Title: 3D Computational Cannula Fluorescence Microscopy enabled by Artificial Neural Networks

Authors: Rajesh Menon, Ruipeng Guo, Zhimeng Pan, Andrew Taibi, Jason Sheperd

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3D Computational Cannula Fluorescence Microscopy enabled by Artificial Neural Networks: Supplementary Information

RUIPENG GUO,1 ZHIMENG PAN,2 ANDREW TAIBI,3 JASON SHEPERD,3 RAJESH MENON1,*

1Department of Electrical and Computer Engineering, University of Utah, Salt Lake City, UT 84112, USA.
2School of Computing, University of Utah, Salt Lake City, UT 84112, USA.
3Department of Neurobiology & Anatomy, Biochemistry & Ophthalmology & Visual Sciences, University of Utah, Salt Lake City, UT 84112, USA.
*rmennon@eng.utah.edu

1. CCM Hardware Description.

The schematic and photograph of our Computational Cannula Microscope (CCM) is shown in Fig. S1. Excitation from a blue LED (center wavelength D 470 nm, M470L3, Thorlabs) is conditioned and focused onto the top face of the cannula via a 20X objective (PLN 20X, Olympus). The cannula guides the excitation to its bottom face and uniformly illuminates a sample placed in close proximity (~100 µm in this paper). The fluorescence from the sample is collected by the same cannula and guided to its top face, which is then imaged onto a sCMOS camera (C11440, HAMAMATSU). We set up a 520 nm–35 nm filter and a 472 nm–
30 nm filter in the optical path to separate the fluorescent signals from the source beam. The cannula is made by removing the sheath of the fiber (FT200EMT, Thorlabs) and placing it in a stainless-steel ferrule (SFLC230, Thorlabs). The cannula diameter and the length are 220 µm and 7 mm, respectively. The numerical aperture of the cannula is 0.39, which is close to those of the objectives. A reference fluorescence microscope was equipped to image the same sample from underneath with objectives that have the same magnification as the one in CCM. The field of view (diameter of circle in the CCM image) is 200 µm, and that of the reference microscope is ~260 µm.

**Choice of depth of layers:** The physics of CCM is quite different from 2D to 3D. The excitation light diffracts outward from the cannula either with scattering (inside phantom) or with no scattering (free space). The diffraction pattern is complex as it depends intimately on what combination of the thousands of supported modes are excited inside the cannula. Therefore, the excitation light distribution changes its average intensity as well as spatial extent in the different planes in very complex manner. Next, each fluorophore that is excited, may then emit fluorescent photons in approximately the same manner. However, the probability of emission is directly proportional to the excitation intensity, which has a very complex distribution from one plane to the next. Secondly, the probability that the emitted photons are collected by the cannula depends in a complex fashion on the (x,y) and z position of the location of the fluorescence relative to the optical axis of the cannula. Please see Fig. S2 for an illustration of these concepts. Since it is very difficult to model these phenomenologically, we decided to show experimental results to illustrate this point in Fig. S3 below. Due to the complex space-variance of this system, the captured CCM frames have very complex dependence on not only the transverse location (x,y), but also the longitudinal location (z) of the fluorophore. We have added a brief note to the revised paper to explain this point.

![Fig. S2: Physics of light propagation after the cannula is complex in 3D due to the diffraction and scattering of excitation and the dependence of the collection efficiency of the emitted light with depth, z measured from the face of the cannula.](image)
Fig. S3: Experimental CCM images of the same neuron (reference image is shown to the left) at different gaps from the cannula face (z), all collected in air are shown. One can see that not only is the image getting much dimmer, but the details of the intensity distribution are changing in complex manner. The images are not normalized, but all taken with the same camera conditions, so the frames can be directly compared to one another.

2. Samples preparation

2.1 Fluorescent beads sample

We utilized 4µm green FluoSpheres sulfate microsphere (505/515) in this paper. Spectrum of the micro-bead is shown in the Fig. S4. To make samples with desired density, diluted the bead solution (2% solid) to 1:500 high purity water, then vortex the diluted solution for 2 minutes. The 1:500 solution is diluted once more to 1:10 high purity water to make 1:5,000 solution, followed by vortex. Then aliquot 50uL of 5,000 solution onto a glass slide, until it is completely dry. We do not use coverslip to locate the bead sample as possible as close to the distal end of cannula. Once prepared, we check the distribution of beads on the slide under the reference microscope. The slide should include both sparse beads area and dense beads area to build a good dataset.

