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Microwave Fixation: Understanding the Variables to Achieve Rapid Reproducible Results

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ABSTRACT The use of microwave irradiation for rapid chemical fixation of tissues in electron microscopy is a subject of current interest. The effects of water load size and location, sample placement in the oven cavity (hot or cold spots), and time on tissue preservation were examined. The use of a microwave container (4 dram vial) encased in 60 ml of ice in a 100 ml polyethylene beaker and a 0% power setting between two 100% power settings (time interval) provided reliable control of temperature during microwave irradiation. High brightness neon lights provided a quick and easy method to identify and map hot and cold spots within the oven cavity. Using microwave irradiation for rapid glutaraldehyde and osmium tetroxide fixation of tissues (Pacific yew needle and mouse kidney and liver) for electron microscopy yielded preservation equal or better than routine immersion fixation when a time interval, a cold spot (as the sample location), and an ice-encased vial were used during microwave fixation. These adaptations provided reliable control of fixation conditions in an 800 watt laboratory microwave oven.

INTRODUCTION

The interest in and use of microwave irradiation (MWI) for fixation of tissue dates back to Mayer (1970) in the UK and has remained primarily in the UK (Hopwood et al., 1984) and Europe (Kok et al., 1988; Wild et al, 1989), with additional research in Australia (Leong et al., 1985), Asia (Chew et al., 1984), and the US (Login and Dvorak, 1985).

The major emphasis for most of the microwave research has been in histology (see Histological Journal, 1988, 1990; Kok and Boon, 1992; Leong, 1993; Login and Dvorak, 1994), with additional studies on electron microscopy (Login and Dvorak, 1985, 1988, 1993). Taking the information from the literature and applying it in the laboratory, however, has not been altogether successful, with the major problem being the reproducibility of results (Login and Dvorak, 1994).

The intent of this work was first to understand the variables associated with MWI and then control as many of these as possible. With this approach, we designed a protocol, which in our laboratory has produced consistent reproducible results in the fixation of three different tissues.

MATERIALS AND METHODS

We used a programmable microwave oven designed for laboratory use with an exhaust fan, a stainless steel oven cavity, short magnetron cycle time (<3 seconds), high power output (800 W), magnetron prewarming, and automatic water load cooling (model 3400; Ted Pella, Inc., Redding, CA).

The determination of sites of sample placement prior to the actual microwave fixation was a modification of Login and Dvorak (1993, 1994) and Login (personal communication). An indexed grid was centered in the oven cavity and taped to the oven floor, and a high brightness neon bulb array (Ted Pella, Inc.) was centered on top (Fig. 1). Standard brightness (striking voltage 45–65 V a.c.) and high brightness (striking voltage 70–95 V a.c.) neon bulbs (Chicago Miniature Light, Sunnyvale, CA) were tested in microwave ovens ranging from 455–800 watts (unpublished data).

The bulb array was irradiated at 100% power for 10 seconds with no water load present. At least 90% of the bulbs should flicker or shine brightly during irradiation. The whole area covered by the bulb array was considered to be one large hot spot under these conditions. Next the water load(s) was positioned and the water volume adjusted so that an area comprising, at a minimum, four bulbs did not light or flicker during 10 seconds of irradiation at 100% power. This area was designated as a cold spot. Water load locations were outlined and the cold spot center was indicated on the grid with an indelible marking pen. An automatic water load cooling device (Ted Pella, Inc.) was used to recirculate and maintain a single water load below 43°C, eliminating the need to change heated water loads between runs.

A final check was made on the cold spot. An agar/saline/Giemsa (ASG) block (Login and Dvorak, 1993, 1994) was placed in the specimen container with 4 ml of water. Other authors (Login and Dvorak, 1993) recommend using the actual fixative. We found in our experience that it was not necessary. The water and ASG block were then irradiated for the desired time

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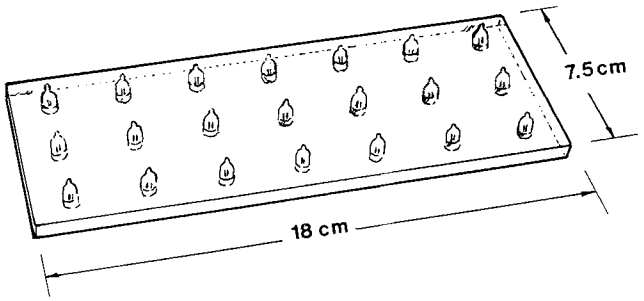


Fig. 1. High brightness neon bulb array.

interval. The ASG block is light purple in color prior to irradiation. At most a blue bull's eye should be present immediately after irradiation (temperature <55°C) (Fig. 2). Too much microwave energy showed up as a greater area of blue, agar clearing, and/or agar melt (>65°C). Results could be varied by repositioning the water load or increasing its volume.

