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Optimization of Automated Immuno EM for Both Pre- and Post-Embedding Labeling

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Immuno electron microscopy (IEM) enables the study of the interrelationship between the cellular content of biomolecules and their proposed function at high resolution. It concurrently provides sensitive antigen detection and detailed cellular structure information. However, IEM is considered one of the most challenging techniques in cell biology [1]. In order to ensure high signal/noise ratio, optimization of incubation/washing regimen and antibody dilutions are critical. Furthermore, IEM experimental procedures are labor-intensive and involve frequent maneuvering of fragile grids or tiny specimens at frequent 5 to 15 minute intervals. The standard post-embedding protocols performed manually in the Electron Microscopy Core Imaging Facility (EMCIF) at the University of Maryland include nearly 50 liquid exchange steps. As a result, experimental outcomes are prone to variation.

An automated post-embedding immunogold labeling procedure using a newly developed automated specimen processor ASP-1000 (Microscopy Innovations, WI, USA) was recently reported [2]. ASP1000 holds tissue specimens or grids in specially designed mPrep capsules [3]. An 8-channel fluid handling system and a three–dimensional robotic platform are used to perform all liquid exchanges and mixing. Researchers only need to handle grids or specimens at the initial loading and final unloading steps. Solution changes and mixing are pre-programed and performed automatically, thus increasing the reproducibility of the labeling outcome. With strategic planning, labeling can be set up as an overnight run leaving the instrument free for other usage during the day. Labile reagents such as silver enhancement solution can be appropriately timed and added during a brief pause in the reaction.

We report here an enhanced procedure for automated post-embedding immunogold labeling in which the pumping speed and frequency of mixing were reduced in order to minimize peeling and folding of resin sections and loss of particulate specimens (Figure 1). Furthermore, the volume of each washing solution was increased by adopting deeper multi-well plates. These changes improved the consistency and the quality of the grids and increased the signal-to-noise ratio (Figure 2). Figure 2 illustrates the detection of bacterial flagellar antigen of *Pseudomonas aeruginosa* freshly applied to EM grids (Figure 2A), of a major chlamydial surface protein on infected Hela cells embedded in unicryl (Figure 2B). A pre-embedding labeling protocol was also developed using mPrepS capsules [3] to hold tissue pieces for labeling and subsequent embedding. We have compared the labeling outcomes of manual and automated labeling. Although both methods resulted in similar labeling efficiency, the automated labeling method consistently yielded lower background noise.

In summary, we have developed automated IEM methods for both post- and pre-embedding labelling using the ASP1000 automated specimen processor. This has not only increased the reproducibility of the immuno labelling results, but also drastically reduced effort and dexterity required to conduct these challenging techniques. The modern demand for efficiency and fast throughput has led to instrument-

assisted automation being increasingly adopted by EM facilities. The ASP-1000 is a versatile instrument that can be programed to perform EM specimen processing, staining and immunolabelling. The current model is limited to processing 8 specimens at one time and there is no temperature control. However, a new deck design with heating and cooling module is being developed. We are currently developing additional protocols to accommodate diversified specimens so as to broaden the range of application for this instrument [4].

References:

[1] R Melo *et al*, Nat Protoc **9** (2014), p. 2382.
[2] E Frankel *et al*, Microsc. Microanal. **22 (3)** (2016), p. 1010.
[3] S Goodman *et al*, Microscopy Today **23** (2015), p. 30.
[4] The authors wish to acknowledge that the ASP-1000 purchase was funded via Defense University Research Instrumentation Program (Proposal number 70183-LSRIP), Department of Defense.

