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A Confocal Reflection Super-Resolution Technique to Image Golgi-Cox Stained Neurons

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Abstract

Metal-based Golgi-Cox (GC) staining is an established method used to visualize neurons with great morphological detail. While GC stained samples are imaged routinely under transmitted light microscopy, this method is unable to yield information on the three-dimensional structure of dendrites and neurons and thus help reveal the connective properties of the central nervous system. While a few studies have attempted simultaneous visualization of GC staining and antigen-specific fluorescent labeling under a confocal reflection technique, the resolution of both confocal reflection and fluorescence modalities used to acquire GC reflection and fluorescently stained antibody signals are still limited by the diffraction limit of light at about 220nm. Here, we report a confocal reflection super-resolution technique (CRSR) to break this diffraction barrier, which is achieved by minimizing the pinhole size from 1 airy unit (AU) to 0.1 AU. This is achieved by minimizing or closing the confocal pinhole size and is possible in this reflection modality, unlike fluorescence, because it is not a photon limited technique. Utilizing the lowest wavelength of light available in the system (405 nm), the CRSR technique results in ~30% lateral and axial resolution improvement. We also show that the CRSR technique can be used in conjunction to visualize both GC and immunofluorescence targets to create precise and improved three-dimensional visualization and analysis. In addition, using these super-resolution confocal reflection data sets

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Author contributions

MS conceived the idea and developed the confocal reflection super-resolution (CRSR) concept and technique, MI developed new protocols for simultaneous staining of GC and fluorescently tagged immune cells/neuron/glia for confocal and CRSR microscopy, MI and YMK performed the experiments, prepared samples, MS and YMK acquired the all the data in IGB microscopy and imaging facility and analyzed the images and data, MS and YMK wrote the paper which was edited by MI.

Conflict of interest

The Microscopy Facility at the Carl R. Woese Institute for Genomic Biology, University of Urbana-Champaign is a Carl Zeiss Labs at Location Partner which has a priority to access before market Zeiss systems for testing, evaluation and reporting.

from GC in CRSR mode significantly reduced the data overestimation, improving the accuracy of statistical analysis of dendritic spine density and average spine dimensions. Combining the 0.1 AU setting with deconvolution routines, the signal-to-noise ratio and resolution could further be improved an additional ~20-25%, yielding CRSR images with resolutions up to two-fold over the diffraction limit both laterally and axially. The improved precision of both visualization and quantification of sub-diffraction limited dendritic spines using the CRSR technique may prove to be critical in investigations that concern changes in detailed neuron morphology under central nervous system disease conditions such as multiple sclerosis and Alzheimer's disease.

Keywords

Golgi-Cox; Confocal Reflection Super-Resolution; 3D neuron visualization; dendritic spine quantification; Neuron Filament Tracing

Introduction

While diffraction limit is surpassed in fluorescence imaging (Gustafsson, 2000; Hell and Wichmann, 1994; Sheppard, 1988), resolution improvement in other conventional optical techniques such as reflection and other modalities are constantly attempted (Azeredo and others, 2016; Brakenhoff and others, 1979; Brakenhoff and Muller, 1996; Brakenhoff and others, 1984; Cornelesetenvelde and others, 1989; Cox and others, 1982a; Cox and others, 1982b; Koerten and others, 1979; 1980; Ploem, 1975; Ploem and Prins, 2017; Prins and others, 1993; Prins and others, 2006; Sivaguru and others, 2017b; Sivaguru and others, 2018a). Super-resolution techniques such as stimulated emission and depletion (Hell and Wichmann, 1994), structured illumination (Gustafsson, 2000), Airyscan (Huff, 2015; Sheppard, 1988) and PALM/STORM (Betzig and others, 2006) have recently been commercialized to go beyond diffraction limit of resolution in biological samples labeled with fluorescent targets. However, it is important to note that fluorescent techniques are inherently photon limited (Pawley, 2006b; Wilson, 1995). One has to collect as much, if not all, photons to faithfully describe the sample at hand. In general, techniques that reject the photons from fluorescence targets from in focal plane or from out of focus focal planes usually result in poor resolution and low signal-to-noise (SNR) ratios (Brakenhoff and others, 1992; Pawley, 2006a; b; Sivaguru and others, 2012; Wilson, 1995). While this photon-limitation holds is true in fluorescence imaging, it does not apply to photon-unlimited imaging modalities such as confocal reflection microscopy involving metallic material samples or biological samples labeled with metallic targets (gold and silver) nanoparticles (Azeredo and others, 2016; Kim and others, 2006; Prins and others, 1993). In the early 70s and 80s another reflection-contrast microscopy (RCM) technique was developed to bridge optical and electron microscopies. This technique has remarkably enhanced resolution and provides an alternative to transmitted light microscopy to image objects that otherwise could not be resolved with conventional transmitted light techniques (Cornelesetenvelde and Prins, 1990; Hoefsmits and others, 1986; Koerten and others, 1980; Ploem, 1975; Ploem and Prins, 2017; Prins and others, 1993; Prins and others, 2006). RCM microscopy was primarily developed as a wide-field epi-illumination technique involving polarizers and wave plates (Ploem, 1975). Later, Turner and Prins (Prins and others, 1993;

Turner and others, 1990) were the first to perform the confocal reflection contrast microscopy using lasers to obtain monochromatic images of ultra-thin sections including central nervous system samples stained with Golgi staining (Szarowski and others, 1992; Turner and others, 1990). Previously, we have demonstrated that using smaller pinholes combined with lowest wavelength of light (405 nm) in a confocal microscope, one could achieve theoretical resolutions in material samples (Azeredo and others, 2016).

