Robotic optimization of specimen preparation protocol for astrocytes seeded on coverslips for transmission electron microscopy (TEM)

Benjamin K. August[1] Linghai Kong[1] Randall J. Massey[1] Su-Chun Zhang[1] Thomas E. Strader[1,2]
1. University of Wisconsin-Madison 2. Heartland Biotech LLC Email: tom.strader@heartlandbiotech.com

Astrocytes are an important type of glia which support neurogenesis, neuron growth and neuron maintenance. They serve as intercellular scaffolding to provide physical support within the CNS and aid in the transport of nutrients to their associated neurons. Scientists at the UW-Madison routinely differentiate human pluripotent stem cells (hPSCs) into astrocytes to study their physiology. These cells are often seeded onto glass coverslips in 24-well polystyrene culture plates.[1] To optimize visualization of ultrastructural details of these cells when chemically fixed for imaging by Transmission Electron Microscopy (TEM), we asked two questions: Are there detectable differences between cacodylate or phosphate buffer used during fixation, and does post-fixation in osmium tetroxide or osmium tetroxide reduced with potassium ferrocyanide affect contrast in terms of membrane preservation?

Reliable and consistent initial TEM fixation and subsequent specimen preparation of astrocytes cultured on glass coverslips is vital for generating reproducible and timely experimental results. Traditional methods often involve tediously moving liquid reagents to and from cumbersome containers to perform numerous manual liquid reagent exchanges. Glass coverslips can also be damaged with excessive handling.

Improved sample preparation protocols are universally desired however time and resource limitations often force TEM laboratory technicians to use the “tried-and-true” traditional methods rather than perform head-to-head comparisons of potentially better performing reagents. The ability to simply and easily prepare multiple identical specimens to test various fixation and staining protocols is now possible using robotic processing, whereas it would be very difficult to compare these variables using the traditional manual technique.

Here, we demonstrate the use of a laboratory robot to compare various conditions used to prepare cells cultured on glass coverslips in the same polystyrene plate in which they were cultured.

Procedure

The astrocytes grown on glass coverslips in the Zhang lab at UW-Madison were cultured at 37°C with an atmosphere maintained at 5% CO2. Human astrocyte progenitors were differentiated from embryonic stem cell line H1 with an established protocol (Krencik et al., 2011). For our study, astrocyte progenitors were plated on laminin coated glass coverslip and matured with BMP4 (Stemgent, 10 ng/ml) and CNTF (R&D Systems, 10 ng/ml) for 7 days before fixation. [2]

In total, eight identical coverslips holding astrocytes were fixed directly in the same 24 well polystyrene plate in which they were cultured according to the following schedule:
The 4 coverslips in rows A and B were fixed with freshly prepared 2.5% glutaraldehyde/2.0% paraformaldehyde in 0.1M Sorensen's Phosphate (Pb) buffer pH 7.4,
The 4 coverslips in rows C and D were fixed with freshly prepared 2.5% glutaraldehyde/2.0% paraformaldehyde 0.1 M Sodium Cacodylate buffer pH 7.4. (See Figure 1 and Table 1 below.)