We used a microwave container (MC) from Ted Pella, Inc. (Fig. 3a). This container was a 4 dram glass vial plus a flow-through basket attached to a Teflon® plunger. The basket fit snuggly in the glass vial. After initial trials, the glass vial was frozen in 60 ml of tap water in the center of a 100 ml polyethylene beaker (Fig. 3b). After each irradiation, a fresh frozen MC was used, ensuring that the sample was always surrounded by ice.

In the literature, temperature rise of the fixative was the most common determinant for the fixation time chosen (Heumann, 1992; Hopwood et al., 1990; Kang et al., 1991; Kok and Boon, 1992; Login and Dvorak, 1985, Wild et al., 1989). Times which yielded final fixative temperatures between 40° and 60°C were typical. Initially we used 8, 12, 16, and 20 seconds at 100% power with one 200 ml water load positioned in the left rear corner (Login and Dvorak, 1985). These times were representative of those found in the literature noted above. The sample was placed in a hot spot (as determined by the neon lights). No temperatures were recorded for this series.

Next, two time intervals were introduced, and water load volumes were increased to control heating. Intervals (all hyphenated time intervals correspond to the following: first number—seconds at 100% power; second number—seconds at 0% power; third number—seconds at 100% power) of 6-10-6 and 8-16-8 (no ice) were tried. Two water loads (300 ml on the left and 200 ml on the right) were placed 9 cm apart in the center of the oven with the sample placed in a hot spot in front of the water loads. Temperatures were recorded for this series. Immediately after MWI the sample was removed from the vial, its contents stirred, and the temperature recorded with a mercury thermometer.

Further control of microwave heating was attempted by encasing the MC in ice (Fig. 3b) and changing to a cold spot. Time intervals of 8-16-8 and 10-16-10 were used, the sample location was changed to a cold spot, and two 250 ml water loads were placed 9 cm apart in the central oven cavity. Temperatures were recorded in the same manner as the previous two intervals.

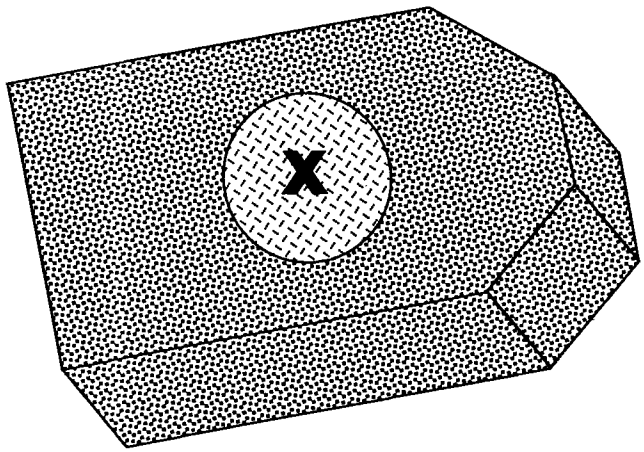


Fig. 2. Agar/saline/Giemsa block showing blue bull's eye (X) indicating proper temperature for microwave fixation. Overall dimensions are 14 × 7 × 3 mm.

Finally, two time intervals were run: 8-20-8 and 10-20-10. The water load, a water driven magnetic stirrer, was externally connected to a load cooler (Fig. 4). This device circulated and cooled the water (~250 ml) while operating the magnetic stirrer. The top center of the magnetic stirrer was positioned in a cold spot. The ice-encased MC was used. Table 1 is a synopsis of the experimental conditions for each time microwave trial.