Golgi staining has a long-held history in the field of neuroscience as an important tool to characterize neuron morphology. It remains a superior neuron staining method with unsurpassed clarity. Golgi staining is based on a metallic reaction which forms mercury- or silver-based aggregates in neurons and, more rarely, glia of the central nervous system. This central nervous system staining method was originally invented by the Italian physician and scientist Camillo Golgi (Golgi, 1873) who named this procedure a “black reaction” to show the details of the nervous system. The original technique has been modified and given multiple names such as Golgi-Cox, rapid Golgi, Golgi-Kopsch, and so on. The conventional method of visualizing Golgi staining is by using transmitted light, otherwise known as bright-field microscopy. Since the light is transmitted through the sample, extended depth of focus images are possible, while pure optical sections with black background are not possible as in confocal fluorescence microscopy. However, efforts have been made to improve the contrast (Ai and others, 2015).

Turner and Spiga et al. (Mancuso and others, 2013; Spiga and others, 2011; Turner and others, 1990) demonstrated that Golgi staining stain can be visualized using the confocal microscope under the reflection modality. Using confocal reflection microscopy instead of the transmitted light microscopy, GC stained neurons can be visualized in a three-dimensional modality better than the extended depth of focus images with the ability to optically section (image z-Stacks) thick brain slices with a black background, which is necessary for better contrast and resolution as in RCM microscopy (Ploem and Prins, 2017). In addition, Spiga and others demonstrated that central nervous system tissue samples impregnated with Golgi staining can also be marked with protein-specific fluorescent tags to allow for the simultaneous visualization and acquisition of Golgi and antibody targeted fluorescence signals in additional channels. This discovery created a new avenue for the 3D-rendering of the biochemical and morphological details of neurons and glia. However, the resolution of the reported confocal reflection technique is limited by the diffraction limit of light. Although super-resolution techniques with improved resolution have been developed in the past couple of decades (see above) and used for fluorescence modalities, apart from efforts by Ploem and Prins (Ploem and Prins, 2017) in reflection contrast and confocal reflection contrast techniques in a range of stained ultra-thin sectioned samples, there has been no attempt made to our knowledge to improve resolution beyond the diffraction limit in GC stained *thick slices* of brain tissue.

Over two decades ago, it was proposed (Centonze and Pawley, 2006) that in confocal fluorescence microscopy one could achieve ~40% improvement in resolution over Abbe's diffraction limit when the pinhole is closed all the way to 0.1 AU (Centonze and Pawley, 2006; Sheppard, 1988). However, Centonze and Pawley have also pointed out that closing the pinhole cuts off ~95% signal, which results in extremely poor SNR if one has to use the

same settings such as laser power and gain used for 1 AU (Huff, 2015; Sivaguru, 2018). To overcome this and bring the SNR to the level of images acquired at 1 AU settings, one has to increase the laser power and/or gain, in addition to doubling the traditional averaging from 4 line average to 8 to effectively remove noise (Huff, 2015; Kolossov and others, 2018). While this could be done easily on both material and biological samples in reflection mode, in fluorescence mode the sample physiological integrity will be compromised, potentially rendering the sample not useful to collect an accurate representation and to answer the scientific question.

In the confocal reflection mode, however, which we employed here, there is no excitation and emission state conversions as in fluorescence. Since there is no Stokes shift (lengthened emission wavelengths compared with excitation wavelengths) in confocal reflection modality as described above, resolution is inherently better than in the fluorescence technique. Though the incoming reflection signal is the same wavelength of light with improved resolution, the signal does decrease as in fluorescence when closing the pinhole to 0.1 AU. However, laser power can be increased without compromising the sample, which we have shown previously in the confocal reflection modality in sub-diffraction targets on material surfaces (Azeredo and others, 2016). In this manuscript, we have extended a similar approach of using smaller pinholes in confocal reflection mode to improve resolution. In combination with deconvolution, this technique can improve resolutions up to two-fold over the diffraction limit in visualizing metal based targets in biological samples with gold nanoparticles as well as GC in thick brain slices. This improved resolution is critical as it has enabled characterizing dendritic spine length, morphology and diameter which are indicative of neuronal function in synaptic communication with better accuracy and substantially reduced over estimation. We hope the simple yet robust CRSR technique may prove to be critical in investigations that concern changes in detailed neuron morphology under central nervous system disease conditions such as multiple sclerosis and Alzheimer's disease.

Materials and Methods

Animals and Institutional Study Approval

Healthy male and female mice of the C57BL/6 background of 6-8-week-old were randomly selected and used in this study. C57BL/6 mice were purchased from Jackson Laboratories. All the mice were kept group-housed (3-5 mice per cage) in a specific pathogen free facility with 12h-12h light-dark cycle in the Veterinary Medicine Basic Sciences Building at University of Illinois at Urbana-Champaign. This study was approved by the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee (Protocol number 16114).

Experimental autoimmune encephalomyelitis induction

To visualize activated astrocytes in the brain, we induced 7-week old mice with an established rodent model of multiple sclerosis termed experimental autoimmune encephalitis (EAE) by one subcutaneous injection of myelin oligodendrocyte glycoprotein (MOG35-55) peptide (100µg/mouse) emulsified in complete Freund's adjuvant including heat-killed

mycobacteria (BD Difco, 200 µg/mouse) on day 0, and i.p. injection of pertussis toxin (200 ng/mouse) on days 0 and 2.