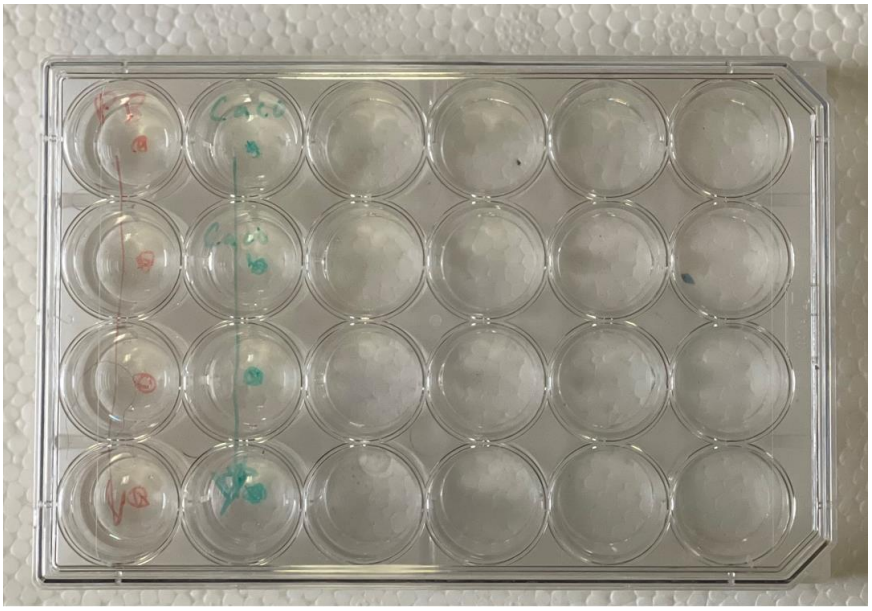

Figure 1. Wells A1, A2, B1 and B2 were post-fixed with 1% Osmium Tetroxide without reduction. Wells C1, C2, D1 and D2 were post-fixed with 1% Osmium Tetroxide reduced with 1% Potassium Ferrocyanide.

	1	2	3	4	5	6
A	Phosphate Buffer	Cacodylate Buffer				
B	Phosphate Buffer	Cacodylate Buffer				
C	Phosphate Buffer	Cacodylate Buffer				
D	Phosphate Buffer	Cacodylate Buffer				

Table 1. Location of buffer used for initial fixation in culture plate

After initial fixation, the 24 well polystyrene plate was placed on the robot's temperature-controlled specimen dock set at 5°C. The remainder of the protocol (see table 2 below) was fully automated using precise robotic pipetting until resin embedding. Fully automated robotic pipetting ensured that all reagents were delivered to their respective sample wells which, in turn, ensured highly reliable and reproducible results.

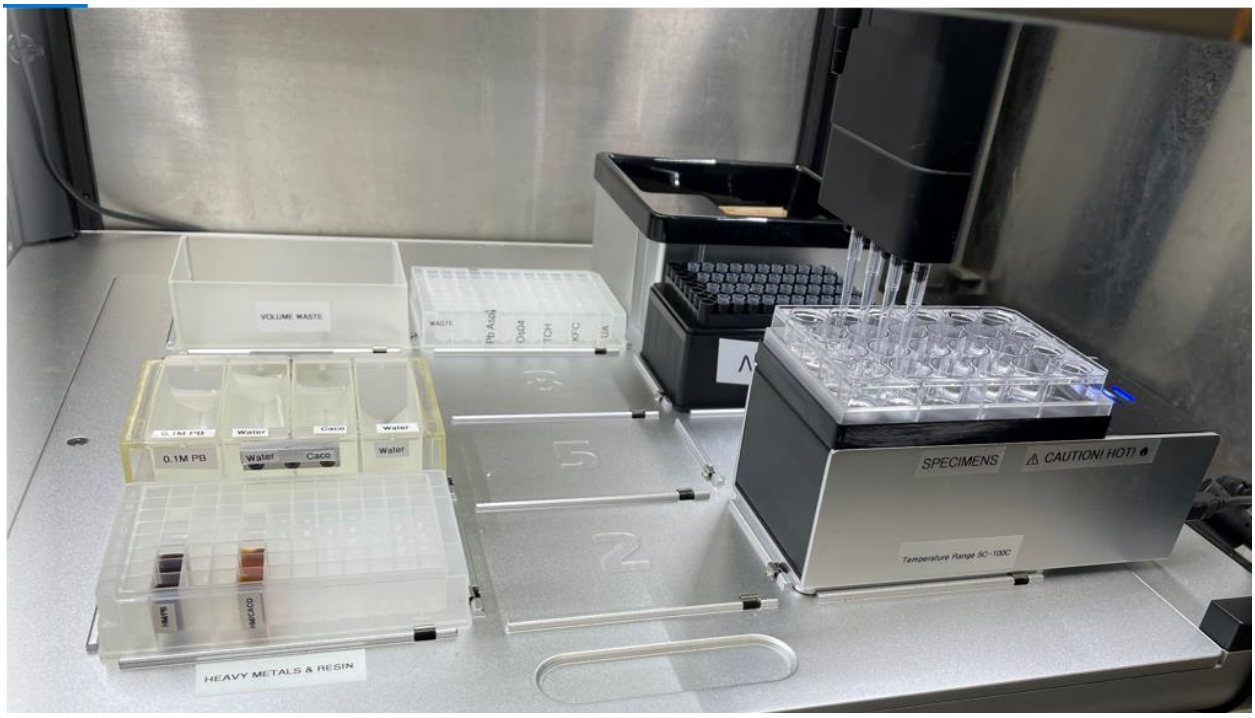

Figure 2 Deck of the Opentrons OT-2 laboratory robot. Note the darker color of the reduced osmium in the reservoir plate in the lower left corner.

