of contrast differences between the two treatments. Contrast is higher in uncleared brains.
Figure S2. Light sheet microscope (LSMF) set-up. A. Spatial distribution of the components of the SPIM microscopy and beam steering unit. B. The cuvette with the tube and the sample is in the transparent box in the center, the illumination objectives are on the left and the detection objectives are behind the cuvette. The components are listed in Table S2.
Figure S3. Stripped light sheet microscope images. A, Horizontal stripe artefact (stationary noise) is seen along the direction of illumination and is caused by absorption by particles in the laser beam pathway. B, After the "Rolling Ball" algorithm is applied to the image, stripes are removed.
Figure S4. CUBIC-cleared embryo brains reproduce histological results shown by conventional methods. **Left panel,** A composite drawing of the distribution of orexinergic neurons in E16 chick embryos, based on Godden et al (2014). **Central panels,** A 40 µm histological section from an E16 embryo studied by Godden et al (2014), taken from the area of the brain indicated by the arrow (Top). A projection view in the same orientation from an E16 embryo brain perfused with 4% PFA in PBS, dissected out and post-fixed overnight, cleared with CUBIC, and stained with the anti-orexin antibody used and validated for chick embryos by Godden et al (2014) (Bottom). Coronal images were acquired with a 5x objective. Note the identical distribution of cells seen in both the drawing and the 40 µm histological section. **Right panels,** Higher magnification views of single orexinergic neurons obtained from conventional histology (Top) and 3-D reconstructions from light-sheet microscopic images of CUBIC-cleared material (Bottom).
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<td>017-000-121</td>
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<tr>
<td>Anti-Goat Alexa-Fluor 594</td>
<td>Molecular Probes</td>
<td>A-11058</td>
<td>1:300</td>
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<td>Anti-Mouse Alexa-Fluor 647</td>
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<td>Anti-Rabbit Alexa-Fluor 488</td>
<td>Molecular Probes</td>
<td>A-11058</td>
<td>1:300</td>
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**Table S1. Immunohistochemistry reagents.** Primary and secondary antibodies and other reagents used for the immunostaining are listed with the maker, catalogue number and dilution they were used at. All secondary antibody is made in donkey.
<table>
<thead>
<tr>
<th>COMPONENT</th>
<th>COMPANY</th>
</tr>
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<tbody>
<tr>
<td>Lasers</td>
<td>CNI</td>
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<tr>
<td>Camera</td>
<td>Andor</td>
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<tr>
<td>Translation Motors</td>
<td>Zaber</td>
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<tr>
<td>Cuvette</td>
<td>Hellma</td>
</tr>
<tr>
<td>Lenses, filters and other optics</td>
<td>Edmund Optics</td>
</tr>
<tr>
<td>Objetives 2X, 5X, 10X</td>
<td>Mitutoyo</td>
</tr>
</tbody>
</table>

Table S2. Optical components used to build the SPIM microscope with the corresponding maker.
Movie 1. Light sheet composite volumetric image of the telencephalon and diencephalon of an E16 chick embryo brain from its right lateral edge to the midline. An E16 embryo was perfused, fixed with 4% PFA, and the brain was dissected out. The isolated brain was CUBIC-cleared, and immunostained with anti-orexin (green) and anti-c-Fos (red) antibodies. Nuclei were counterstained with DAPI (blue). Sagittal images were acquired with a 2x objective. Sagittal stack animation, rostral is to the left, dorsal is up.
Movie 2. **Light sheet composite volumetric image of orexinergetic neurons in and E16 chick embryo hypothalamus** An E16 embryo was perfused, fixed with 4% PFA, and the brain was dissected out. The isolated brain was CUBIC-cleared, and immunostained with anti-orexin (red). Nuclei were counterstained with DAPI (blue). Coronal images were acquired with a 5x objective. The movie is a rotation of a maximum intensity projection 3D render of the volume, rostral is up.
Movie 3. Light sheet volumetric image of an E16 chick embryo brain thick section. An E16 embryo was perfused, fixed with 4% PFA, and the brain was dissected out and embedded in agarose. One-millimeter thick sections were cut with a vibratome, cleared with CUBIC, and immunostained with anti-orexin (green) and anti-TH (red) antibodies. Nuclei were counterstained with DAPI (blue). Images were acquired with the light sheet microscope using a 5x objective. The movie is a rotation of a maximum intensity projection 3D render of a 1mm thick coronal slice, dorsal is to the left in the first frame.
Movie 4. Light sheet volumetric image of a whole E4 chick embryo. An E4 embryo was dissected out and fixed with 4% PFA, CUBIC-cleared, and stained with DAPI (blue). A 2x objective was used to acquire sections of the whole embryo. The movie is a sagittal stack animation; the head of the embryo is at the top left.
Movie 5. Higher-magnification volumetric image of a portion of the same E4 chick embryo seen in Supplementary movie 4. A 10x objective was used to acquire movies of the whole embryo. The movie is a sagittal stack animation in the same orientation as in Supplementary movie 4.
Movie 6. Light sheet volumetric image of the hypothalamus of an E16 chick embryo. An E16 embryo was perfused, fixed with 4% PFA, and the brain was dissected out. The isolated brain was CUBIC-cleared, and immunostained with anti-orexin (red), anti-TH (white) and anti-c-Fos (green) antibodies. Nuclei were counterstained with DAPI (blue). Coronal images were acquired every 5µm using a 5x objective. The movie is a coronal stack animation from dorsal to ventral; the third ventricle runs diagonally from the upper left-hand-side corner to the bottom-center.
Movie 7. Light sheet volumetric image of the hypothalamus of an E18 chick embryo. An E18 embryo was perfused, fixed with 4% PFA, and the brain was dissected out. The isolated brain was CUBIC-cleared, and immunostained with anti-orexin (red), anti-TH (white) and anti-c-Fos (green) antibodies. Nuclei were counterstained with DAPI (blue). Coronal images were acquired every 5µm using a 5x objective. The movie is a rotation of a maximum intensity projection 3D render of the volume, dorsal is to the left in the first frame.
Movie 8. Light sheet volumetric image of the hypothalamus of an E20 chick embryo. An E20 embryo was perfused, fixed with 4% PFA, and the brain was dissected out. The isolated brain was CUBIC-cleared, and immunostained with anti-orexin (red), anti-TH (white) and anti-c-Fos (green) antibodies. Nuclei were counterstained with DAPI (blue). Coronal images were acquired every 5µm using a 5x objective. The movie is a rotation of a maximum intensity projection 3D render of the volume, dorsal is to the left in the first frame.