Fig. S4. Green FluoSpheres microsphere (diameter is 4µm) is used in the experiment. The blue dash line is excitation curve and the red line is emission curve.

2.2 Cultured neurons sample
Same neurons sample was used as the previous work[1]. Primary neurons were taken from dissociated hippocampi of E18.5 Sprague-Dawley rat pups. Hippocampi were dissociated using 0.01% DNase (Sigma-Aldrich) and 0.067% papain (Worthington Biochemicals) prior to trituration through glass pipettes to obtain a single-cell suspension. Cells were then plated at $8 \times 10^4$ cells/ml in the Neurobasal medium (Thermo-Fisher) supplemented with 5% horse serum, 2% GlutaMax (Thermo Fisher), 2% B-27 (Thermo Fisher), and 1% penicillin/streptomycin (Thermo Fisher) on coverslips (No. 1, Bioscience Tools) coated overnight with 0.2 mg/ml poly-L-lysine (Sigma-Aldrich) in 100mM Tris-base (pH 8). Neurons were grown at 37°C/5% CO2 and fed via a half-media exchange every third-day with astrocyte-conditioned Neurobasal media supplemented with 1% horse serum, 1% GlutaMax, 2% B-27, and 1% penicillin/streptomycin, with the first feeding containing 5 µM β-D-arabinofuranoside (Sigma-Aldrich) to limit the overgrowth of the glial cells. The neurons were grown for 12–14 days in vitro prior to transfection, fixation, and imaging.

The neurons were transfected after 12 days in vitro with 0.5 µg of pCAG-eGFP (Addgene: 89684) using lipofectamine 2000 at a 3:1 ratio when complexed with plasmid DNA. The spectrum of pCAG-eGFP is shown in Fig. S5. Then neurons were transfected over the course of 1 h at 37°C in pH 7.4 Minimum Essential Media (Thermo Fisher) supplemented with 2% GlutaMax, 2% B-27, 15 mM HEPES (Thermo Fisher), 1 mM Sodium Pyruvate (Thermo Fisher), and 33 mM Glucose. After the transfection, the neurons were given 24 hrs in growth media at 37°C/5% CO2 to allow a sufficient recovery and expression of the plasmid prior to fixation in 4% formaldehyde (thermo fisher)/4% sucrose (VWR) in phosphate buffered saline for 15 min at room temperature. After fixation, the neurons were mounted in a Prolong Gold Aqueous Medium (Thermo Fisher) and imaged.

2.3 Agarose phantom preparation

We uses the similar method with that used in Kim’s paper[2]. Imaging phantom used for experiment was made in 2% agarose gel, prepared by the following steps. First, mix 0.1g of agarose powder (EZ BioResearch S-1020-500) and 5mL of high purity water in a flask and stir it until agarose disperses uniformly in the solution. Then heat it in a microwave oven, using 100% power for 10 seconds and repeat heating with 10 seconds interval until agarose completely dissolves. Gently stir between intervals to suspend agarose. Before agarose solidify, mix 50uL of 1:500 diluted bead mixture into the agarose and pour the agarose solution into a mold on a glass slide. When agarose solidify, fix agarose gel on the glass slide with glue. The thickness of phantom we used in this paper were 1-1.5mm and 5mm. Additional results from the agarose phantom are shown in supplementary fig. S10-S13.

3. Description of ANN2
We modified the reconstruction ANN by translating output of model from one single image to three images, corresponding to three z-positions, as shown in Fig. S6. To adapt this model, we changed recorded reference images to three images by adding two images with zero values. The sequence of recorded reference images in the 3D array are different, related to the z-position. Once the model is trained, it can output three reconstructed images (including the reconstructed image on one layer) with one CCM image input. More results from both ANN1 and ANN2 are shown in Fig. S7-S9.

Fig. S6. The designed ANN2 system: ANN2 architecture is similar with the reconstruction ANN in ANN systems, but size of output is modified to 128*128*3.