The robot was programmed to perform the following procedure:

Robotic Protocol			
Step	Reagent	Time (mins)	Mixing cycles
1	Fixative rinse 1 (Caco or PB, per table below)	3	30
2	Fixative rinse 2 (Caco or PB, per table below)	3	30
3	Fixative rinse 3 (Caco or PB, per table below)	3	30
4	Fixative rinse 4 (Caco or PB, per table below)	3	30
5	Fixative rinse 5 (Caco or PB, per table below)	3	30
6	Heavy Metal (Os04 or Os04-KFC, per table below)	30	300
7	Heavy Metal rinse 1 (Water)	3	30
8	Heavy Metal rinse 2 (Water)	3	30
9	Heavy Metal rinse 3 (Water)	3	30
10	Heavy Metal rinse 4 (Water)	3	30
11	Heavy Metal rinse 5 (Water)	3	30
12	30% EtOH	2	20
13	50% EtOH	2	20
14	70% EtOH	2	20
15	80% EtOH	2	20
16	90% EtOH	2	20
17	95% EtOH	2	20
18	100% EtOH	2	20
19	100% EtOH	2	20
Total time		76	

Table 2. Robotic protocol

After robotic rinsing in their respective buffers, the robot delivered aliquots of osmium tetroxide for post-fixation; two samples for each of the two buffer groups were treated with 1% osmium tetroxide in its respective buffer (PB or cacodylate according to the initial fixation schedule) reduced with 1% potassium ferrocyanide and other two samples from each buffer group were treated with 1% osmium tetroxide without potassium ferrocyanide reduction (See Table 3 below). All sample groups were postfixed for 30 minutes.

	1	2	3	4	5	6
A	Phosphate OsO4	Cacodylate Os04				
B	Phosphate OsO4	Cacodylate OsO4				
C	Phosphate KFC/Os04	Cacodylate KFC/Os04				
D	Phosphate KFC/Os04	Cacodylate KFC/Os04				

Table 3 Location of reagents used for post-fixation in culture plate.

After post-fixation, the robot rinsed the specimens 5 times for 3-minutes in deionized water. The samples were then dehydrated in a graded ethanol series as follows: 30%, 50%, 70%, 80%, 90%, 95%, 100%, 100%.

Following the last 100% ethanol step, the coverslips were removed from the robot and placed in polypropylene 'medicine cups' for 2 additional rinses in 100% extra-dry acetone at 4°C, followed by infiltration and embedding in Durcupan™ epoxy resin. Because the specimens were processed in a polystyrene plate, which is not compatible with acetone, the solvent transition (to acetone) and infiltration/embedding steps were done manually.

If polypropylene 24-well plates are used, all subsequent steps can be done on the robot prior to final embedding and polymerization. At the time if this experiment, polypropylene 24 well plates were not available at our facility.

Results

No differences in overall image quality were detected between cacodylate buffer and phosphate buffer when combined with either experimental osmium conditions.
Clear differences were observed, especially in membrane preservation, overall contrast, and the ability to discern cytoskeletal elements, (i.e., microtubules and neurofilaments) in the samples post-fixed with potassium ferrocyanide reduced osmium as compared with unreduced osmium.