Golgi-Cox staining protocol

Mice were deeply anesthetized with isoflurane and perfused intracardially with phosphate buffered saline (PBS) (15 ml) followed by 4% paraformaldehyde (pH 7.4) (25 ml). Brain and spinal cord were removed and stained with Rapid Golgi according to kit protocol (FD Rapid Golgi-Stain Kit, FD Neuro-Technologies, INC), described as follows. Brain was placed in 5 ml of Solution A and B of ratio 1:1 for 24 hours and then immersed in an identical volume of fresh Solution A and B for an additional 14 days in dark. The samples were then transferred to solution C for 3 days, with a replacement of fresh solution after 24 hours. To attain cryoprotection, brains were then transferred in a 30% sucrose solution in PBS for 1-2 days. Afterward, samples were snap frozen in optimal cutting temperature compound (SakuraTek, Japan) on dry ice and kept frozen at -80°C to be cut into 100 µm thick coronal slices using Cryo-cut 1800 (Reichert Jung) at -14°C. Cut slices were stored in 24-well plates with 0.02% azide in PBS at 4°C. To develop GC staining, brain slices were washed in distilled water (2 x 4 minutes) and submerged in solution D and E according to kit protocol. Slices were rinsed in distilled water (2 x 4 minutes) again.

Immunofluorescence procedure

GC stained tissue slices were permeabilized with 0.5% Triton X-100 overnight at 4°C. To prevent unspecific binding, slices were washed with 0.05% Tween-20 in tris-buffered saline (TBS-T) (3 x 10 minutes) and then pre-incubated in 3% bovine serum albumin in TBS for 3 hours. Slices were then incubated in mouse monoclonal GFAP antibody, 5C10 (Novus Biologicals) at the dilution of (1:500) in TBS for 2 days and overnight at 4°C, respective to their thicknesses. All sections were washed (3 x 10 minutes) in TBS-T and incubated in Alexa Fluor 546 Goat Anti-Mouse (1:500) for 2 hours at RT. Rinsed slices were mounted on poly-lysine coated slides with Pro-Long Gold Antifade-Mounting media (Thermo Fisher Scientific) with 0.17 mm thick (#1.5) coverslips. Edges of coverslips were sealed with nail polish. Slides were stored at -20°C until confocal microscopy visualization.

Gold nanoparticle preparation

100 nm gold nanoparticles (GNP) were purchased from Nanocomposix under the catalog # AUCN100-25M and lot number of HKE0007. According to manufacturer's certificate of analysis, GNPs dimensions were measured at 103 ± 10 nm using transmission electron microscope and around 90% of particles are in the range of 100-120 nm. Gold nanoparticles were supplied in aqueous 2mM sodium citrate; an aliquot of 100 microliter of this solution was dispensed in the center of a Zeiss high performance cover glass (No. 1 1/2, Catalog # 22 x 22 mm-0107052) and kept in a fume hood and allowed to dry. A very small droplets of a quick dry translucent nail polish is applied on four corners and a microscopic slide is brought on the cover glass and left in the hood until completely dried. The sample is imaged through the cover glass slide for GNP reflection measurements.

Confocal reflection and fluorescence microscopy

Samples of GNP and GC stained brain slides were imaged under a Zeiss LSM 880 Laser Scanning Microscope set up on an AxioObserver stand (Carl Zeiss, Oberkochen, Germany). A continuous wave laser of 405 nm was used to image GNP particles as well as GC metallic stain and a 561 nm laser was used to excite Alexa 568 labeled GFAP antibodies targeted to astrocytes as shown in the light path diagram in Figure 1. While the 400-410 nm bandwidth centering 405 reflection was collected with 405 laser source for CRSR, 565-615 nm bandwidth of emission signal collected for GFAP fluorescence from Alexa 568 was excited by the 561 nm laser. One could also use longer wavelengths for CRSR such as 488, 561 and 633 in the absence of 405 nm UV line in the system, but the resolution would be lower with increasing excitation wavelength (Table 1) according to the Abbe's diffraction limit $0.61 \cdot \lambda / \text{NA}$ principle (Abbe, 1873). As indicated in Fig. 1, the guiding mirror was removed to allow light to be reflected on the main beam splitter (MBS) at a 90° angle to collect the maximum excitation light of the same wavelength that illuminated the samples. We used throughout a Plan-Apochromat 63x/ 1.40 NA Oil for CRSR modality and two other objectives for contextual visualization (larger fields of view with lower resolution) such as a Plan-Apochromat 10x/ 0.45 NA and a Plan-Apochromat 20x/0.8 NA. The MBS 690+ was used as a main beam splitter for CRSR modality to avoid laser suppression from MBS. For GFAP fluorescence, MBS 561 was used, to optimally reflect the 561 laser line. The guiding mirror was used to send the light at a 45° angle to avoid reflection from the excitation light in the emission path in fluorescence detection (Fig. 1). Digital zoom was set at 3.2x when imaging under 63x magnification. Master gain at the GaAsP photomultiplier was kept at 516 and 522 (lowest gain setting is 500) for 1 and 0.1 AU. Pinhole size for acquisition was set at 1AU (38 microns) and 0.1 AU (4 microns). Laser power was set at 0.2% (lowest laser power setting available) when acquiring images under 1 AU pinhole size; laser power was set at 2.0% when acquiring images under 0.1 AU pinhole size, indicating a drop in signal while closing the pinhole (Supplementary Data Table 1). GFAP fluorescent signals were acquired under 1 AU pinhole size. These settings are very close to the lowest laser power and gain in the system and indicate that in the reflection mode obtaining an image without saturating the pixels is a challenge; the range indicator in the program was used to make sure the pixels are not saturated. A stack (40-50 slices) of optical sections were acquired at $0.10 \mu\text{m}$ (Z steps) increments with XY pixel dimensions of 0.04×0.04 microns were obtained to reveal the 3D structure of the GC neuron staining. For all acquisitions, images were integrated with a $1.03 \mu\text{s}$ pixel dwell time with a raw data dimension of $42 \times 42 \times 4.9$ microns from a 1024×1024 in XY and ~49 slices in Z (Voxel sizes of the images are $0.04 \times 0.04 \times 0.1$ microns in X, Y and Z axes, respectively). The signals were integrated in 8 bit, line average= 4, digital gain= 1.00 with a digital offset set at 0.00. Screen shots of the 1 and 0.1 AU CRSR instrument settings for raw data are presented in Supplementary Figs. 1 and 2.