4. Dataset and training of ANNs

To build 3D dataset, first we fixed z position and stepped the slide using a stage in a raster fashion with the step size of 50µm. Then we changed the z position of slides by 50µm and the reference microscopy was refocused to record reference image. In this paper, we chose 3 z-positions to build the dataset. Both reference images (1024*1024) and CCM (340*340) images were reshaped to 128*128 to fit the ANN and also to speed up the training process for proof of principle. 16700 images from each layer are used for training and testing the ANN systems. All the images were mixed together and separated into training data, evolution data and testing data. 1000 images were used for testing and 10% percent of other images were used as evolution data. All the images were normalized into range [0,1] before training and testing. The batch size was 16 in all the training of ANNs. And the training began overfitting after about 15 epochs. Once the model was trained, it could be saved for further prediction. For the ANN that used for phantom test, we pre-process the 3 layers dataset with the equation below:

\[
\text{CCM}_{\text{image}} = \text{CCM}_{\text{image}}(\text{layer1}) + \text{CCM}_{\text{image}}(\text{layer2}) + \text{CCM}_{\text{image}}(\text{layer3})
\]

\[
\text{Ref}_{\text{image}} = \text{Ref}_{\text{image}}(\text{layer1}) + \text{Ref}_{\text{image}}(\text{layer2}) + \text{Ref}_{\text{image}}(\text{layer3})
\]

We generated synthetic images that included 3-layers information in both CCM images and reference images with these equations. During the phantom test, first we put the sample under the cannula and moved it close to the end of cannula. Then we moved the sample up...
with step 50 µm and recorded the CCM images simultaneously. The cannula was inserted into
phantom step by step till all the tip of cannula got into the sample. In this test, we can insert
cannula into sample as depth as 0.75mm. Note that, this limitation comes from that the length
of cannula tip in this test was 0.75mm.

5. More Results

![Image](https://via.placeholder.com/150)

**Fig. S7.** The reconstructed results are from ANN system that consists of reconstruction ANN
and classification ANN. (a) The results are from neurons dataset. SSIM and MAE for training
dataset are 0.9213 and 0.0082. SSIM and MAE for testing dataset are 0.8974 and 0.0104. The
accuracy for classification ANN could reach 0.9980. The layer column is the predicted layer
numbers, which correspond to z-positions. (b) Dataset built with beads sample. SSIM and
MAE for training dataset are 0.9791 and 0.0017. SSIM and MAE for testing dataset are 0.9878
and 0.0012. The accuracy for classification ANN is 0.9665.
Fig. S8. The reconstructed results are from ANN system that output three images with single ccm image input. The reference images in left red square come from the reference image by adding two images with zero values. The results in right red square are reconstructed from the ccm images in left column. (a) Results come from dataset built with neurons sample. SSIM and MAE for test dataset are 0.9700 and 0.0031. SSIM and MAE for test dataset are 0.9639 and 0.0036. (b) Results come from dataset built with beads sample.

Fig. S9. Comparison of reconstructed results in different layers: the results are three single bead images come from the three different layers. The resolution was similar for the three layers.
Fig. S10. More phantom result: the thickness of phantom used in this test is about 1 mm. The beads were sparse in this sample. (a) We can track the beads in view field with reference microscope. Three beads were in view field, two beads were on same z plane and the other one was on different z plane. From the left image, we can see that the beads were pushed aside as the cannula was inserted. (b) The cannula touched sample from the first images. We got similar images from reconstructed results.

Fig. S11. More phantom result: when the cannula was inserted into phantom as depth as 700µm, we tried to record the beads distribution with reference microscope, as shown in (b). We increased the exposure time to make sure all the beads could be seen. Most beads were reconstructed as z = 650µm, but the two aside bead in red circles.
Fig. S12. More phantom result: the thickness of phantom used in this test is 5mm. We couldn’t track the beads distribution with reference microscope. The cannula began to be inserted into phantom at the red arrows position.

It was difficult to capture the reference images at the various depths through the scattering phantom. However, we were able to capture some exemplary reference images for validation, two from one sample are shown below in Fig. S13, all at the same scale.

Fig. S13: Preliminary validation of ANN1_r outputs with reference images.
References

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