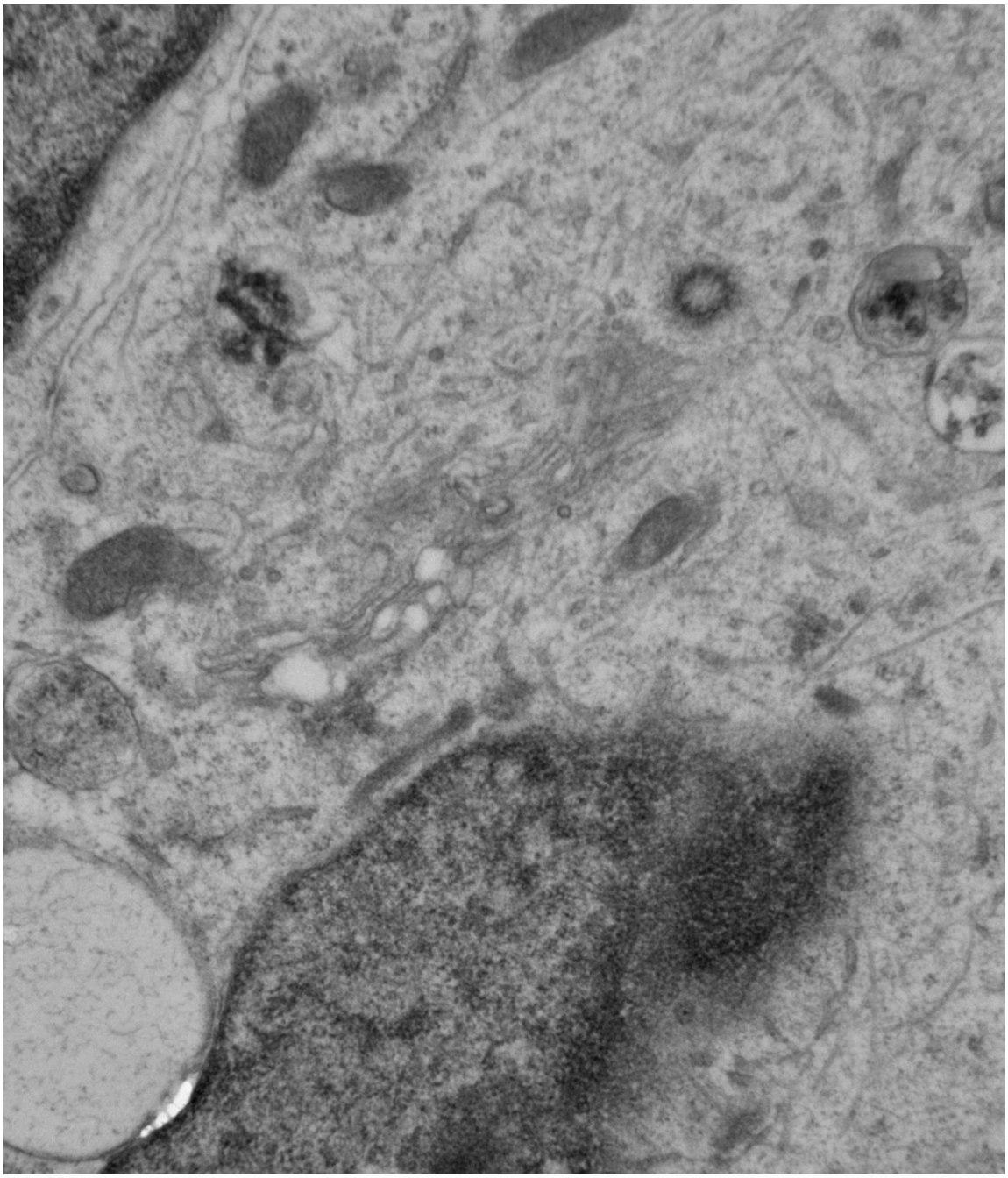

Image 1 (Phosphate buffer/Os04) Phosphate buffer combined with unreduced osmium tetroxide produces clearly identifiable Golgi and centrosome.