The tissues, Pacific yew (*Taxus brevifolia*) needles and mouse (*Mus musculus*) liver and kidney (1 mm^3) were preserved in various fixatives (see Table 1, fixative), dehydrated in ethanol or acetone (50, 70, 90% for 30 min each), infiltrated with LR White Hard Grade (Ted Pella, Inc.) (three changes of 100% resin within 24 h), and polymerized overnight at 65°C in a Teflon® flat embedding mold covered with an ACLAR® film (Ted Pella, Inc.). Control tissues were fixed in buffered 2.5% glutaraldehyde followed by a buffer rinse and then postfixed in buffered 2% osmium tetroxide, each for 1 h. The buffer was 0.1 M sodium cacodylate, pH 7.0. After fixation the controls were processed the same as microwave-fixed tissue.

RESULTS

Figures 5 and 6 show the preservation typically achieved with immersion fixation of yew needle. The cell wall preservation is satisfactory. Figure 5 is an immature chloroplast, while Figure 6 depicts a mature chloroplast. Chloroplast preservation is adequate in both, but cytoplasmic extraction is greater in Figure 6.

Figures 7–10 show the effects of MWI with increasing time using a 200 ml water load and a hot spot for fixation of yew needle (Table 1, times 8, 12, 16, and 20). The sample placement and water load size are similar to those used by Benhamou et al. (1991) to chemically fix plant tissue employing MWI. The bubbles in the chloroplasts may be artifacts due to heat or may be extracted lipids. They are evident in all figures. Fixation quality is difficult to evaluate. Cytoplasmic extrac-

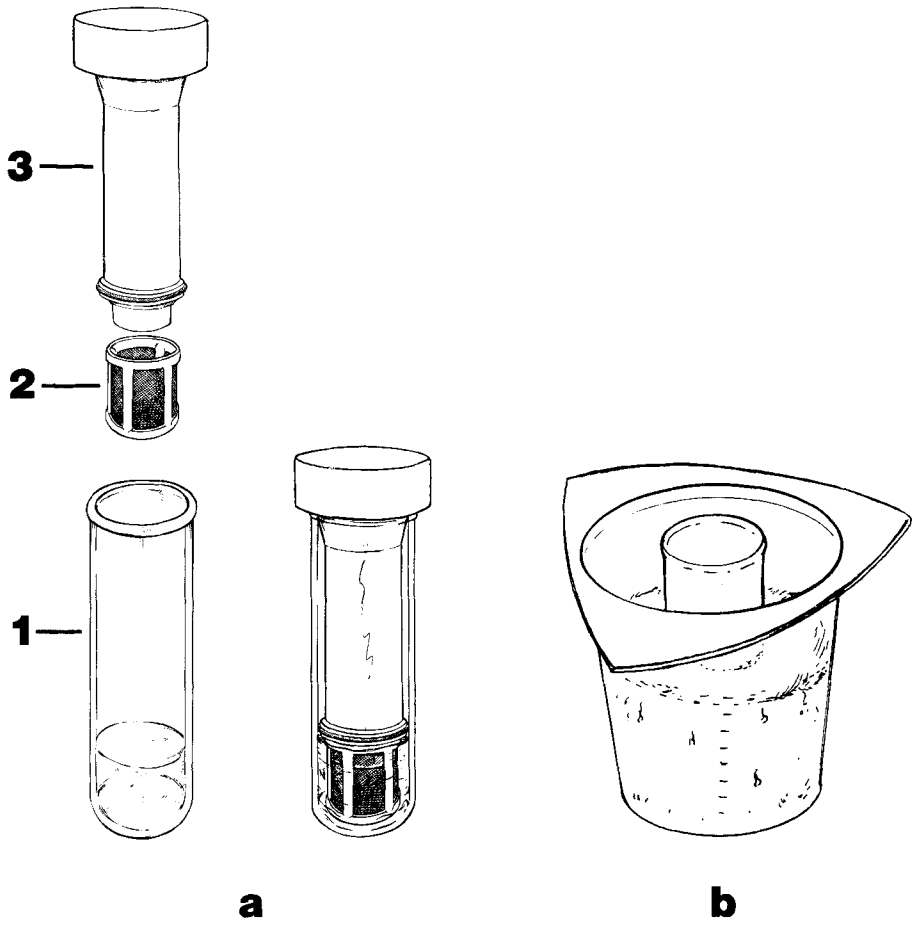


Fig. 3. Drawing of the microwave container (MC): 1, four dram shell vial; 2, flow-through specimen basket; 3, Teflon® plunger. **a:** Container without ice. **b:** The shell vial has been frozen in a 100 ml polyethylene beaker.

tion is severe, but cell wall preservation appears to improve with longer fixation times.

