Automated Pre-Embedding Immunogold Labeling for TEM

mPrep™ System

Applications Note #605

Introduction

Immunogold labeling (IGL) for transmission electron microscopy (TEM) is a powerful imaging technique which uses gold-conjugated antibodies to localize biomolecular structures at the ultrastructural level.

Here we present an automated method for "pre-embedding" or "en bloc" IGL. (For post-embedding IGL on TEM grids, see Application Note 606.)

Performing IGL is considered one of the most challenging techniques in cell biology.[1] Experimental procedures are labor-intensive with repeated maneuvering of small specimens or fragile grids at 5-15 minute intervals.[2] Dozens of sequential reagent treatments must be accurately delivered to achieve reproducible results. These protocols often require multiple days to perform.

This application note describes automated pre-embedding IGL using the mPrep ASP-1000 Automated Specimen Processor (Figure 1) to label hemagglutinin-tag (HA-tag) overexpressed Sirtuin 1 (SIRT1) in murine brain, using silver enhancement of ultra-small gold particles. This methodology was developed and optimized at the University of Maryland, Baltimore.[2]

Automated IGL greatly reduces hands-on time compared with manual IGL methods, while also providing robotic consistency for reproducible results.

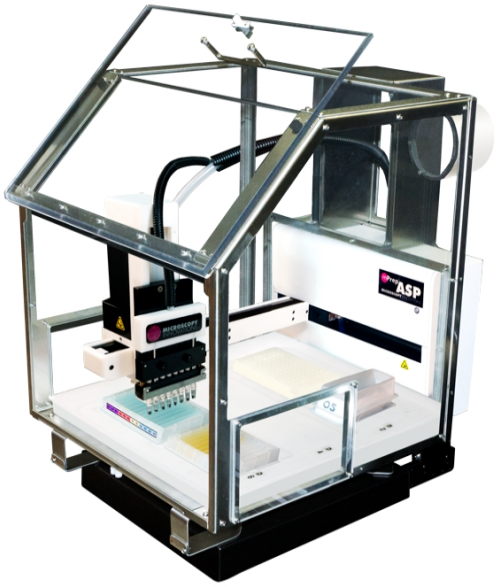

Figure 1: mPrep ASP-1000 Automated Specimen Processor.

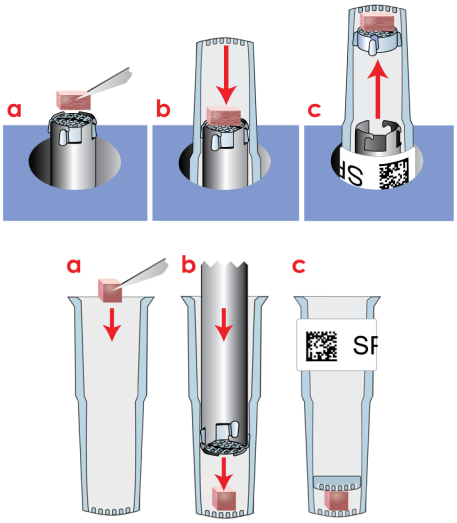

Figure 2: Inserting specimens into capsules. TOP: The mPrep/s Workstation (blue) enables easy specimen orientation. BOTTOM: One-touch manual insertion of specimen into capsule.

Methods and Materials

Mouse brain was cut from 30-µm thick vibratome sections into 2-mm diameter discs using a biopsy punch. The discs were then inserted into mPrep/s™ capsules (Figure 2) and mounted on the ASP-1000 for automated processing (Figure 3). Reagents were pre-dispensed into 96-well plates and loaded onto the ASP-1000 deck (Figure 3).

The Automation Protocol with reagent steps and process details is shown in Figure 4. Rinse reagents (PBS, incubation buffer, deionized water) were placed in 1.2-mL deep-well microplates filled to 1 mL. All other reagents were aliquoted in 35-100 µL volumes into 300-µL microplates in the original work.[2] (Microscopy Innovations recommends 500-µL microplates, rather than 300-µL ones. See Ordering Information.) To avoid inactivation, the plate containing glutaraldehyde was strategically placed in the far corner of the ASP deck away from the antibody labels.

