PREFACE

Microwave Techniques and Protocols brings to the clinical and research community a how-to manual based on contributions from laboratories that are using microwaves as a means to facilitate biological sample processing. This project actually began in 1992 when Ted Pella Inc. decided to develop microwave technology for clinical and research applications. A number of questions arose almost immediately: What can microwave technology do to improve sample processing for microscopy applications? How does it work? And, most importantly, why use it?

In 1992 there was adequate literature to engender confidence that microwaves could be used to facilitate the following: (1) microwave-assisted stabilization or chemical fixation for light and electron microscopy, (2) enhanced special stain protocols for histology, (3) accelerated decalcification, (4) lower incubation times during immunocytochemistry, and (5) reduced processing times for small tissue biopsies into paraffin. The problem was where to begin when you didn't know anyone in the field. Microwave-assisted chemical fixation presented itself as the place to start to the literature interest at that time and owing to the fact that success or failure could be determined relatively quickly. It took almost three years before publication of our first paper on microwave fixation for electron microscopy (Giberson and Demaree, 1995). By 1995 we still could not locate anyone using the microwave to routinely fix tissue, and based on our experience, we could understand why. Success had come with a lot of effort, but few fundamental answers as to how or why the process worked.

Much has changed since that original paper. Protocols now exist for microwave-assisted chemical fixation for both light and electron microscopy that can be done rapidly, reproducibly, and routinely. However, fixation is only a fraction of the time required in overall sample processing. The original 1995 paper was the seed for moving forward and demonstrating that the microwave could be used for each step in processing for electron microscopy (Giberson et al., 1997). That 1997 paper described a four-hour protocol that has since been shortened to two hours (*see* Chapter 2). A microwave workshop series that began in the summer of 1995, and continues to this day, is the basis for the contributions to this book.

The contributors to this manual are uniformly from those laboratories routinely using microwave technology to facilitate their processing methods in the various fields of microscopy. The methods and results these authors describe are the tangible evidence that microwaves can be used routinely as the basis for improved sample processing for microscopy applications. These applications include complete sample processing protocols for light and electron microscopy, decalcification, and immunocytochemistry. The overall time savings, ease of use, and quality of results serve as justification for using microwaves in the laboratory. The question as to whether there is a "microwave effect" is alluded to, but not discussed in any great detail. When the term microwave technology is used, it is generic and intended to mean equipment designed for laboratory versus household use.

Microwave Techniques and Protocols is designed for anyone with a background and experience in sample processing for immunocytochemistry, decalcification, light microscopy, or electron microscopy, and clearly demonstrates that microwave technology has a place in today's laboratory.

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2 Vacuum-Assisted Microwave Processing of Animal Tissues for Electron Microscopy

Richard T. Giberson

INTRODUCTION

Microwave (MW) technology has found a permanent niche in the world of surgical pathology, biomedical research, and allied disciplines, for a few simple reasons: It can reduce the processing times for tissue samples destined for evaluation by light, scanning, or electron microscopy (EM); it is relatively inexpensive; and it is applicable to a wide range of applications that extend beyond tissue processing. MW-assisted processing of tissue samples, sections, or slides is a new tool in the way the art and science of tissue processing is approached. Understanding of the technology and how to apply it are probably the two most critical factors in determining overall individual success or failure. This report may provide a better understanding of the methods of MW-assisted processing, and introduce a new methodology for tissues being processed for evaluation by EM.

The first reported use of a MW in tissue processing was by Mayer (1970), which described MW heating as a direct means of tissue stabilization. Since that first report, MW stabilization has become known as a process in which tissue samples, either fresh or taken from formalin, are placed in a solution (typically, normal saline), and heated to final solution temperatures (temps) between 45 and 70°C in the MW (Leong, 1991, 1994; Hopwood, 1993; Kok and Boon, 1992).

From: *Microwave Techniques and Protocols*
Edited by: R. T. Giberson and R. S. Demaree Jr.

MW-assisted chemical fixation was the next process to evolve, (first reported in the late 1970's [Login, 1978]), and it has become a method for rapid fixation of tissue being processed for EM (Login and Dvorak, 1988, 1993; Login et al., 1990; Benhamou et al., 1991; Giberson and Demaree, 1995). Rapid fixation was followed by the first report (to the author's knowledge) of MW energy being used for epoxy resin polymerization (Giammara, 1985).