Figure 11 demonstrates the effects of using a time interval (8-16-8, no ice) vs. continuous time with the sample placement in a hot spot. Water load volume has also been increased (see Table 1). Preservation is improved over that shown in Figures 7–10. Cytoplasmic density is better, and membrane continuity is improved. Figure 12 shows the effects of using a time interval (8-20-8) with the ice-encased MC in a cold spot. Preservation is improved over that seen in Figures 7–11. The time interval, however, was not long enough to adequately preserve the starch granule. Shrinkage of the starch granule is evident in Figure 12.

The best fixation parameters for this particular tissue (yew needle) were those associated with the 10-20-10 fixation time. Note the excellent preservation and lack of shrinkage associated with the starch granules (Figs. 13, 14). Consistent satisfactory preservation was achieved with the 10-20-10 processing time.

The introduction of an ice-encased MC in a cold spot during microwave fixation led to a decrease in final fixative temperature (see Table 1) and the disappearance of the bubbles within the chloroplasts (Figs. 12–14) seen in all previous plant tissues (Figs. 7–11). The only ice melt seen in the MC was adjacent to the vial corresponding to the fixative level.

Based on our experience with the plant material we decided to try the 8-20-8 and 10-20-10 fixation times for animal tissues. Microwave fixation of both mouse kidney (8-20-8) (Fig. 15) and liver (10-20-10) (Fig. 16) compared favorably with standard immersion fixation (Figs. 17, 18). The time intervals were not reversed to see if they had any effect on fixation results. The only observable difference seen between MWI and immersion fixation was with the mouse kidney. The usual trilaminar appearance of the basal lamina in the glomerulus was not apparent in the microwave fixed tissue. Basal lamina density was significantly higher as well.

DISCUSSION

Microwaves are electromagnetic waves with an output frequency of 2.45 GHz (billion cycles/second) for

TABLE 1. Microwave fixation parameters

Fixation parameters	Microwave trials identified by time or time interval									
	8	12	16	20	6-10-6	8-16-8	8-16-8	10-16-10	8-20-8	10-20-10
Tissue processed in trial										
Pacific yew needle	+	+	+	+	+	+	+	+	+	+
Mouse liver										+
Mouse kidney									+	
Sample placement in microwave										
Hot spot	+	+	+	+	+	+				
Cold spot							+	+	+	+
Water load size										
200 ml	+	+	+	+						
300/200 ml					+	+				
250/250 ml							+	+		
Load cooler (~250 ml)									+	+
Fixatives used in trial (All fixative volumes were 3.75 ml)										
Para./glut[1]	+	+	+	+	+[8]	+[8]				
Reduced osmium[2]	+[6]	+[6]	+[6]	+[7]	+[8]	+[8]				
Uranyl acetate[3]	+[6]	+[6]	+[6]	+[7]	+[8]	+[8]				
Glutaraldehyde[4]							+[9]	+[9]	+	+
Osmium tetroxide[5]							+[9]	+[9]	+	+
Fixative temperature (°C) after irradiation										
Para./glut.					55	58				
Reduced osmium					40	49				
Uranyl acetate					40	50				
glutaraldehyde							22	24		
Osmium tetroxide							21	24		
Microwave container										
4 dram vial only	+	+	+	+	+	+				
Ice-encased vial (Fig. 3b)							+	+	+	+

[1]2.0% paraformaldehyde + 0.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.0.
[2]1% osmium tetroxide + 1.5% potassium ferricyanide in 0.1 M sodium cacodylate buffer, pH 7.0.
[3]2% aqueous uranyl acetate.
[4]2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.0.
[5]2% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.0.
[6]time = 10 seconds.
[7]time = 20 seconds.
[8]Temperature of fixative prior to microwave irradiation = 20 ± 2°C.
[9]Temperature of fixative prior to microwave irradiation = 3°C.