Mouse brain was labeled using rabbit anti-HA-tag (500x dilution from stock) and goat anti-rabbit IgG conjugated to ultra-small gold (100x dilution from

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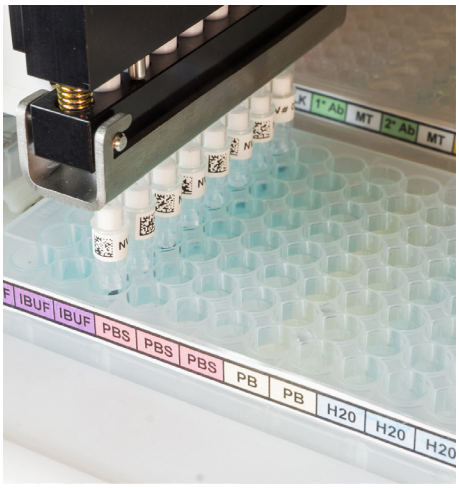

Figure 3: mPrep/s capsules mounted on ASP-1000 draw reagents from microplates on the autoprocessor deck.

Automation Protocol

Pre-embedding IGL / mPrep™ ASP-1000

Step(s)	Reagent	Aspirate Hold (sec)	Dispense Hold (sec)	Step repeats x rows	Time: minutes x rows
1-3	PBS	15	0.5	8x3	5x3
Pause	**Add quench & restart**				
4	Sodium borohydride quench	30	0.5	20	18
5-7	PBS	30	1	15x3	13.5x3
8	Permeabilization buffer	30	0.5	30	27
9-11	PBS	30	0.5	15x3	13.5x3
12	Block buffer	30	0.5	60	54
13-15	Incubate buffer	30	0.5	15x3	13.5x3
16	1° Antibody	60	0.5	250	350
17-20	Incubate buffer	30	0.5	15x4	13.5x4
21	2° Antibody (ultra small gold)	60	1	100	140
22-26	Incubate buffer	30	1	15x5	13.5x5
27-28	PBS	30	1	15x2	13.5x2
29	Glutaraldehyde	30	1	15	13.5
30	PBS	30	1	15	13.5
Pause	**PAUSE in PBS**				
31	Glycine quench	30	1	15	13.5
32-37	Deionized water	30	1	15x6	13.5x6
Pause	**Add silver enhance & restart**				
38	Blot				
39	Silver enhance (Aurion)	60	1	40	49
40	Blot				
41	Deionized water	15	1	15	10
42-46	Deionized water	0.5	1	10x5	4x5
46	Glutaraldehyde	30	1	15	13.5
48	PBS	15	1	15	10
49	Glycine quench	30	1	15	13.5
	Total run time:				~18.5 hrs

Figure 4: Automation protocol and reagent sequence. Reagent steps shown in blue were dispensed from 1.2-mL square-well 96-well plates. Reagent steps shown in black were dispensed from 300-µL microplates. Speed for all steps was 35. Repeats are number of aspirate-and-dispense cycles. Pauses for user intervention are noted in red. Steps 1-30 are typically run overnight. Washing is then restarted the next morning (steps 31-49), with fresh silver enhancement reagent being prepared and loaded into microplates at that time.

stock), which was later silver-enhanced to enable imaging at moderate magnifications. All gold conjugates and reagents for quenching, blocking, and silver enhancement were produced by Aurion (Wageningen, The Netherlands).

The entire pre-embedding IGL required about 18.5 hours run-time. For convenience, reagents were set up for overnight labeling with primary and secondary antibodies, incubations, and washes, followed by glutaraldehyde fixation. The ASP-1000 then automatically paused until morning, as indicated in Figure 4. The next morning, the operator restarted the program at step 31 (quenching and washing), prepared the labile silver enhancement reagent and, at a brief programmed pause (after step 37), dispensed it into the proper microwell row before restarting the instrument to complete the automated protocol.