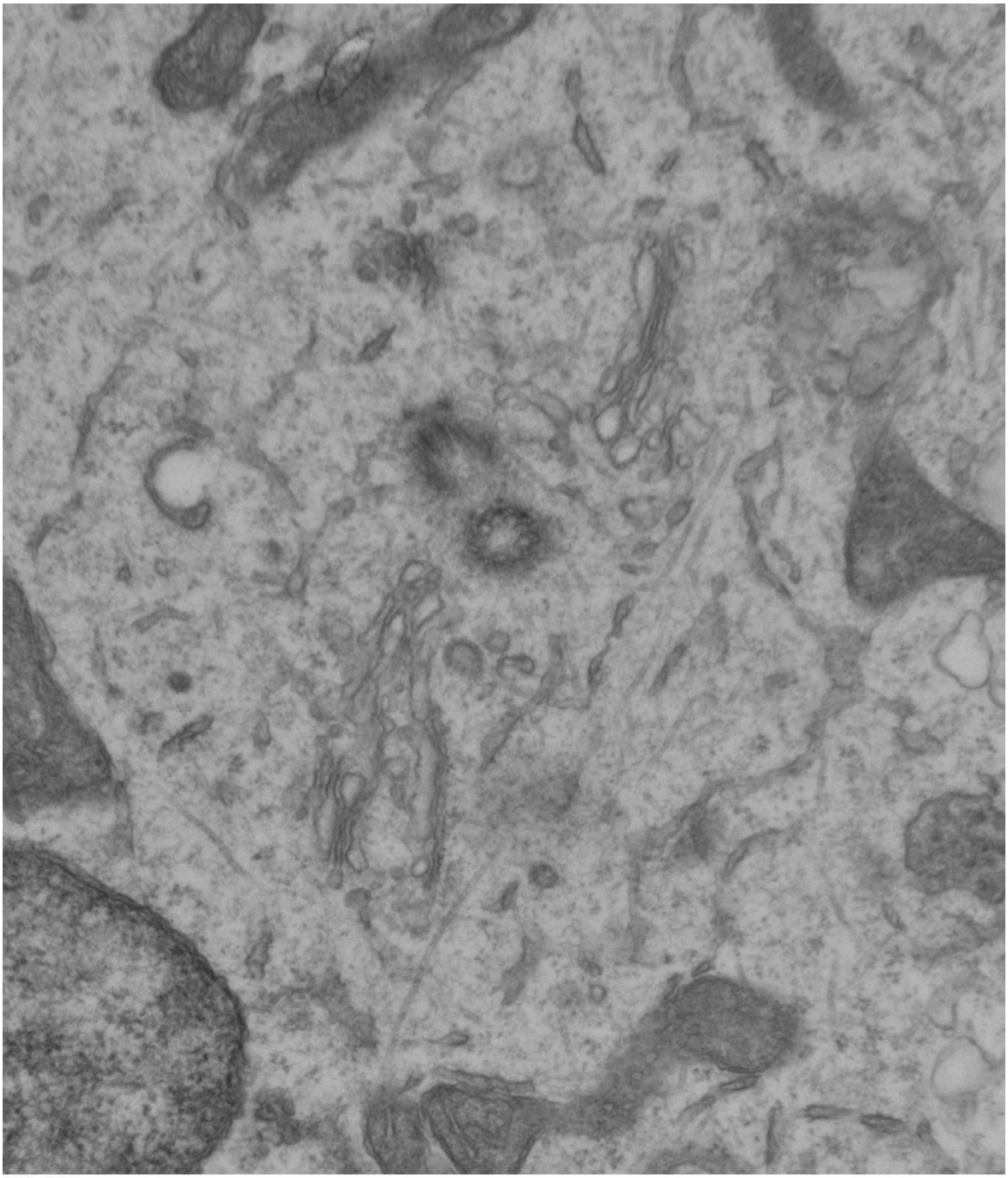

Image 2 (Phosphate buffer/Os04-KFC) Phosphate buffer with reduced osmium tetroxide gives low cytoplasmic background with well-defined centrosome and Golgi apparatus, including clear, well contrasted membrane preservation of the microtubules in the centriole.

