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A novel, modernized Golgi-Cox stain optimized for CLARITY cleared tissue

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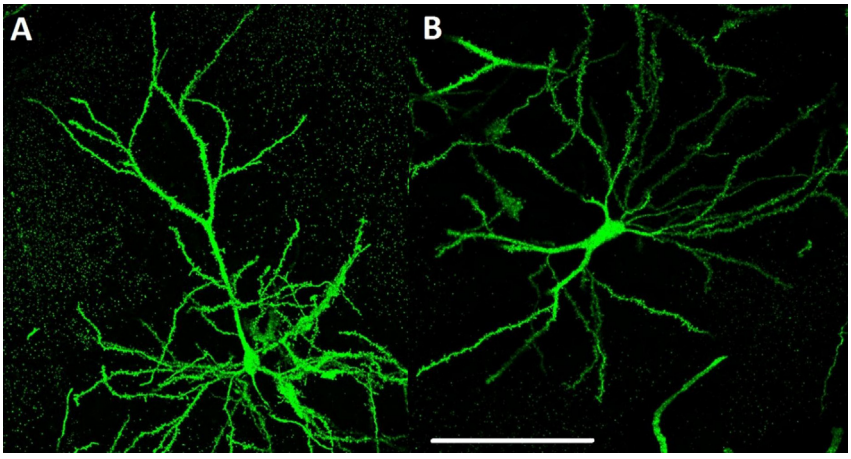
HIGHLIGHTS

- New Golgi-Cox method stains in 48 h within fresh or fixed tissue.
- Compatible with modern tissue clearing techniques speeding clearing to a few days.
- Revealed that neurons stained via this new Golgi method fluoresce.
- Excellent 3D reconstructions overcoming previous limitations of the Golgi stain.

GRAPHICAL ABSTRACT

Use of the Golgi stain has been limited by the need to reconstruct full neuronal morphology from sectioned tissue. Here an improved Golgi-Cox stain is described that stains within 48 h in brains cleared using CLARITY. Using these methods neurons could be observed in 3-D in their entirety in situ.



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ABSTRACT

Background: High resolution neuronal information is extraordinarily useful in understanding the brain's functionality. The development of the Golgi-Cox stain allowed observation of the neuron in its entirety with unrivalled detail. Tissue clearing techniques, e.g., CLARITY and CUBIC, provide the potential to observe entire neuronal circuits intact within tissue and without previous restrictions with regard to section thickness.
New method: Here we describe an improved Golgi-Cox stain method, optimised for use with CLARITY and CUBIC that can be used in both fresh and fixed tissue.
Results: Using this method, we were able to observe neurons in their entirety within a fraction of the time traditionally taken to clear tissue (48 h). We were also able to show for the first-time that Golgi stained tissue is fluorescent when visualized using a multi-photon microscope, allowing us to image synaptic spines with a detail previously unachievable.

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3d reconstruction
Morphology

Comparison with existing methods: These novel methods provide cheap and easy to use techniques to investigate the morphology of cellular processes in the brain at a new-found depth, speed, utility and detail, without previous restrictions of time, tissue type and section thickness.
Conclusions: This is the first application of a Golgi-Cox stain to cleared brain tissue, it is investigated and discussed in detail, describing different methodologies that may be used, a comparison between the different clearing techniques and lastly the novel interaction of these techniques with this ultra-rapid stain.

1. Introduction

Camillo Golgi developed the first histological method to fully delineate entire neurons on clear backgrounds, and it remains one of the best ways to visualise a neuron (Golgi, 1873, Mancuso et al., 2013). The Golgi-Cox stain offers the greatest neuronal resolution, allowing all morphological features of the neuron to be visualised (Golgi, 1873, Mancuso et al., 2013). Since its development, the Golgi-Cox stain has played a critical role in neuroscience, in the investigation of neuronal morphology (Sholl, 1953, Rutledge et al., 1969, Brown et al., 2005, Mychasiuk et al., 2013, Levine et al., 2013, Mitra et al., 2005, Radley et al., 2006). Nevertheless, the Golgi-Cox method is restricted by tissue transparency meaning that imaging is typically limited to 200 μm in depth (Glaser and Van Der Loos, 1981) using confocal microscopes (Graeden and Sive, 2009, Brakenhoff et al., 1988, Castano et al., 1995, Castano et al., 1994). The Golgi-Cox stain also fails to effectively stain fixed tissue, although previously shown to have the ability to stain fixed tissue (Bayram-Weston et al., 2016), the staining usually results in large amounts of artefact, or obscured morphology that is difficult to delineate. The Golgi-Cox method has also been modified over the last century to stain in reduced time. Originally taking months to process (Golgi, 1873), modifications to concentrations and care of the samples brought the time to 8 weeks (Sholl, 1953), to the now current standard of 2 weeks (Levine et al., 2013), with recent research showing that modifications to temperature can change the way in which the Golgi-Cox stain penetrates tissue, leading to reduced staining time (Ranjan and Mallick, 2010, Ranjan and Mallick, 2012), however these authors were still restricted to fresh tissue and sections of 200 μm.