After completion of the IGL protocol, specimens were embedded in resin within the same mPrep/s capsules using the ASP-1000. The two-hour dehydration and resin infiltration protocol was a standard ASP-1000 automated tissue embedding protocol (as shown in Application Notes 601-604). Following overnight resin curing, the labeled and embedded specimens were ready for sectioning on the morning of the third day of processing.

Total operator hands-on time was around 2 hours for the entire preparation process. This consisted of about 1 hour to load specimens into mPrep/s capsules, set up the IGL reagents, and then later add the labile silver enhancement reagent. Then, it took about another hour to set up the dehydration and resin reagents, followed by transferring the infiltrated specimens to an oven for resin curing, and cleaning up.

Imaging used a Tecnai T12 transmission electron microscope (Thermo Fisher) at 80 keV. Images were acquired with an AMT digital camera (Advanced Microscopy Techniques, Woburn, MA).

Results and Discussion

The ASP-1000 demonstrated fully automated immunolabeling of HA-tag overexpressed SIRT1 on murine brain using ultra-small gold and silver enhancement (Figure 5A). Automating the workflow for pre-embedding IGL yielded reproducible labeling results with signal-to-noise ratio and labeling efficiency comparable to manual labeling[3] (Figure 5B).

This automated method provided a significant reduction in hands-on effort compared to manual preparation. Preparing solutions, dispensing them into 96-well plates, and loading specimens into mPrep capsules for IGL required only 60 minutes, compared to 8-16 hours of hands-on effort for manual pre-embedding IGL. Labor requirements were further reduced by using the ASP-1000 for automated resin embedding. By retaining specimens in the same

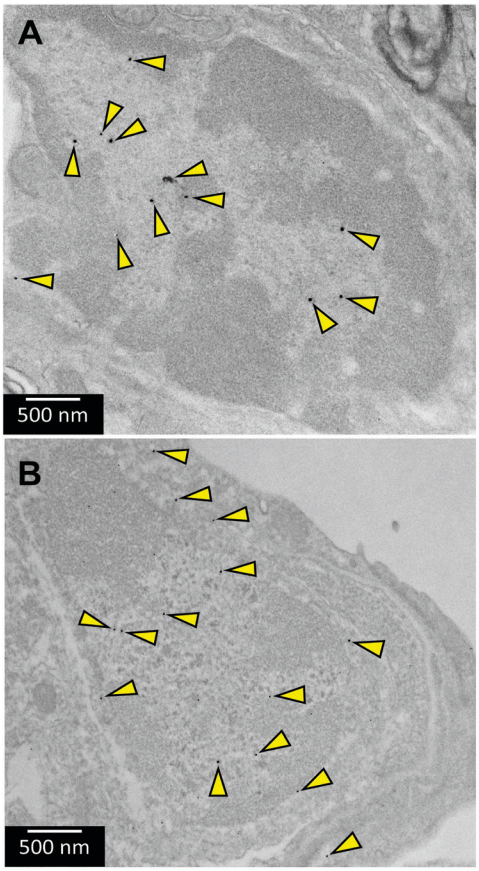

Figure 5: TEM images of murine brain with HA-tag overexpressed SIRT1 labeled with silver-enhanced ultra-small colloidal gold. Tissue was labeled prior to embedding with rabbit anti-HA-tag antibody and goat anti-rabbit IgG conjugated to ultra-small gold, followed by silver enhancement to enable viewing the small gold labels at this moderate magnification (yellow arrowheads). A) ASP-1000 prepared, and B) manually prepared.

mPrep/s capsules for both the immunolabeling and embedding processes, no additional handling was required, thus reducing risk of sample damage or loss.