By the mid 1990's, MW technology was gaining some converts in the EM field, as a method to facilitate the fixation process. Tissue processing for EM, however, is a time-consuming process that routinely requires 36–48 h to complete. The fixation steps (aldehyde followed by osmium tetroxide [OsO_4]) usually account for <10% of the total time. Boon et al. (1986) had reported on MW-assisted dehydration, clearing, and paraffin infiltration for histology. The author et al. applied a similar approach to the remaining steps (dehydration; resin infiltration; resin polymerization) of tissue processing for MW (Giberson et al., 1995, 1997). A unified MW-assisted processing protocol evolved from this work. The time required to process fresh tissue to a polymerized resin block was effectively reduced from 36–48 h to <4 h. During this period, the combining of vacuum (vac) and microwave irradiation (MWI) was reported on as a histoprocessing technique (Kok and Boon, 1994, 1996; Kovács et al., 1996).

Chapter 3 describes the original vac MW-assisted processing of botanical tissues for EM. This work describes vac MW-assisted processing of animal tissues for EM. Vac is applied during the fixation and resin infiltration steps in the MW. This protocol was designed around commercially available equipment (Ted Pella, Redding, CA), and has reduced tissue-processing times for EM to 2 h.

MATERIALS AND METHODS

Equipment and Materials Required

The MW used was a Model 3451 Microwave Processor (Ted Pella) which comes equipped with the following: a water-load recirculation device (load cooler), variable power controller (supplies continuous power output from 250 to 750 W), accessories for tissue processing and MW oven calibration, and a temp-restrictive (TR) temp probe to control sample temp maximums during MWI. A vac chamber (Ted Pella, no. 3435) was used for the vac MW steps. Standard fixatives (2% glutaraldehyde in 0.1 *M* sodium cacodylate, pH 7.2–7.3; 2% aqueous OsO_4), dehydrating agents (50, 70, 90, and 100% acetone), and resins (Eponate 12, Ted Pella) were used. Rat liver, kidney, and lung were the tissues processed.

Processing Protocol

1. MW oven calibration. The first step in processing is the determination of hot and cold spots within the MW cavity (MW calibration) (Giberson and Demaree, 1995; Login et al., 1996). This process is necessary to ensure uniform MW heating of the microcentrifuge tubes (MTs) during the fixation steps. A neon bulb array is normally used to identify hot and cold spots; however, it is too large to fit into the vac chamber. Two 100-mL water loads are positioned inside the vac chamber, as well as an additional load (1-L plastic beaker containing ~800 µL water) positioned behind the vac chamber (Fig. 1). This load is recirculated and cooled to a constant temp during MWI. A trial fixation run (without samples) is done (*see* step 2 below) with the aldehyde fixative, and the same number of MTs that will be used during the actual fixation run. Temp maximums among all tubes should be within 3–4°C of each other after MWI. If not, increase water-load volumes, and/or reposition the vac chamber relative to the 1-L water load (*see* Fig. 1).
2. Aldehyde fixation. The vac chamber with water loads is positioned as shown in Fig. 1. The MTs, containing the samples, are placed in a Teflon® holder (Fig. 2), and filled with 600 ± 100 µL fixative. Fixative temp should be <20°C prior to MWI. The MW is programmed for the following time sequence: 1 min. 0% power; 40 s 100% power at 650 W; 3 min, 0% power. The holder is placed in the vac chamber, as shown in Fig. 1, and a vac of 20" Hg is drawn. The programmed time sequence is initiated.
3. Buffer rinse. Remove the holder with MTs from the vac chamber, and replace the fixative with buffer. There are two buffer changes during a 5-min period outside the MW.
4. OsO_4 fixation. The same series of steps used during the aldehyde fixation are repeated for Os fixation.
5. Acetone dehydration. The tissue samples are transferred to baskets, for dehydration and resin infiltration. 1–7 baskets can be placed in the 55 mm diameter polypropylene Petri dish (Ted Pella). The Petri dish is filled with ~15 mL 50% acetone prior to tissue transfer from the MTs. A second water load is placed in the MW cavity, prior to starting MW-assisted dehydration. This is done to create a cold spot that can be checked using the neon bulb array. The samples are placed in the MW cavity, and the temp probe is placed in the acetone (Fig. 3). The continuous power output of the MW is set for 650 W, MWI at 40 s, and the temp restriction at 37°C, for each dehydration step. The following graded series is run: 1 × 50%; 1 × 70%; 1 × 90%; 2 × 100%.
6. Resin infiltration. The vac chamber is returned to the MW chamber without the two 100-mL water loads, and the water load added for the dehydration steps is removed. The Petri dish, containing 100% resin, is