3D Rendering and 2D and 3D Image Analysis

All images were processed for line intensity profile analysis in the programs Zen (LSM 880 confocal microscope manufacturer's software) or Autoquant (Deconvolution software). 3D image visualization of GC and quantification of dendrites morphometric parameters was done using Imaris (Bitplane Inc., Zurich, Switzerland). Images of GNPs and GC speckles were deconvolved using the Autoquant blind deconvolution algorithm to test the impact of

deconvolution in addition to the smaller pinholes, where the PSF is estimated from the supplied image using 20 constrained iterations, medium noise setting as shown in supplementary Fig. 3 and described in detail in (Sivaguru and others, 2017b) and references cited in that article. Full Width at Half Maximum (FWHM) values of individual GC speckles or gold-nanoparticles in both XY and XZ dimensions were generated by conducting line profile analysis in Zeiss' Zen software and/or ImageJ (NIH open source software, (Rueden and others, 2017)). Image properties were calibrated to appropriate raw data voxel dimensions as described above. For statistical purposes, FWHM measurements were made on at least 4-6 distinct locations of each objects (GNP, GC speckle, GC dendrite) to confirm the statistical range of resolution improvement. The mean of means and standard error of means are presented. The experimental PSF determinations for GNP and GC were made using the same Zen program using the manufacturer's routine by selecting point objects with multiple factors of quality as described in Supplementary Fig. 4 and Fourier Transforms were also performed after creating the experimental PSF to check the range of frequencies obtained in the same program. Both individual optical sections and optical section from the middle of the Z stack are presented for PSF and their respective FFTs (Supplementary Figs. 5–8). The images were finally composed in the program Adobe Photoshop with appropriate intensity adjustments in most cases min/max or when gamma is adjusted to 0.45, it has been mentioned in the image legend itself (Cromey, 2010; Sivaguru and others, 2018a). Most acquisition and processing information is provided in main text and SI figures; any additional request for information will be fulfilled by the corresponding authors for both microscopic image acquisition and sample preparation. Raw data files and PSD files showing the adjustments for each figure are uploaded in the open source program figshare.com and could be accessed using this link. <https://figshare.com/s/c02626d8a76f7dcb544>

As mentioned above, 3D rendering and surfaces were created using the program Imaris under the Surpass mode (Bitplane Inc., Zurich, Switzerland). 3D projections were generated from individual optical sections as described before (Goldraj and others, 2006). Using the filament tracer algorithm, a new filament was manually traced and created using the Autopath mode (Swanger and others, 2011). A single dendrite starting point was assigned at the edge of the ROI. Automatic thresholds were used for assigning dendrite end points and dendrite surface rendering. The minimum dendrite end diameter, maximum spine length, and minimum spine end diameter were measured in 2D slicer view and entered into the filament tracer algorithm. Automatic thresholds were also used for generating spine seed points and surface rendering. Filament statistics were exported into Microsoft Excel program and finally graphed in the program SigmaPlot 13 (Systat Inc., San Jose, CA). Representative images were presented after adjusting and optimizing the intensities in a similar way between 0.1 AU and 1AU in the program Imaris as well as in Photoshop for visualization purposes without pixel saturation.

Statistical analysis

To determine the statistical significance of the data between 1 and 0.1 AU settings, GraphPad prism or Sigmaplot was used to conduct paired student t-tests.