Total elapsed time to perform IGL was also reduced because the ASP-1000 can process unattended overnight. When desired, the instrument's software provides program pauses to enable timely preparation of labile reagents, such as fresh silver enhancement solutions. It can also be programmed to alert the operator at any step, via SMS texting or email, for timely response.

The ability to operate unattended provides walk-away convenience, allowing busy labs to spend their valuable time on other tasks such as microtomy, imaging and analysis. Automation with the ASP-1000 provides uniform processing times to deliver consistent results within each batch of specimens and across batches.

Benefits of ASP-1000 Processing in mPrep/s Capsules

- Walk-away automation frees time to work on other projects.
- Long, tedious protocols are easily handled by robotics.
- Uniform processing times across multiple samples ensure consistent results.
- Reagent dispensing from microplates provides accurate control and simplifies setup and cleanup.
- ASP-1000 deck holds up to six standard microplates or reservoirs, allowing up to 72 reagent or rinses.
- Many ready-to-use protocols are available for the flexible ASP-1000.
- Easily customizable ASP-1000 control software allows an unlimited number of processing steps.
- Sends SMS text messages to operator when intervention is required or to notify when protocol is completed.
- Capsule-based processing reduces specimen handling and potential for errors.
- mPrep/s capsules allow users to orient a specimen during placement into the capsule without further manipulation in later steps.

Acknowledgements

We gratefully acknowledge Patricia X. Marques, John Strong, and Ru-ching Hsia of the University of Maryland, Baltimore Electron Microscopy Core Imaging Facility for performing this study[2] and for providing permission to share their work.

Ordering Information

Product #	Item Description/Catalog Information
41000	mPrep ASP-1000 Automated Specimen Processor
22200	mPrep/s capsules in storage box - 8 capsules, 12 screens, 8 blank label sets
22500	mPrep/s capsules - bulk pack: 96 capsules, screens & blank label sets
31500	mPrep/f30 Standard filter-couplers in capsule storage box, 16/pk
32010	mPrep/s Insertion Tool
34000	mPrep/bench 96-well rack, silicone
51010	96-well plates,1.2ml, square well polypropylene, 10/SLV
51001	96-well plates, 500µl, round well, polypropylene, 10/SLV
42100	mPrep/s Workstation
32010	mPrep/s Insertion Tool
53010	X-Pierce hairline cross-cut pierceable film for automation
53050	Self-adhesive, foil plate sealing sheets, pierceable, 100/box
53070	Pierce Heat Seals, pierceable foil heat sealing sheets, 100/pk

Reagents

- PBS: 0.1 M phosphate buffered saline, pH 7.4
- Sodium borohydride quench: 0.1% $NaBH_4$ in PBS
- Permeabilization buffer: 0.05% Triton X-100 in PBS

- Block buffer: 2.5% BSA, 0.5% fish gelatin, 0.05% NaN_3 in PBS
- Incubate buffer: 0.2% acetylated BSA, 0.1% fish gelatin, and 0.05% NaN_3
- 2% glutaraldehyde in PBS
- Glycine quench: 50 mM glycine in PBS
- Immunolabels: primary (1°) antibodies, gold-conjugated to secondary (2°) antibodies, silver enhancement reagent

References

[1] Melo RCN, Morgan E, Monahan-Earley R, Dvorak AM & Weller PF. (2014) Pre-embedding immunogold labeling to optimize protein localization at subcellular compartments and membrane microdomains of leukocytes. *Nat Protoc* 9, 2382-94. doi:10.1038/nprot.2014.163

[2] Marques N, Strong J, Strader T, Hsia R-C. (2018) Optimization of Automated Immuno EM for Both Pre- and Post-Embedding Labeling. *Microsc. Microanal.* 24 (Suppl 1), 2018: 1300. doi:10.1017/S1431927618006980

[3] Marques N, Strong J, Strader T, Hsia R-C. (2018) Optimization of Automated Immuno EM for Both Pre- and Post-Embedding Labeling. Poster presented at Microscopy and Microanalysis 2018 (ibid)

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