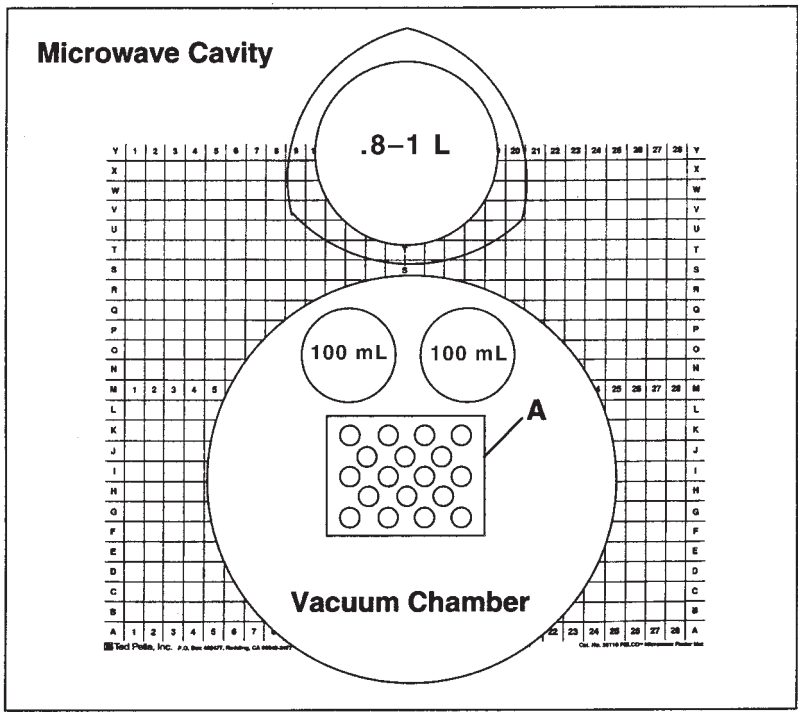

Fig. 1. The location of the MTs (A) inside the vac chamber relative to the recirculated water load (.8–1 L) in the MW cavity and the two water loads (100 mL each) placed inside the vac chamber.

placed in the vac chamber, and the temp probe is positioned in the resin. The lid to the vac chamber has a special port to allow the temp probe to monitor and control solution temps while under vac. The continuous power output of the MW is set for 650 W, MWI at 2 min, and the TR at 43°C. The following series is run: 3 × 100% resin.

8. Resin polymerization. This is done underwater, in a 1-L container, with BEEM™ capsules, according to Giberson et al., (1997). BEEM capsules, containing resin and sample, are capped with a piece of Parafilm™ in the lid. The capsules are placed in a Teflon holder, which is placed in a Rubbermaid™ (no. 5) 940-mL container, which is filled with water (Fig. 4). The original recirculated water load (~800 mL) is left in the MW during polymerization. The continuous power output of the MW is set for 750 W, MWI for 75 min, and the TR is raised from 60, 70, and 80°C at 10-min intervals, for the first 30 min, then is raised to >100°C for the remaining 45 min.