Results and Discussion

Imaging GC under CRSR and conventional transmitted light at varying resolution

Figure 2 shows images of GC stained murine hippocampal neurons acquired using the CRSR and transmitted light modalities under increasing magnifications, depth of field and resolution. Plan Apochromat objectives of 10x, 0.3 NA (Fig. 2 A–B), 20x, 0.8 NA (Fig. 2 C–D), and a 63x, 1.4 NA with a digital zoom of 3.2 (Fig. 2 E–F) were used to understand the quality of GC stained neurons with increasing magnifications and numerical apertures. Fig. 2 B, D and F show the CRSR images while A, C and E show transmitted light images. While comparing the two modalities, the presented data reveal that the optical sectioning ability of CRSR reduced out of focus blur, increased SNR, and improved resolution with increasing magnification, numerical aperture and smaller depth of fields. Similar observations were made with confocal reflection microscopy using the GC stained neurons (Spiga and others, 2011) as well as widefield based RCM microscopy and confocal based CRCM microscopy in a wide range of stained and unstained samples on both metallic (gold nanoparticles) and non-metallic labeled targets in nature (Deitch and others, 1990; Ploem and Prins, 2017; Prins and others, 1993; Szarowski and others, 1992; Turner and others, 1990). Though using a 633 nm laser line has been shown to be better than 405 and 488 to avoid autofluorescence and reduce scattering and absorption of a given sample (Sivaguru and others, 2018a; Szarowski and others, 1992), we chose to use a 405 nm laser line to gain the maximum resolution (Table 1).

Evaluation of CRSR technique performance with gold nanoparticles and GC speckles

To understand the resolution improvement, a spherical object such as a fluorescent beads of sub-diffraction limit is routinely used to evaluate and test the optical system's point spread functions (PSF) under a given modality (Juskaitis, 2006; Sivaguru and others, 2012; Sivaguru and others, 2018b; Vandervoort and Strasters, 1995). In addition, such spherical objects could be actually dispersed in the sample itself for a very realistic scenario in evaluating PSF of an optical system at the same time imaging actual samples of interest. Here, we used 100 nm gold nanoparticles (GNPs) as such point objects. The metallic nature of GNP is ideal to test the resolution limit of reflection based CRSR technique. In addition, we have also used a second fine point like targets (unknown size) of GC metallic labels adjacent to neurons in the sample itself as point objects. Lateral and axial resolution of both targets were evaluated under CRSR technique in this study at pinhole settings of both 1 and 0.1 AU (Fig. 3). When comparing the central focal plane of the acquired Z stack in both cases, lateral resolution improved 32% and 26% in GNP and GC targets, respectively (Fig. 3C and J). In addition, closing the pinhole clearly reduced the cover glass derived reflection fringes seen as ring patterns as evidenced from the obtained images in two settings (Fig. 3A and B). Creation of an experimental PSF from multiple point objects of entire Z stacks showed smaller PSFs in XY and a smaller number of focal planes (indicative of smaller PSF in axial direction as well) occupied by PSF from 0.1 AU setting compared to 1 AU settings (Supplementary Figs 5–8). On the other hand, their respective Fourier transformations (FTs) showed the opposite, i.e., the 0.1 AU setting images showed higher retrieved frequencies and they occupied more focal planes compared to 1 AU, indicative of retrieval of higher frequencies in axial dimension. These relationships are presented for a single extracted focal

plane from the center in both XY and XZ dimensions (Fig. 3). To summarize, 0.1 AU produced relatively smaller PSFs and better band width compared to its 1 AU counterpart (Fig. 3D, F, K, M and Supplementary Figs 5–8). These improvements in smaller PSFs and better retrieved FT frequencies were more pronounced in GC data (Fig. 3E, G, L and N), affirming that the CRSR technique effectively increases resolution of GC staining. In addition to the line profiles and full width at half-maximum (FWHM) shown in graphs in Fig. 3, we also provided a consolidated performance analysis of differences and % resolution improvement in CRSR technique in 1AU and 0.1 AU settings of GNPs, GCs and GC dendritic spines of multiple locations presented with statistical analysis in Table 2. Such improvements have been shown while using smaller pinholes in confocal fluorescence microscopy previously in a range of samples (Cox and others, 1982a; Cox and others, 1982b; Wilson, 1989; 1995; 2011). This approach has also been adapted in a confocal fluorescence based Leica Lightning Super-Resolution modality (Leica Microsystems CMS GmbH, Germany), where smaller pinholes are combined with deconvolution to achieve better resolution than conventional confocal modality.

Since the resolution is improved while minimizing the pinhole at the cost of losing signal (see Introduction), higher laser power was required to visualize GNP and GC under the 0.1 AU pinhole setting in contrast to the 1 AU settings. If laser power is unadjusted when acquiring images at 0.1 AU pinhole size, there was an 87% decrease in peak intensity (Supplementary Table 1). Hence, laser power was adjusted (increased ~10 fold) while acquiring all 0.1 AU setting images irrespective of sample target (Supplementary Figs 1 and 2, Supplementary Table 1). Resolution improvements in CRSR were also evident in maximum intensity projected multiple particles of GNPs, GC speckles and GC dendritic spots (Table 2). Results indicate that the CRSR technique at 0.1 AU setting consistently produced higher resolution anywhere from 21% improvement of GC dendrites to 37% improvement of GNPs resolution in terms of FWHM. The same target GC dendrites showed 31% improvement of 1 AU in axial resolution (XZ) and the GNPs yielded 36%, indicating that the extent of resolution improvement is better and higher in the Z dimension compared to XY, which is also supported by higher frequency retrieval in the FT images in axial dimension (Fig. 3G and N; Supplementary Figs. 5–8; Table 2). These results are consistent with the in-focus optical transfer functions reported previously and the resolution improvement with smaller pinholes (Cox and others, 1982a; Cox and others, 1982b; Juskaitis, 2006; Pawley, 2006a; Wilson, 1989; 1995; 2011)

Simultaneous visualization of CRSR-acquired Golgi-Cox stained neurons and fluorescently tagged astrocyte

