Automated Post-embedding Immunogold Labeling on TEM Grids

mPrep™ System

Applications Note #606

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.

This application note presents an automated method for post-embedding IGL or on-grid IGL, using the mPrep ASP-1000 Automated Specimen Processor. The method in this application note was developed and optimized at the University of Maryland, Baltimore.[1,2] (For pre-embedding IGL, see Application Note 605.)

Performing IGL requires that dozens of sequential reagent treatments must be accurately delivered every 5-15 minutes to achieve reproducible results. Not surprisingly, IGL has been described as one of the most challenging techniques in cell biology[3], thus making it well-suited for automated processing. Further, given the expense of primary antibodies and colloidal gold conjugates, it is advantageous to utilize robotics to precisely deliver these reagents in the required microliter volumes.

This application note provides an automated IGL protocol in which thin-sectioned rat liver tissue and whole-mount *Pseudomonas aeruginosa* bacteria on filmed grids were prepared using the mPrep ASP-1000 Automated Specimen Processor (Figure 1). The protocol can be performed while lab personnel are occupied with other tasks, or even overnight to have labeled grids ready in the morning.

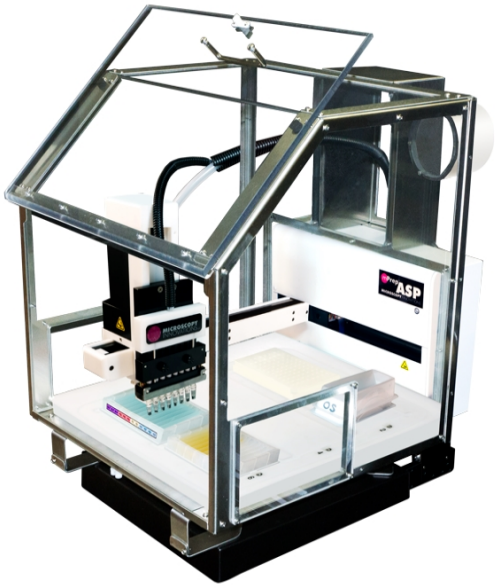

Figure 1: mPrep ASP-1000 Automated Specimen Processor.

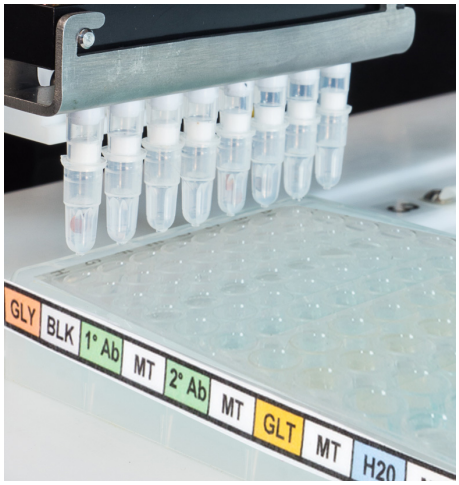

Figure 2: mPrep/g capsules mounted on ASP-1000 with reagents in microplates on autoprocessor deck.

Methods and Materials

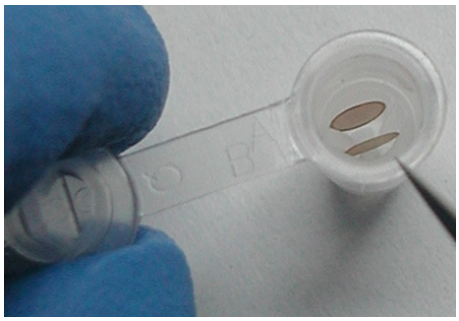

Figure 3: Capsule loading. Grids with thin sections or whole-mount specimens are inserted into mPrep/g capsules.

Rat liver tissue pieces were fixed in 4% paraformaldehyde, dehydrated, and embedded in Unicryl™ resin for post-embedding IGL. Ultrathin sections were then collected on 300 mesh Pioloform®-coated Ni grids for labeling. *Pseudomonas aeruginosa* bacteria were directly applied to Pioloform®-filmed grids for labeling.