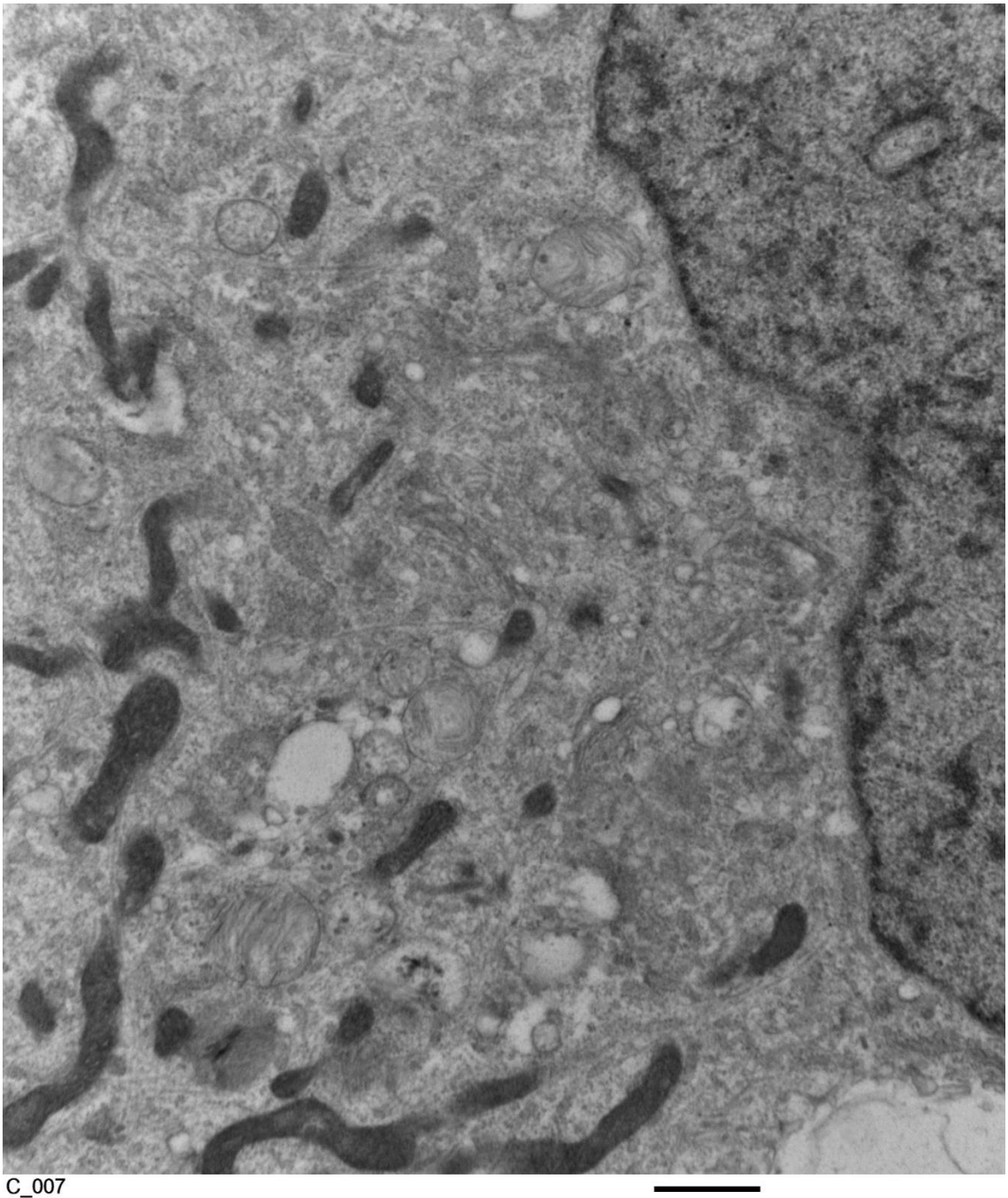

Image 3 (Cacodylate Buffer/Os04) Cacodylate buffer with unreduced osmium tetroxide shows Golgi apparatus however lack of clear and easily discernable membranes makes it difficult to resolve against the dense background cytoplasm.