As originally developed, tissue clearing used a series of dehydration and bleaching steps (Spalteholtz, 1914) that, although effective, damaged fixed tissue in a manner that prohibited further histological investigation (Steinke and Wolff, 2001). In contrast, modern clearing techniques involve an aqueous-based approach, such as CUBIC (clear, unobstructed brain imaging cocktails and computational analysis) (Tainaka et al., 2014, Susaki et al., 2014) and CLARITY (Clear Lipid-exchanged Anatomically Rigid Imaging/immunostaining-compatible Tissue hYdrogel) (Chung et al., 2013, Chung and Deisseroth, 2013), that achieve transparency via the removal of lipids and/or increasing the tissue's refractive index (Richardson and Lichtman, 2015) allowing greater depth penetration. Applying the Golgi-Cox stain to brain tissue cleared using these more recent techniques would allow complete neurons to be 3D rendered within an intact brain, offering the most accurate data on neuronal morphology. Our aim was, therefore, (1) develop the ability to apply the Golgi-Cox stain to fixed tissue, (2) develop it for use with modern clearing techniques, and (3) for use with multiphoton microscopy, together offering the greatest laser penetration (2 mm) and neuronal detail (Theer and Denk, 2006, Dufour et al., 2006). Such a development will, therefore, rectify the restrictions currently imposed by the Golgi-Cox stain, providing significant improvements to 3D rendering and morphological visualisation. As described below, we have been able to successfully develop this approach and, in the course of this development also observed that the Golgi-Cox stain can be made to fluoresce to provide considerably more cellular detail, something that has not previously been reported.

2. Materials and methods

2.1. Experimental model and subject details

A total of 72 adult male hooded Wistar rats (Laboratory Animal Services, University of Adelaide, Australia) weighing approximately 250–350 g were used across the development of these methods, with 56 rats used in replication of methods. The animals were split into replication of the URG stain on CLARITY and CUBIC tissue, tissue used for two-photon, and tissue visualised via brightfield and at 200 μm sections in uncleared tissue. Animals were housed in groups of 2–4 in 12 h light/12 h dark cycles, with food and water *ad libitum*. All methods and protocols were performed in accordance with The University of Sydney Animal Ethics Committee (AEC No. 5960). For an illustration of this experimental model please see Fig. S1.

2.2. Ultra-rapid Golgi stain (URG)

URG stock solution was prepared 1 day before incubation. The solution may be stored for up to 6 months. The final solution is mercury chloride, potassium chromate and potassium dichromate, each at 1% concentration. A stock solution of 500 ml would be made as follows; 5 g mercury chloride is first dissolved in 100 ml of distilled water at 80 °C, allowed to cool. 5 g potassium dichromate is dissolved in 100 ml of distilled water and added while stirring to the above solution. The resultant solution is then added while stirring to 5 g potassium chromate dissolved in 300 ml distilled water. Ensuring the solution is protected from light.

The animals were deeply anesthetized (Lethalbarb ™ 325 mg Pentobarbital Sodium/mL) and transcardially perfused with either 0.9% NaCl (250 ml) followed by 4% paraformaldehyde (pH 7.4, 200 ml) followed by 0.1 M (10X) PBS (300 ml), or with 0.9% NaCl (300 ml) followed by 0.1 M (10X) PBS (250 ml). Brains were removed and placed in light sensitive (100% UV blocked) polypropylene 50 ml tubes with 20 ml of URG solution, ensuring the samples are completely submerged. The containers were then placed in an incubator set to 37 °C or 42 °C for PFA fixed brains, and left for 36 h. Brains were then rinsed twice in distilled water for 5 min each, and developed in 30% Ammonium Hydroxide for 20 min. Brains were rinsed for another 5 min in distilled water before being left in a 10% Sodium Thiosulfate solution for 20 min, ending development. Development was performed in a dimly lit room, with aluminium foil used to keep samples in the dark. Development was performed either immediately following incubation or following clearing. In which case after incubation brains were cleared following the protocols described below, and then developed the same as above.