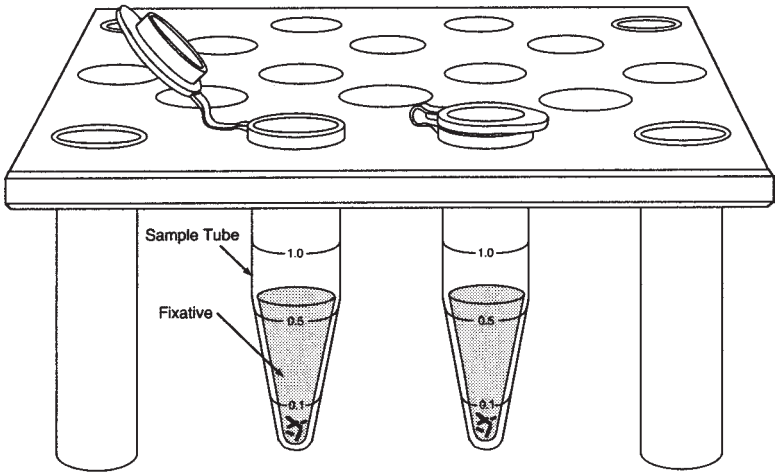

Fig. 2. The MT holder is MW-transparent (i.e., does not heat), and can hold up to 14 sample tubes. The fixative vol (600 ± 100 µL) and tissue samples are depicted in two representative MTs placed in the holder.

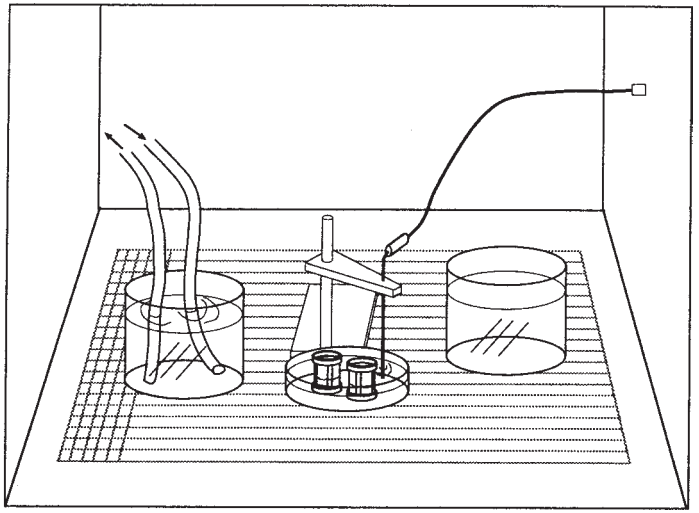

Fig. 3. A 55-mm polyethylene Petri dish, containing two tissue-processing baskets is shown relative to the water load placements in the MW cavity. The TR temp probe tip is placed into the acetone in the Petri dish, to control temp maximums during MWI.

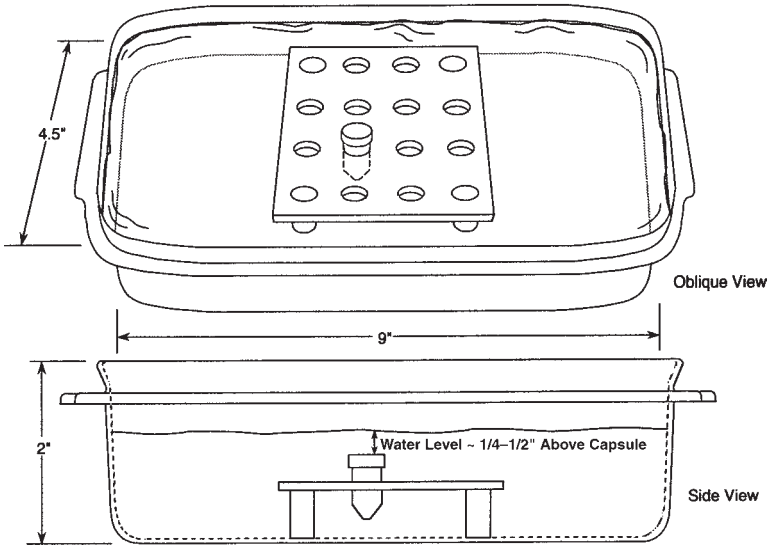

Fig. 4. BEEM capsules (or similar) containing resin and sample are capped with a piece of Parafilm in the lid, placed in a Teflon holder, which is centered in a Rubbermaid (no. 5) 940-mL container. The container is filled with tap water, as shown, and the TR temp probe placed in water to control temp maximums during the first 30 min of MW-assisted polymerization.