TEM grids with the above specimens were inserted into mPrep/g™ capsules (Figures 2-3) and mounted on the ASP-1000 for automated processing

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Automation Protocol

Post-embedding IGL / mPrep™ ASP-1000

Step(s)	Reagent	Aspirate Hold (sec)	Dispense Hold (sec)	Agitations x rows	Time in reagent x rows (min)
1	Glycine quench	2	0.5	30	5
2-3	PBS	2	0.5	15x2	2.5x2
4	Block	2	0.5	120	20
5-6	Incubate buffer	2	0.5	15x2	2.5x2
7	1° Antibody	5	0.5	300	60
8-13	Incubate buffer	2	0.5	15x6	2.5x6
14	2° Antibody (ultra small gold)	5	1	300	60
15-18	Incubate buffer	2	1	15x4	2.5x4
19-21	0.1 M PB	2	1	15 x 3	2.5x3
22	Glutaraldehyde	2	1	30	5
23-24	0.1 M PB	2	1	15 x 2	2.5x2
25-28	Glycine quench	30	1	15	13.5
32-37	Deionized water	2	1	15x4	2.5x4
29	Uranyl acetate (aq)	2	1	30	5
30-33	Deionized water	2	1	15x4	2.5x4
34	Blot	-	-	-	-
	Total run time:				~4 hrs

Figure 4: Automation protocol and reagent sequence. Multiple step(s) indicate repeated rows of same reagent. Aspiration hold was 2 seconds for all steps except primary and secondary antibodies (steps 7 and 14) where the hold was 5 seconds. All steps used an aspirate and dispense speed of 30 and dispense hold of 0.5 seconds. Agitations are (aspirate-dispense cycles) x (rows of same reagent). Time is (minutes in each row) x (number of rows holding same reagent). Blotting (step 34) of mPrep/g capsules was done on a folded Kimwipe placed in a 12-well trough plate.

Plate Map

Deck Position/Labware/Reagents

row	Position 1	Position 2
	Not used	96-well 300-µL PS
1		2% Glut (22) 100 µL
2		PB (23) 200 µL
3		PB (24) 200 µL
4		dI H_2O (25) 200 µL
5		dI H_2O (26) 200 µL
6		dI H_2O (27) 200 µL
7		dI H_2O (28) 200 µL
8		1% UA (29) 100 µL
9		dI H_2O (30) 200 µL
10		dI H_2O (31) 200 µL
11		dI H_2O (32) 200 µL
12		dI H2O (33) 200 µL

row	Position 3	Position 4
	not used	12-well trough plate
1		Kimwipe (34)

row	Position 5	Position 6
	96-well 300-µL PS	96-well 300-µL PS
1	Quench (1) 100 µL	1° Antibody (7) 50 µL
2	PBS (2) 200 µL	Incubate buffer (8) 200 µL
3	PBS (3) 200 µL	Incubate buffer (9) 200 µL
4	Blocking (4) 100 µL	Incubate buffer (10) 200 µL
5	Incubate buffer (5) 200 µL	Incubate buffer (11) 200 µL
6	Incubate buffer (6) 200 µL	Incubate buffer (12) 200 µL
7	0.1M PB (19) 200 µL	Incubate buffer (13) 200 µL
8	0.1M PB (20) 200 µL	2° Antibody (14) 50 µL
9	0.1M PB (21) 200 µL	Incubate buffer (15) 200 µL
10		Incubate buffer (16) 200 µL
11		Incubate buffer (17) 200 µL
12		Incubate buffer (18) 200 µL

Figure 5: Reagent layout on ASP-1000 deck. Each cell indicates the reagent, step number, and fill volume as Reagent (xx) yy µL. Note that the plate containing glutaraldehyde (2% Glut), aqueous uranyl acetate (1% UA), and their rinses are in deck position 2, which is positioned away from antibody labeling reagents.

using the Automation Protocol shown in Figure 4. Reagents were dispensed into 96-well plates and loaded onto the ASP-1000 deck (Figures 2 and 5).