After evaluating the resolution performance of CRSR using point objects with reflection properties, we compared the resolution of GC staining in actual brain slices under the same pinhole setting used for 1 AU and 0.1 AU (Fig. 4 A–D). In addition, we have also labeled the samples with fluorescently tagged astrocytes using a standard immunofluorescence staining method (pseudo-colored red) after completing GC staining in the same sample in a sequential manner. We imaged these astrocytes (pseudo-colored red) using routine confocal fluorescence microscopy technique together with GC stained neurons (pseudo-colored green) using the CRSR technique, simultaneously in the same location. The two z-stacks

were then merged to visualize the spatial distribution and localization of two signals from different modalities (Fig. 4 E–F'). Technically one can combine CRSR imaging with multiple fluorescent targets using 488, 561 and 633 nm laser excitations with the application of appropriate second antibodies with fluorophores excitable at those wavelengths. We present the data for one probe (GFAP) for demonstration purposes. From the presented results in figure 4, it is evident that the CRSR technique provided images with both better resolution and SNR in both XY and XZ dimensions, closely following the resolution improvements recorded from GNPs and GC speckles (Fig. 3 and Table 1). Further, images generated using CRSR can be combined with fluorescence visualization to offer a powerful visualization technique to study cell-to-neuron interactions (Fig. 4E–F'). To exemplify this point, it was shown that astrocytes mediate dendritic spine maturation in a contact-dependent manner (Nishida and Okabe, 2007). In line with this, encephalitogenic T cells have been shown to induce a lethal increase in neuronal calcium levels upon direct contact with neurons, causing detrimental damage to neurons (Nitsch and others, 2004). Because the background is black, the contrast is higher as in RCM microscopy (Hoefsmit and others, 1986; Ploem and Prins, 2017; Prins and others, 1993). One could create 3D rendering and visualization because of the optical sectioning ability. The image intensities could be converted in to solid objects (Surface objects) which could be analyzed with higher precision (Fig. 4E'–F'). While we did not observe major inconsistencies between hippocampal staining patterns in our slice samples, it is important to note that one must conduct GC staining with care and consistency to ensure reproducible results (Kassem and others, 2018; Ranjan and Mallick, 2010; Vints and others, 2019; Zaqout and Kaindl, 2016). In addition, the shading of one signal over another (GC shading the underlying GFAP fluorescence signal at a different Z location or vice versa) is present to an extent; the minimal loss of intensity can be compensated by incrementing laser power and/or gain through the Z-stacks automatically (linear extrapolation as in Zen LSM software) during image acquisition or applying restoration routines post processing (Biggs, 2009; Biggs and Andrews, 1995; Sivaguru and others, 2017a; Vandervoort and Strasters, 1995).

Precision of dendritic spine quantification enhanced by resolution and contrast improvement in CRSR technique

The logical next step was to examine quantitatively whether the resolution improvement from CRSR helped improve the accuracy of neuron-derived data from brain slices labeled with GC, such as changes in neuronal dendritic spine data. To answer this question, we present Figure 5, which shows data derived from five independent hippocampal neuron dendrite segments visualized using the CRSR 0.1 AU and 1 AU settings. To reconstruct the complete morphology of each dendrite segment, we used the Filament Tracer, an algorithm in the program Imaris (as described above) that has been used and tested to quantify and characterize (Fig. 5A–A' and B–B') neuronal dendritic spines (Dumitriu and others, 2011; Swanger and others, 2011). Dendritic spines are neuronal projections that receive presynaptic signals from other neurons, providing feedforward excitatory input to the cell. Golgi-staining is commonly used to extract these structural features of dendritic spine population and characterization which provide valuable clues regarding neuronal function (Harris and Kater, 1994; Hayashi and Majewska, 2005; Sala and others, 2001). Figure 5 C and D shows that spine density was overestimated by approximately 200% under the 1 AU

setting in comparison to the CRSR 0.1 AU. While taking a closer look at each individual data point, we found that the overestimation of dendritic spines under the 1 AU setting occurred predominantly in dendrite segments that were densely populated with spines (Figure 4). This implies that the improved resolution of GC staining under the CRSR modality significantly reduced the overestimation of dendritic spine count/density and can add precision to the rapid spine quantification routines proposed earlier (Risher and others, 2014). As shown in figure 5E, dendritic spines reconstructed from z-stacks acquired under the 1 AU setting has a statistically significant 2-fold increase in area size. Similarly, we found that the mean diameter of dendritic spines reconstructed from z-stacks under the 1 AU setting had a statistically significant 1.5-fold increase in mean diameter (Figure 5F and G). Spine area, size and diameter are key parameters in determining the functional characterization of dendritic spines (Harris and Kater, 1994; Hayashi and Majewska, 2005; Risher and others, 2014; Vangindertael and others, 2016). In the hippocampus CA1 region, crucial relationships have been revealed between the dimensions of dendritic spines and its functional properties. Studies have shown that the area of active zones per synapse is proportional to spine volume (Racca and others, 2000; Ramirez-Leon and others, 2001; Vangindertael and others, 2016).