Comments on the Processing Protocol

Each step in the MW is an independent event, which means that a step done on the bench can be followed by a step or steps in the MW, or visa versa. Although the buffer rinse is not routinely a MW-assisted step, because of its short duration, it can be done in the MW as a 40-s step identical to the parameters outlined for dehydration. For difficult-to-fix tissues, the best results come from using a low, continuous power setting (i.e., 250 W) for an extended time period (5–45 min) (unpublished results and personal communication), using the temp probe to control the temp maximum of the fixative. When using paraformaldehyde as the fixative, or in combination with glutaraldehyde, the 40-s MW step is done at 250 W. The 3-min 0% power period is then followed by 10 s at 650 W (*see* Chapter 16 for details regarding the influence of MW radiation on formalin).

Polymerized resin blocks were sectioned, picked up on 200-mesh copper grids, poststained with uranyl acetate, and lead citrate, and viewed by a Philips T/EM 400 transmission EM. Photography was done by routine darkroom methods.

RESULTS

The three tissues processed by vac MW-assisted processing were rat lung, liver, and kidney. The samples were processed together, and polymerized resin blocks were ready for sectioning within 2 h, as measured from the aldehyde fixation step. Rat lung (Fig. 5A,B) demonstrates good preservation of the phospholipid in the lamellar bodies of the type II aveolar epithelial cells. Ribosomes are abundant and distinct, and mitochondrial density is good. Rat liver is shown in Fig. 6A,B. The cytoplasmic density is uniform, there is no extraction evident, rough endoplasmic reticulum is well-preserved and abundant; and the mitochondrial density and structures are well-preserved. Rat kidney is shown in Fig. 7A,B. Structural preservation of the glomerulus is good, and Bowman's capsule is evident in Fig. 7A, as are capillary loops. In Fig. 7B, good mitochondrial preservation is evident, as well as that of the basement membrane, capillary endothelium, and podocytes. Microtubules are evident in the podocytes (Fig. 7B).

The quality of preservation, resin infiltration, and cutting properties were equal to, or better than, that achieved using routine bench processing protocols requiring 36–48 h to complete (results not shown). Electron beam stability of the MW-cured resin is excellent, as are the post-staining properties (uranyl acetate followed by lead citrate).

DISCUSSION

Vac MW-assisted processing of tissue for EM introduces tissue-processing flexibility that has not existed before (Giberson et al., 1995, Giberson, 1997). Each step (aldehyde fixation; OsO_4 fixation, graded dehydration series, resin infiltration) can be completed in <10 min for the majority of tissues the author has tried to date, including: *Chlamydomonas* cells, yeast with cell walls, nematodes, and botanical tissues (unpublished results). MW-assisted resin polymerization is the time consuming step in the process, requiring 75 min for epoxy resins and 40 min for LR White (Giberson et al., 1995, 1997; Demaree et al., 1995). The polymerization step differs from other published methods (Giammara, 1985, 1993) in that polymerization is equal among all specimen blocks, does not require any MW oven calibration, and can be done as a routine procedure.

The number of different samples that can be processed at one time is up to the individual. The author has done as many as 50 at one time, after the fixation steps, and prefers to fix no more than eight samples at one