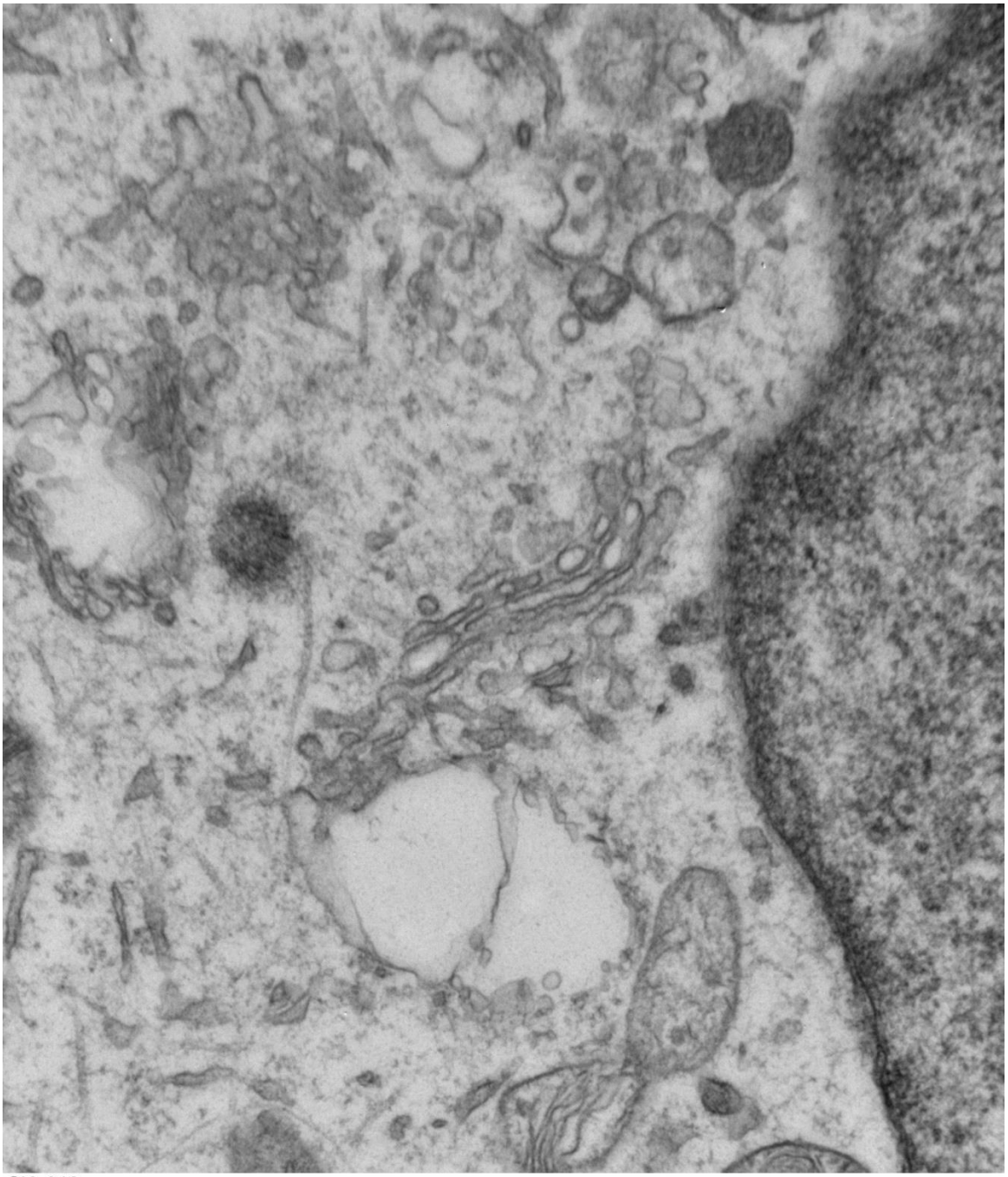

Image 4 (Cacodylate buffer/Os04-KFC) Cacodylate buffer with reduced osmium tetroxide shows beautifully preserved Golgi apparatus membranes, low cytoplasmic background, and easily visible microtubules.

Conclusion

All combinations produced generally acceptable images, however the osmium reduced with potassium ferrocyanide, in both buffer applications, appeared to produce better membrane contrast and lower cytoplasmic background along with more easily discernable cytoskeletal elements.

The use of phosphate buffer vs. cacodylate buffer did not appear to have any significant effect on membrane preservation, contrast, or ultrastructural details.

The laboratory robot enabled us to easily test 4 variables in one simple experiment using computer-controlled precise automated liquid handling with zero possibility of human error induced from trying to manually distribute a complicated array of reagents to each individual sample properly. This revolutionary increase in performance is due to the benefit of a programmable lab robot having zero possibility of decision-making errors, plus liquid handling accuracy and precision unattainable by a human being.

References:

1. https://www.waisman.wisc.edu/stem-cell-research-program/zhang-lab/
2. Nature Biotechnology, volume 29, pp528–534 (2011) https://www.nature.com/articles/nbt.1877