All reagents were aliquoted in 50-200 µL volumes into 300-µL polystyrene microplates in the original work[1]. (Microscopy Innovations recommends 500-µL microplates rather than 300-µL ones. See Ordering Information.) To minimize potential epitope inactivation by glutaraldehyde, the plate containing toxic reagents (glutaraldehyde and uranyl acetate) was placed far from the antibody labels.

Rat liver peroxisomes were labeled with rabbit anti-catalase primary antibody (Thermo Fisher Scientific LF-PA0060) using a 50x to 200x dilution with incubation buffer, and goat anti-rabbit IgG conjugated to

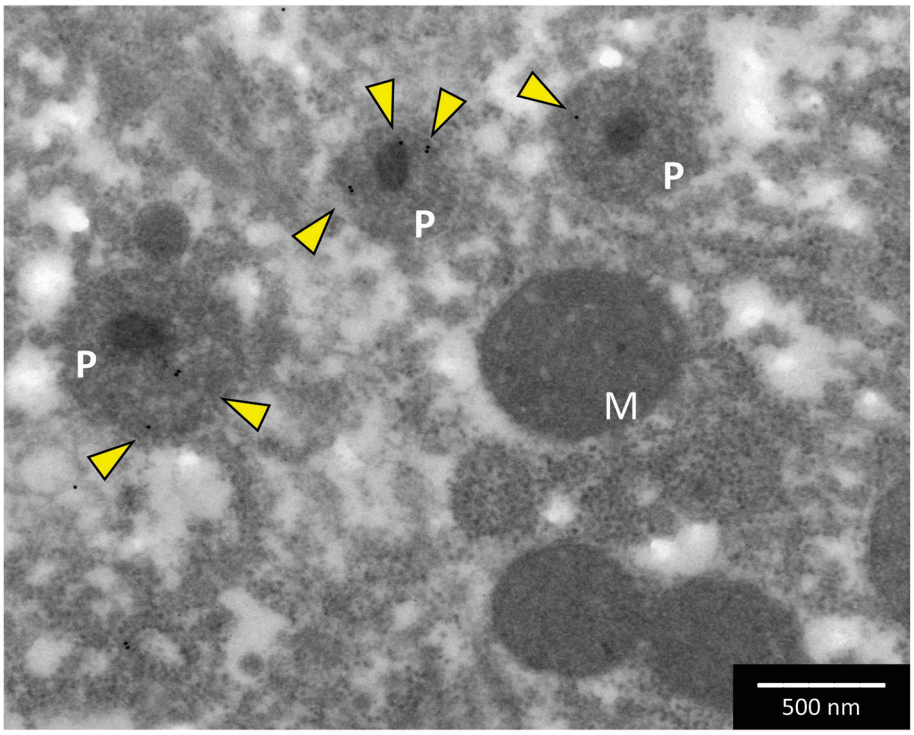

Figure 6: ***Rat liver embedded in Unicryl resin.*** Catalase in peroxisomes labeled with 10 nm gold (arrowheads). P: Peroxisome, M: Mitochondria

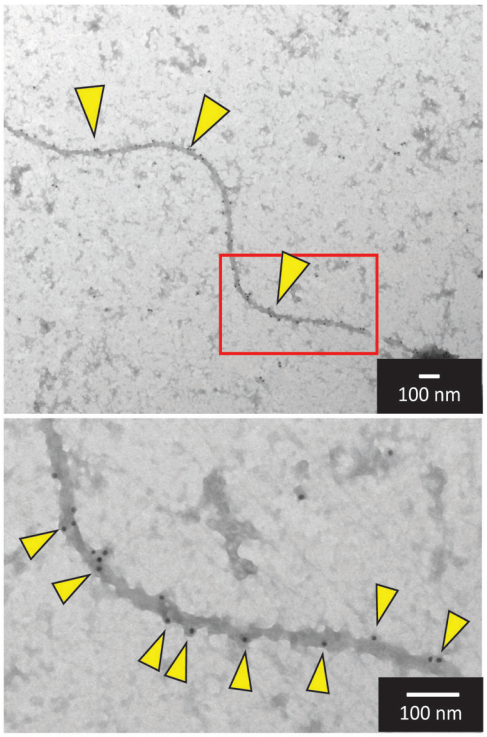

Figure 7: Whole-mount bacteria on grid. Flagella labeled with 10 nm gold (arrowheads). Selected region (red box) at higher resolution on bottom.