Using a post-acquisition deconvolution routine to further improve the CRSR resolution

As a final processing step, we applied blind deconvolution to GC images (0.1 AU) obtained from CRSR and compare them with 0.1 AU data without this step both laterally and axially (Fig. 6 and Table 1). We show that the added blind deconvolution routine to 0.1 AU setting data improved the resolution laterally and axially and conferred up to two fold enhancement over the diffraction limit (Fig. 6) compared with a conventional confocal setting (220 nm). In addition to resolution improvement in CRSR that led to sub 100 nm resolution (Table 1 and Fig. 6D and H) in FWHM of the deconvolved 0.1 AU datasets, the SNR in these data sets was also substantially improved (Fig. 6 C, D, G, and H). In Table 1, we summarize the results of 0.1 AU with and without deconvolution for the 405 nm laser used in this study and project how this will be slightly different when longer wavelengths are used. Overall the data indicate an additional 25-30% improvement in resolution. These improvements are possible only if the images are acquired carefully without any saturation, which is one of the fundamental requirements for deconvolution (Biggs, 2009; Biggs and Andrews, 1997a; b; Vandervoort and Strasters, 1995) and as indicated in materials and methods, it is easy to saturate the reflection signal. Most optical super resolution techniques in use now (Structured Illumination and Airyscan) are also coupled with a deconvolution routine at the end to give an extra push in the SNR and resolution (Vangindertael and others, 2018). It is our intention to work closely with deconvolution industry partners to develop a PSF for reflection modality so that this could be provided to current and future users as an upgrade to an existing license. Recent developments in correlative light and electron microscopy (CLEM) efforts will be benefitted by CRSR, as it adds another tool in super resolution in addition to fluorescence (SR-SIM and Airyscan) before taking the samples to SEM and TEM modalities.

Taken together, utilizing the optical sectioning ability of the confocal and the ability to keep the background dark to improve contrast, resolution and SNR, the presented CRSR

technique is useful in providing 3D information of the metal stained biological structures. By closing the pinhole size of the confocal microscope from 1AU to 0.1AU and adding a deconvolution routine, we achieved around two-fold improvement GC lateral and axial resolution using the CRSR technique. We hope that this improved resolution of GC will help in visualizing and quantifying investigations that concern changes in detailed neuron morphology from central nervous system diseases such as multiple sclerosis and Alzheimer's disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Light path diagram for CRSR and transmitted light.

Schematic description of the light path and the optical components used in a confocal microscope LSM 880 for CRSR imaging. The 45 degree reflection mirror in front of the main beam splitter is the key for the CRSR technique. By removing this mirror, the excitation light hit the MBS at 90 degree angle, which allows the same wavelength to be detected using a different MBS which will not cut off 405nm. In fluorescence mode, this mirror will be used and an appropriate MBS 405 will be selected to suppress the excitation light in the emission path. In dual labeled samples, the same MBS for fluorescence (at 488, 561 and 633) could be used as well as for the 405 reflection beam sequentially without any hard ware movement. The representative beam path describes this scenario with using such an MBS both 561 excitation of GFAP image and GC GC stained hippocampal neurons could be imaged but spectrally separated by the spectral dispersion grating in front of the detectors. We have used the GaAsP detector to collect both signals. The bandwidth is of collection is controlled by a set of prism and sliders. At the same time a transmitted light image is also collected using the same 405 nm laser using the transmitted light PMT after setting the Koehler illumination.

Figure 2. Imaging GC under conventional transmitted light and CRSR modalities at increasing magnifications.

(A, C, E), Transmitted light field images of GC staining in murine hippocampal region under increasing magnifications (10x 0.3 NA, 20x 0.8 NA, and 63x 1.4 NA objective with 3.2x digital zoom). (B, D, F), CRSR images of the same focal plane. Note out of focus light was eliminated with increasing numerical aperture and decreasing depth of field. For example, compare E and F, the dendrite at 90 degrees visible in E in the transmitted light but is not visible in F. In addition, having a dark background in CRSR images gives inherent increase

in contrast. All images are acquired using 405 nm laser light. Scale bar is 100 μm for (A and B), 50 μm for (C and D) and 10 μm for (E and F).

Figure 3. Evaluation of CRSR technique performance with PSFs of gold nanoparticles and GC Speckles.

All images are acquired with 63x Plan Apochromat 1.4 NA objective with 3.2 times zoom. (A) Center focal plane (optical section) of a Z stack from GNP acquired under 1 AU setting. (B) Same field of view of GNPs at center focal plane of acquired Z Stack under 0.1 AU pinhole setting. (C) Line profile analysis of the GNP particle inside the white box in A and B also showed in insets and their indicated FWHMs. Experimental PSFs created from the same representative GNPs (white boxes in A and B) and their PSF shape in XY and XZ (D and F) and their respective Fourier transforms (FTs) (E and G). For comparison of PSF and FTs a

circle (on XY images) or ellipse (on XZ images) are drawn covering the intensities above background (purple) on 1 AU PSF (circle) and its FTs (Ellipse-covering most frequencies in FTs). Then the same circle and ellipse from 1 AU PSF and FTs are copied over to all PSF and FT images from 0.1 AU. As the presented data in Fig. 3 is only for the central focal plane of the respective images, PSFs and FTs for all optical sections are presented in Supplementary Figs. 5–8. The images of both PSFs and FFTs for GNP and GC are presented in 16 bit pseudo-colored intensity profile using “Rainbow” look-up-table in the native Zeiss Zen program with interpolation. The center white pixel in each images represent a value of 65,535 in a 16 bit gray scale. Representative images of GC speckles without interpolation and their respective histograms are presented in Supplementary Figs. 9–12. Similar analysis were done on the GC speckle images (H to N). Images A-B and H-I pairs are set at same min/max intensity levels. Scale bars A, B, H and I are 5 μm .