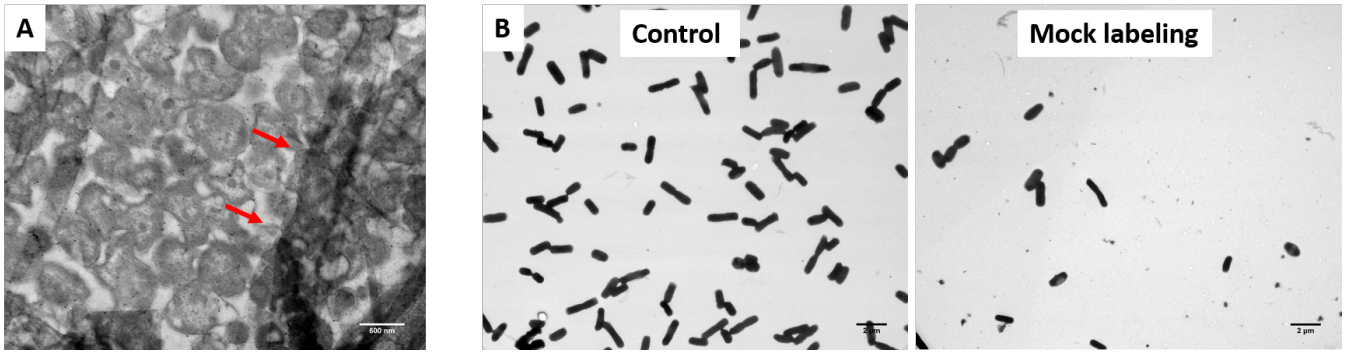

Figure 1. Rigorous mixing of the automated labeling can result in curling and folding (red arrow) of sections (A) and loss of particulate sample (B)

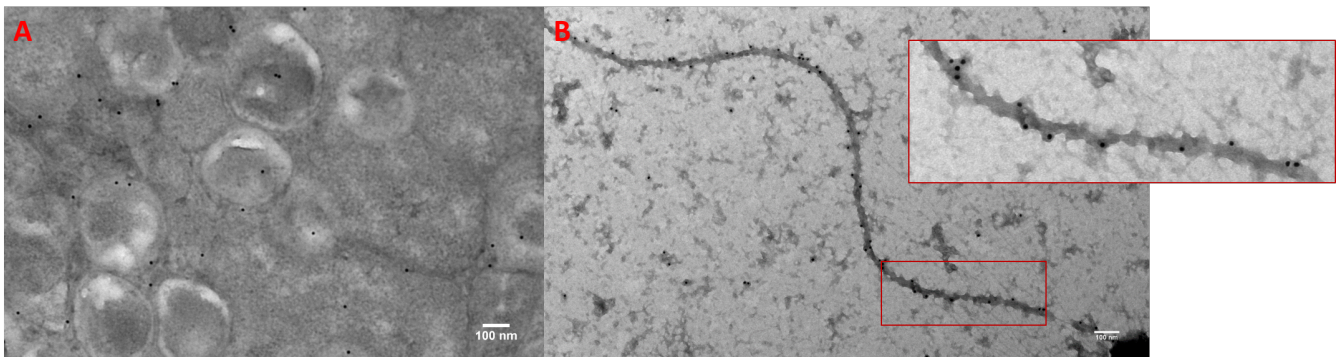

Figure 2. Immunogold labeling results using a modified ASP-1000 program for the detection of a major chlamydial surface protein in infected Hela cells embedded in unicryl (A), and a flagellum specific antigen of *Pseudomonas* (B). *Scale bar* = 100 nm.

Optimization of automated immuno EM for both pre- and post-embedding labeling

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INTRODUCTION

Immuno electron microscopy (IEM) is an important technique to study the interrelationships between the biomolecules contained in cells and their proposed function at high resolution. It also provides specific antigen localization and detailed cellular structural information. However, IEM is considered one of the most challenging techniques in cell biology [1]. The experimental procedures are labor-intensive and involve frequent maneuvering of fragile grids or tiny specimens at frequent (5 to 15-minute) intervals. When performed manually, standard post-and pre-embedding protocols of the Electron Microscopy Core Imaging Facility (EMCIF), University of Maryland Baltimore (UMB), include nearly 50 liquid exchange steps. As a result, experimental outcomes are prone to error and variations in signal/noise ratio.