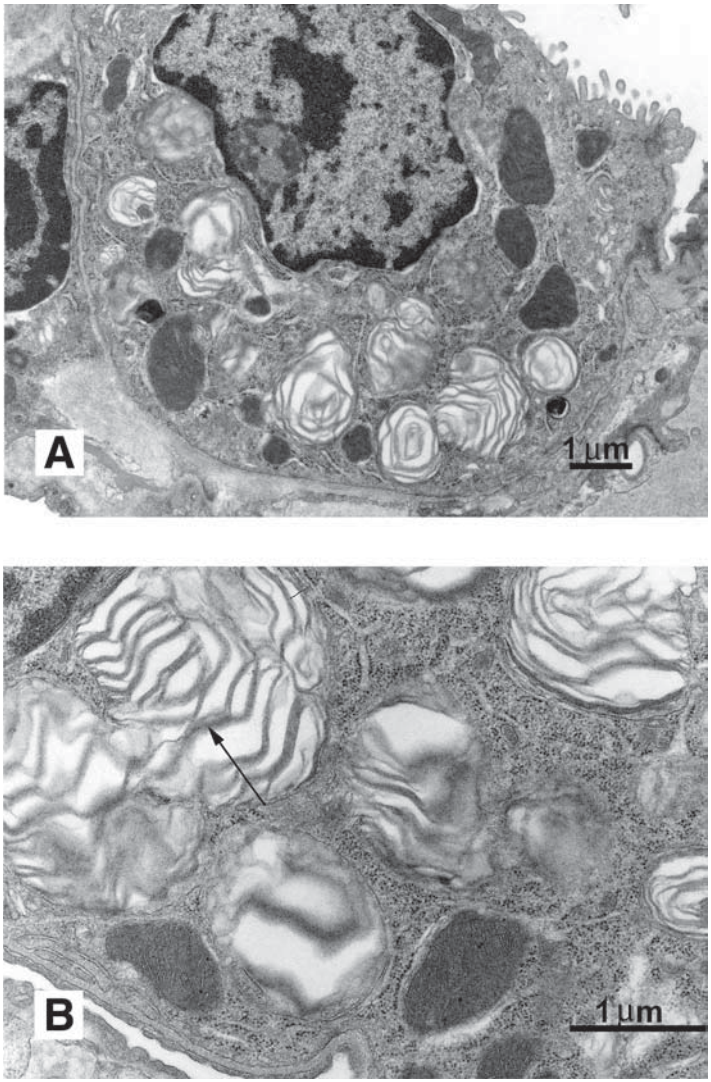

Fig. 5. (A) A low-magnification image of a type II aveolar epithelial cell. **(B)** A higher-magnification image of a type II aveolar epithelial cell, which demonstrates cytoplasmic detail (ribosomes, endoplasmic reticulum) and density. The phospholipid (arrow) in the lamellar bodies is well-preserved.

time, however, after fixation, the number of samples being processed simultaneously is not a major issue, one way or the other. When a rapid answer is desired or required, it is most convenient to use MW-assisted

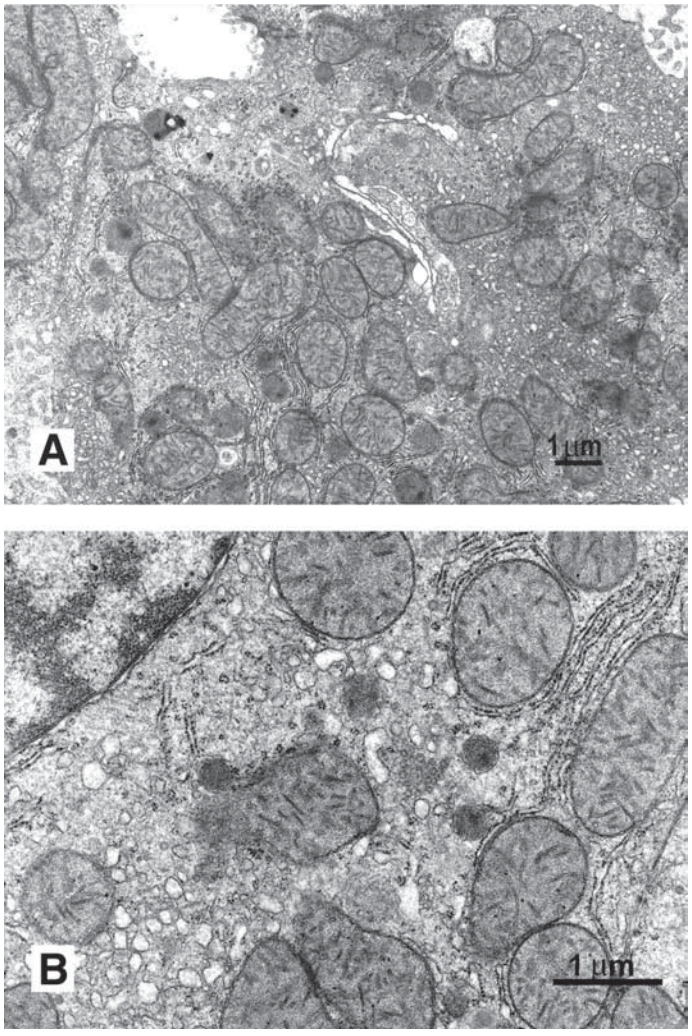

Fig. 6. (**A** and **B**) are low and higher magnifications of the typical preservation seen with vac MW-assisted processing. In both figures, the cytoplasmic density of the hepatocytes is uniformly dense, and the mitochondrial structure is well-defined. Rough endoplasmic reticulum is easy to distinguish.