household or laboratory microwave ovens. These instruments receive approximately 1,200 watts of electrical line power which the magnetron (microwave source) converts to a given number of watts (watt = 14.33 cal/min) of electromagnetic energy in the oven cavity. Microwaves are thought to heat samples, referred to as dielectrics, by dipole rotation and ionic conduction (Neas and Collins, 1988). The electromagnetic field will cause molecules with permanent or induced dipole moments to align with the electromagnetic field and initiate an electrophoretic migration of dissolved ions. Both of these effects result in the production of heat. A dielectric is a material which transmits electric effects by induction. Induction is the appearance of an electric current or magnetic properties in a body because of the presence of another electric current or magnetic field (microwave) (Neas and Collins, 1988).

The dissipation factor of a material is one way to describe how microwaves interact with it. Expressed as tangent ∂, dissipation factors can be found in Kok and Boon (1992), Neas and Collins (1988), and Von Hippel (1954). Materials with high dissipation factors (dielectrics) adsorb microwave energy and heat up (water = 1,570 at 25°C; water + 0.1 M NaCl = 2,400 at 25°C) (Neas and Collins, 1988). Materials which are transparent to microwaves (i.e., Teflon®, borosilicate glass,

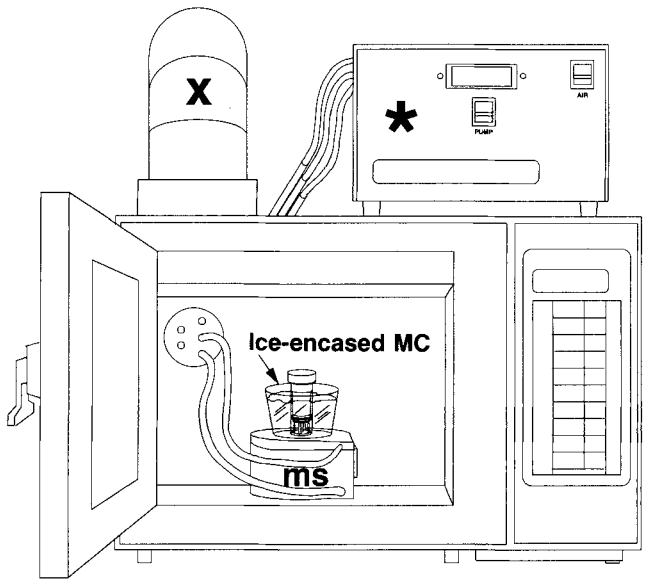


Fig. 4. Drawing of the microwave oven with the magnetic stirrer (ms), ice-encased MC centered on the stirrer, load cooling device (*), and exhaust vent (x).