The aim of our study is to develop and optimize automated post-embedding and pre-embedding immunogold labeling protocols suitable for a broad range of specimens using the automated specimen processor, ASP1000 (Microscopy Innovations, WI, USA). The ASP1000 is equipped with an 8-channel fluid handling system and a three dimensional robotic platform (Figure 1A). All reagents for each step are pre-loaded in 96-well plate at the start of the experiment. The labeling procedures are pre-programmed according to the spatial locations of each reagent. The speed and frequency of mixing and agitation of each reagent at labeling and washing steps can be pre-programmed and adjusted individually.

Here, we report automated pre- and post–embedding IEM labeling workflows that yielded reproducible labeling results with improved signal/noise ratio and labeling efficiency comparable to that of manual labeling. Pros and cons of these automated IEM methods and further improvements are discussed.

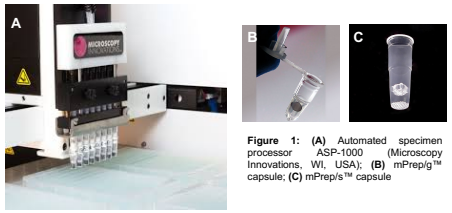

Figure 1: (A) Automated specimen processor ASP-1000 (Microscopy Innovations, WI, USA); **(B)** mPrep/g™ capsule; **(C)** mPrep/s™ capsule

MATERIALS AND METHODS

Post-embedding Immunogold labeling: Different types of specimen including brain, heart muscle, cultured cells and bacteria in suspension were tested for automated immunogold labeling. Specimens were fixed in 4% paraformaldehyde, stained with uranyl acetate, dehydrated and embedded in unicryl using a progressive lowering temperature technique as previously described [2]. Ultrathin sections were collected on 300 mesh pioloform-coated Ni grids and loaded in mPrep/g™ capsule (Figure 1B) and then transferred onto the ASP1000 for labeling. Antibodies used in the IEM experiments are described in the figure legend.

Pre-embedding immunogold labeling: 30 micron thick, 2 mm diameter mouse brain slices were generated using a biopsy punch. The thin tissue slices were then loaded into mPrep/s™ capsules (Figure 1C) and transferred to the ASP1000. The labeling workflow was paused (in bold font in Table 2) at the end of specific steps when a labile reagent (such as sodium borohydride and silver enhancement reagent) is to be used next, thus allowing technical staff to add freshly made reagent and re-start the workflow. After the last washing step, microplate containing reagents for staining, dehydration and resin infiltration were loaded on the ASP1000 robotic platform (not shown in the labeling protocol). The labeled specimens were further processed for resin embedding using a tissue embedding program in the ASP1000 and further processed in the same mPrep/s capsule without manual handling or transfer [3,4].

Funding

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References

[1] R Melo *et al*, Nat Protoc 9 (2014) p. 2382-94.
[2] SA Mojica et al, Mol Biol Cell 26 (2015) 1918-34
[3] E Frankel *et al*, Microsc. Microanal. 22 (Suppl 3), (2016) 1010-11.
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UMB Electron Microscopy Core Imaging Facility

RESULTS

Table 1: Steps and parameters of automated post-embedding IEM

Reagent	Speed	Aspiration Hold (seconds)	Dispense Hold (seconds)	Repeats	Estimated time (minutes)
Quench (Glycine in PBS)	35	30	0.5	15	15
Phosphate buffered saline (PBS)	35	15	0.5	10	6
Blocking	35	30	0.5	30	27
Incubation Buffer x2	35	15	0.5	10	6.5 x2
1° Antibody	35	30	0.5	120	106
Incubation Buffer	35	15	0.5	3	2
Incubation Buffer x3	35	15	0.5	10	6.5 x3
2° Antibody (Gold conjugated)	35	30	0.5	120	106
Incubation Buffer	35	15	0.5	3	2
Incubation Buffer x3	35	15	0.5	10	6.5 x3
PBS x2	35	15	0.5	10	6.5 x2
Glutaraldehyde	35	15	0.5	15	10
Phosphate buffer x2	35	15	0.5	10	6 x2
Water x3	35	0.5	0.5	10	4 x3
Uranyl Acetate	35	30	0.5	15	14
Water x3	35	0.5	0.5	10	4 x3
Blot					
Total run time:					**6hr 45 min**