resin polymerization for only those samples that one intends to section that day. The rest go to the convection oven for overnight polymerization.

Even in workshop situations, in which a number of individuals are learning the technique for the first time, polymerized resin blocks are

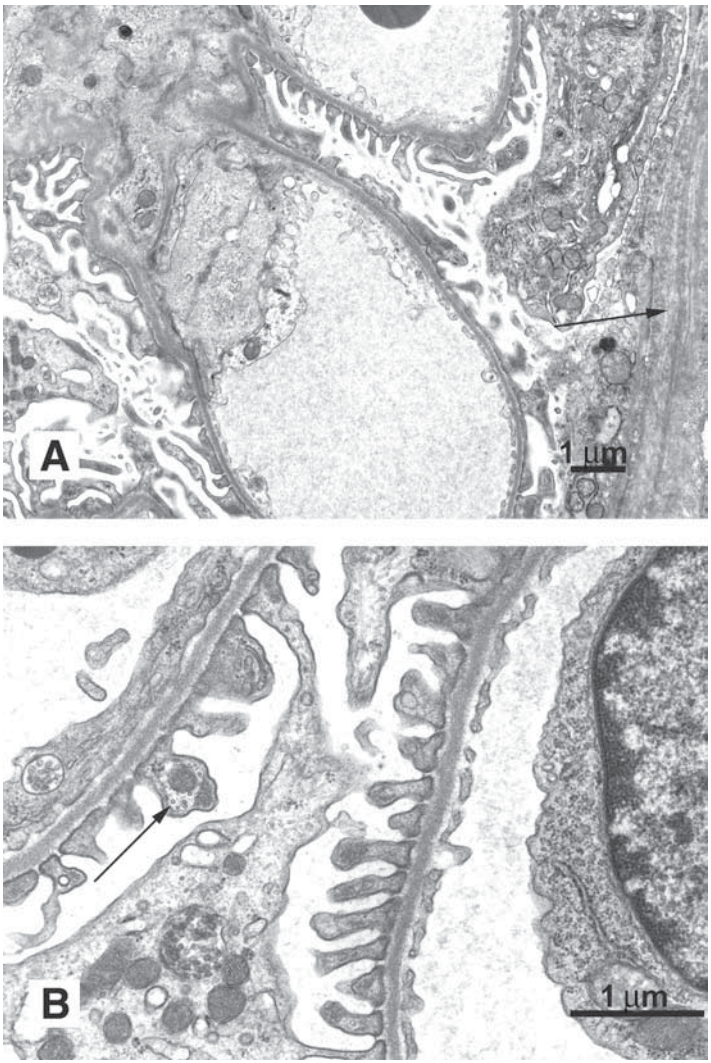

Fig. 7 (**A** and **B**) are low and higher magnifications of the typical preservation seen in the glomerular region. A is typical of the outer regions of the glomerulus next to Bowman's capsule (arrow). B is typical of the preservation seen in the capillary loops. Note the microtubules (arrow) in the podocyte.

obtained by the participants routinely in 3.5–4 h (unpublished results from 18+ national workshops). Vac MW-assisted processing of tissue specimens for EM results in significant time savings, compared to any existing methodology. The author would argue that the quality of the

results (Figs. 5A, B and 7A,B) are comparable to those obtained from routine bench, or other MW-assisted, protocols (Giberson et al., 1995, 1997). The implementation of vac MW-assisted processing for EM samples results in time saving of >30%, compared to previous MW-assisted protocols (Giberson et al., 1995, 1997), and to well over 90%, compared to bench protocols.

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