10 nm gold (Aurion, Wageningen, The Netherlands) with a 25x dilution from stock. *Pseudomonas aeruginosa* flagella were labeled with rabbit anti-flagella (500x dilution) and goat anti rabbit IgG conjugated to 10 nm gold (Aurion) at 50x dilution.

The IGL protocols for labeling on grids required an approximate 4-hour runtime, preceded by 30-60 minutes setup to prepare solutions, dispense them into microplates, and insert grids into the mPrep/g capsules.

After IGL processing, grids were retrieved from mPrep/g capsules, air-dried, and examined with 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

Images of cells prepared using the ASP-1000 autoprocessor demonstrate specific IGL labeling of both thin sections on grids (Chlamydia-infected HeLa cells, Figure 6) and whole-mount specimens (*Pseudomonas aeruginosa* flagella, Figure 7). Improved signal-to-noise ratio was shown, as compared with manual labeling.[1]

Automated IGL offered significant time savings for lab staff. Total hands-on time for the automated method was 30-60 minutes, while manual IGL required about 8 hours. Grids were handled only during initial loading and final unloading from mPrep/g capsules, thus reducing effort and potential damage from repeated handling. All solution delivery and agitation steps were performed automatically and simultaneously for all grids, thereby providing experimental reproducibility. The ASP-1000 can parallel process 16 grids in 8 mPrep/g capsules at one time.

Automated post-embedding IGL procedures integrate smoothly into the laboratory workflow. The ASP-1000 can be set up for overnight processing at the close of the working day to provide grids for analysis first thing the next morning. Alternatively, processing can be set up in the morning and automatically completed by the end of the workday without further operator intervention. Either way, the ASP-1000 greatly reduces hands-on time compared to manual IGL.

Finally, the ASP-1000 can also perform post-labeling contrast enhancement with uranyl acetate in the same mPrep capsule, as shown here, without having to transfer the grids, thus further reducing effort and risk of grid damage.

Benefits of ASP-1000 Processing in mPrep/g 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 controls 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 grid handling and potential for errors.

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[1-2] and for providing permission to share their work.

Ordering Information

Product #	Item Description/Catalog Information
41000	mPrep ASP-1000 Automated Specimen Processor
21300	mPrep/g capsules in storage box - 16 capsules, 16 blank label sets
21500	mPrep/g capsules - bulk pack of 96 capsules & blank label sets
31500	mPrep/f30 standard filter-couplers in capsule storage box, 16/pk
51001	96-well plates, 500uL, round well, polypropylene 10/sleeve
53503	12-channel reagent reservoirs, 5/sleeve
53502	12-channel reagent reservoirs, 25/cs

Reagents

- PBS: 0.1 M phosphate buffered saline, pH 7.4
- Glycine quench: 50 mM glycine in PBS
- Block: 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
- PB: 0.1 M phosphate buffer, pH 7.4 (without salt)

- 2% glutaraldehyde in PBS
- 1% uranyl acetate (UA)
- Immunolabels: primary (1°) antibodies, gold-conjugated to secondary (2°) antibodies

References

[1] 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

[2] Lillehoj EP, Guang W, Hyun SW, Liu A, Hegerle N, Simon R, Cross AS, Ishida H, Luzina IG, Atamas SP, Goldblum SE. (2019) Neuraminidase 1-mediated desialylation of the mucin 1 ectodomain releases a decoy receptor that protects against Pseudomonas aeruginosa lung infection. *J Bio Chem* 249(2) 662-678. doi: 10.1074/jbc.RA118.006022

[3] 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

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