Table 2: Steps and parameters of automated pre-embedding IEM

Reagent	Speed	Aspiration Hold (seconds)	Dispense Hold (seconds)	Repeats	Estimated time (minutes)
Phosphate Buffer x3	35	15	0.5	8	5 x3
STOP- Add Quench					
Quench (Sodium Borohydride)	35	30	0.5	20	18
Phosphate Buffer x3	35	30	0.5	15	13.5 x3
Permeabilization	35	30	0.5	30	27
Phosphate Buffer x3	35	30	0.5	15	13.5 x3
Blocking	35	30	0.5	60	54
Incubation Buffer x3	35	30	0.5	15	13.5 x3
1° Antibody	35	60	0.5	250	350
Incubation Buffer x4	35	30	0.5	15	13.5 x4
2° Antibody (Ultra Small Gold)	35	60	0.5	100	140
Incubation Buffer x5	35	30	0.5	15	13.5 x5
Phosphate buffered saline (PBS) x2	35	30	0.5	15	13.5 x2
Glutaraldehyde	35	30	0.5	15	13.5
PAUSE-Hold until user intervention					
Phosphate buffered saline (PBS)	35	30	0.5	15	13.5
Quench (Glycine in PBS)	35	30	0.5	15	13.5
Water x6	35	30	0.5	15	13.5 x6
STOP- Add Silver					
Blot					
Silver Enhancement (Aurion)	35	60	0.5	40	49
Blot					
Water	35	15	0.5	15	10
Water x5	35	0.5	0.5	10	4 x5
Glutaraldehyde	35	30	0.5	15	13.5
Phosphate buffered saline (PBS)	35	15	0.5	15	10
Quench (Glycine in PBS)	35	30	0.5	15	13.5
Total run time:					**18hr 53 min**

Table 1 and Table 2 list the labeling workflow and the parameters developed for the automated post-embedding and pre-embedding labeling programs respectively. The reagents in blue were dispensed in 1200 µl deep well 96-well plate in 1000 µl volumes. All other reagents were dispensed using a 300 µl microplate in 35 to 100 µl volumes. Microplates containing glutaraldehyde were strategically placed in the far corner of the platform away from other immune reagents to avoid inactivation.

The total experimental time for post embedding labeling is approximately 6 hr 45 min, thus can be started in the morning and completed by the end of the day with only 30 to 60 min actual hands-on time. Pre-embedding labeling can be completed in 18 hr 15 min. We normally set up the reagents and start the program at the end of the day, leave the program to perform primary and secondary antibody incubations and washings overnight, and pause after the glutaraldehyde fixation step (in red). In the morning of the next day, technical staff re-starts the washing and quenching steps, prepares the silver enhancement reagent, re-starts and completes the program in approximately an additional 4 hours. Embedding of the labeled specimens can be performed immediately in the same mPrep capsule in the ASP1000 and completed within two hours. The pre-embedded labeled specimen will be ready for ultrathin sectioning and EM examination in the morning of the third day with total hands-on time of around 2 hours.

RESULTS (CONT)

Post-embedding labeling

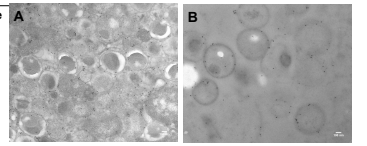

Figure 2: Compares manual **(A)** and automated **(B)** post-embedding labeling of the chlamydial major outer membrane protein (MOMP) on *Chlamydia*-infected Hela cells embedded in unicryl resin. Antibodies Rabbit anti-MOMP (1000x) and Goat anti-rabbit IgG conjugated with 10nm gold (25x) were used for labeling. *Scale bars* = 100 nm.