Figure 4. Resolution and SNR improvement in CRSR technique and simultaneous visualization of GC and GFAP.

(A, C) XY and (B, D) XZ views of GC stained hippocampal neuron dendrites visualized using the confocal reflection technique with 1 AU and 0.1 AU settings under high magnification of 63x with 3.2x digital zoom. Scale bars in A-D represents 10 μm . (E) Representative images of combination of GC (0.1 AU, pseudo-colored green) and the GFAP confocal fluorescence (1.0 AU, pseudocolored red) images of raw data where all optical sections are used to create a maximum intensity projected 3D image. (E') Imaris-filament tracer rendered image of (E) with isosurface algorithm after thresholding. Noise and out of

focus pieces neurons (in the bottom right corner of (E)) are filtered out for clarity. The white boxes indicate the images for F and F'. F is the raw data of the combined channel as in E and F' is the same as in E' but zoomed in to better visualize the spines and GFAP labeled neuron interaction and proximity. See also Supplementary Movie 1. The image scale is shown in labeled grids in E and E'.

Figure 5. CRSR technique results in improved accuracy in dendritic spine quantification and characterization compared to conventional confocal setting of 1 AU.
 (A, B) Close ups of neuronal dendrite and (A', B') are Imaris filament tracer 3D rendering. Color codes in A' and B' indicates; Purple: spine attachment points, Teal: terminal points, Green: dendrites and Blue: spines. (C) Quantification of rendered dendritic spine density (spine count per μm dendrite length). (D) % overestimation of dendritic spine density in 1 AU while using 0.1 AU as baseline, N = 5. (E) Quantification of spine area between the 1 AU and 0.1 AU. (F) Quantification of spine mean diameter between the 1 AU and 0.1 AU. (G) Representative frequency plot of spine mean diameter Vs spine count of one dendrite

showing 0.1 AU almost one third of spine count compared to 1 AU together with much lower spine diameter. Scale bars in A-D represents 0.4 μ m. Error bars represent means $\pm$ SEM. *, $P < 0.05$; Student's t test.

Figure 6. Impact of a deconvolution routine on resolution and SNR of 0.1 AU GNPs and GC Speckles data sets.

For better comparison, the Z stacks from the same data sets from Fig. 3 B (GNPs 0.1 AU) and I (GC speckles 0.1 AU) were used for the deconvolution using a blind deconvolution routine (settings are at Supplementary Fig. 3). (A-D) images are from GNPs and (E-H) are from GC speckles at indicated XY, XZ dimensions. The white boxes in images shows zoomed in images of GNPs or GC speckles in XY dimension and green boxes highlight the zoomed in GNPs and GC speckles in XZ dimension. The line profiles in C and G indicate the FWHM calculated from particles highlighted in white lines across a specific set of

particles in A and E. (D and H) showing statistical analysis of over 4-5 particles quantified for assessing the improvement in FWHM in both lateral and axial directions before and after deconvolution. Representative FWHM values are represented in Table 1 as well for 405 nm and compared with the influence of longer wavelength on resolution. Scale bar in A is 5 μm . Error bars represent means $\pm$ SEM. *, $P < 0.05$; Student's t test.

Table 1.

Potential impact of using longer wavelengths on CRSR resolution based on Abbe's resolution limit. The extent of influence is also compared with and without deconvolution in final resolution of CRSR is also calculated *. See also Fig. 6.

Wavelength (nm)	Abbe's Resolution limit, nm (% reduction, factor)	0.1 AU GNP nm	0.1 AU GNP + Deconvolution nm	0.1 AU GC Speckles nm	0.1 AU GC Speckles + Deconvolution nm
405 *	176	142 *	116 *	118 *	90 *
488	212 (20%, 0.83)	170	139	142	108
561	244 (38%, 0.72)	196	160	163	124
633	275 (56%, 0.64)	222	181	184	140

* Experimental wavelength used in this study and the resolutions obtained

Table 2

Comprehensive statistical analysis of resolution improvement and performance of CRSR technique on GNP and GC targets of interest.

Dimension	Target	1 AU	0.1 AU	Resolution (FWHM) improvement % (factor)	P value (Paired t test)
		Resolution (FWHM in nm)	Resolution (FWHM in nm)		
XY – single optical section	GNP	192 ± 17 (N = 6)	121 ± 5 (N = 6)	37% (1.58)	0.001*
XY - MIP	GNP	180 ± 10 (N = 5)	140 ± 9 (N = 5)	22% (1.28)	0.003*
XZ - MIP	GNP	550 ± 82 (N = 5)	350 ± 28 (N = 5)	36% (1.51)	0.045*
XY – single optical section	GC speckle	191 ± 9 (N = 5)	129 ± 9 (N = 5)	32% (1.48)	0.002*
XY - MIP	GC speckle	150 ± 9 (N = 5)	110 ± 7 (N = 5)	27% (1.40)	0.007**
XZ - MIP	GC speckle	600 ± 45 (N = 4)	450 ± 24 (N = 4)	25% (1.33)	0.050*
XY - MIP	GC dendrite	205 ± 15 (N = 6)	161 ± 12 (N = 6)	21% (1.27)	0.042*
XZ - MIP	GC dendrite	728 ± 44 (N = 5)	503 ± 32 (N = 5)	31% (1.44)	0.004**