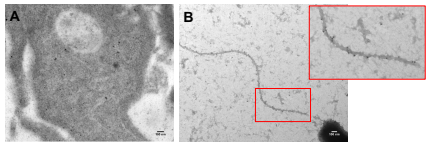

Figure 3: illustrates two other automated post-embedding labeling results using mouse brain tissue **(A)** and unembedded bacteria directly applied onto grids **(B)**. The antibodies used for labeling were chicken anti-GFP (2000x) and goat anti-chicken IgY conjugated with 10nm gold (50x) for **(A)** and rabbit anti-flagella (500x) and goat anti rabbit IgG conjugated with 10nm gold (50x) for **(B).** *Scale bars* = 100 nm.

Pre-embedding labeling

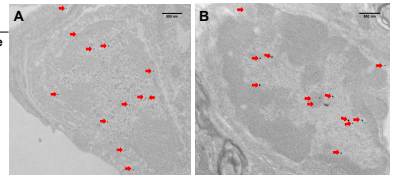

Figure 4: Compares manual **(A)** and automated **(B)** post-embedding labeling of HA-tag overexpressed SIRT1 on mouse brain slices. Red arrows mark the gold particles The manual post-embedding labeling experiment requires nearly a whole week from the start of labeling to the generation of ultrathin sectioned specimens ready for TEM examination. Antibodies Rabbit anti-HA-tag (500x) and Goat anti-rabbit IgG conjugated with Ultrasmall gold (100x) were used for labeling. *Scale bars* = 500 nm.

CONCLUSIONS

- We report here optimized protocols for automated immunogold labeling using the ASP1000 robotic platform for both post- and pre- embedding immunogold labeling. The immunogold labeling efficiency and signal to noise ratio of the automated labeling method were comparable to those of manual labeling,
- During the process of optimization of the labeling protocol, we found that vigorous mixing using fast pumping speed and more frequent agitation (a regimen adapted from the automated embedding program), often resulted in detachment and tearing of resin ultrathin sections. In the case of particulate specimen such as bacteria, loss of specimen was evident. We thus reduced the pumping speed and used a more gentle and less frequent mixing regimen to minimize peeling and folding of resin sections and loss of particulate specimens. Furthermore, we observed that increasing the volume of each washing solution by using deep well plates also improved both the reproducibility of signal-to-noise ratio and the quality of the grids.
- The major advantage of the automated labeling protocol is the drastic reduction of hands-on time required to perform the experiments. Solution preparation, dispensing in 96-well plate and specimen loading in mPrep capsules takes approximately 60 minutes compared to 8 to 16 hours hands-on time required when performing the labeling manually. Furthermore, the ASP1000 can also be used for UA-lead staining and resin embedding. Therefore, contrast enhancement after post embedding labeling and the specimen staining, dehydration and embedding after pre-embedding labeling can all be performed in the same mPrep capsule using the ASP1000 without having to transfer the grids or tissue pieces thus reducing the risk of sample damage.
- Current optimization was focused on labeling quality, reproducibility and reduction in hands-on time with less emphasis on total experimental time. We anticipate the incubation and washing time currently adapted from the manual labeling protocol can be further optimized (reduced) due to the more efficient agitation and mixing of the reagents. However, the current incubation time allows overnight operation which is more conveniently integrated in a busy work schedule.
- A major limitation of the current pre-embedding labeling protocol using the ASP1000 is that the sample must fit in the mPrep-capsule. Therefore, specimens larger than 3mm, grown on coverslips or other solid support cannot be labeled using this automated method. Moreover, the current design of the ASP1000 can only accommodate 8 specimens for processing. Therefore, any experiment consisting of more than 8 specimens must be processed in batches. Loading grids and specimens into mPrep capsules can also be difficult particularly for beginners. A bench top magnifying lamp, dissecting microscope and some practice may be required to overcome this difficulty.