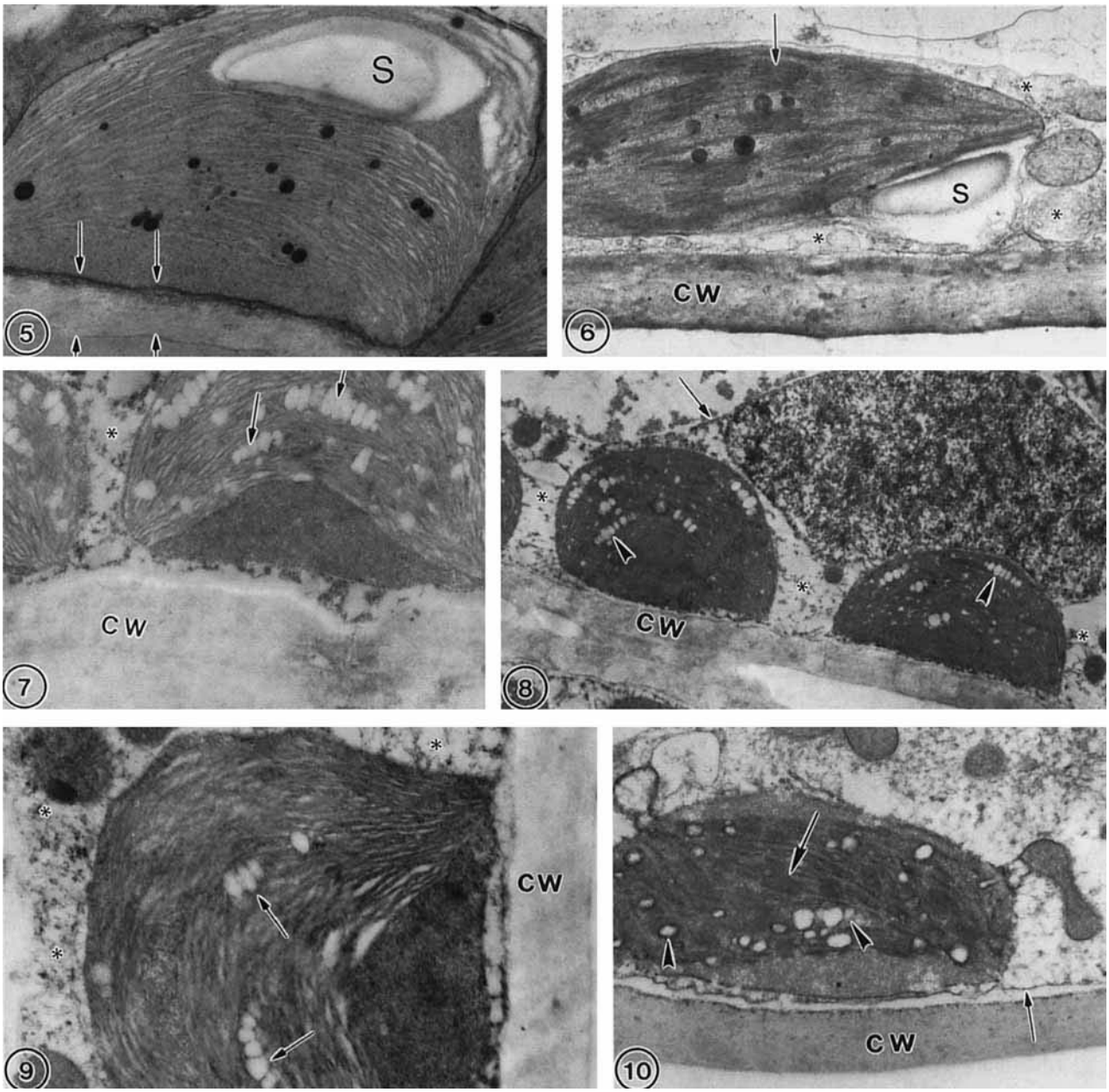


Fig. 5. Normal immersion fixation of yew needle. The chloroplast is immature and lacks the characteristic stacked grana structure. The starch granule (S) is shrunken, and the cell membrane has pulled away from the cell wall (opposing arrows). ×16,250.

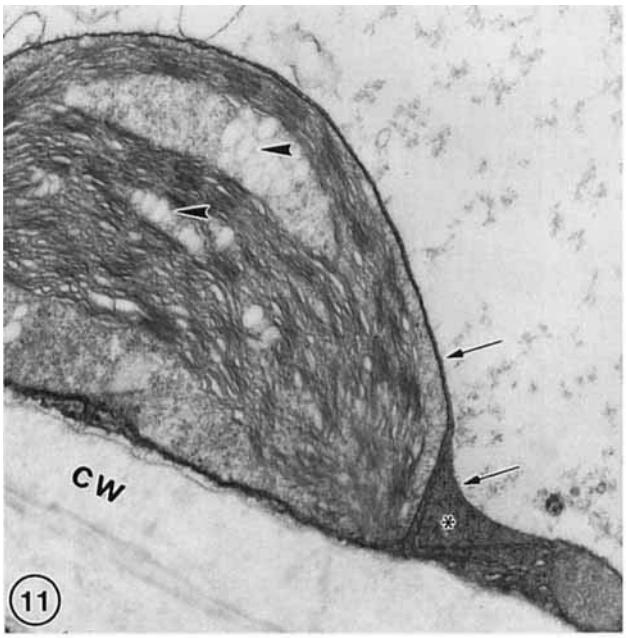
Fig. 6. Normal immersion fixation of yew needle. The chloroplast is mature and has the characteristic stacked grana (arrow). The starch granule (S) is shrunken. Cytoplasmic density (*) is poor. The cell wall (cw) structure is satisfactory. ×22,750.

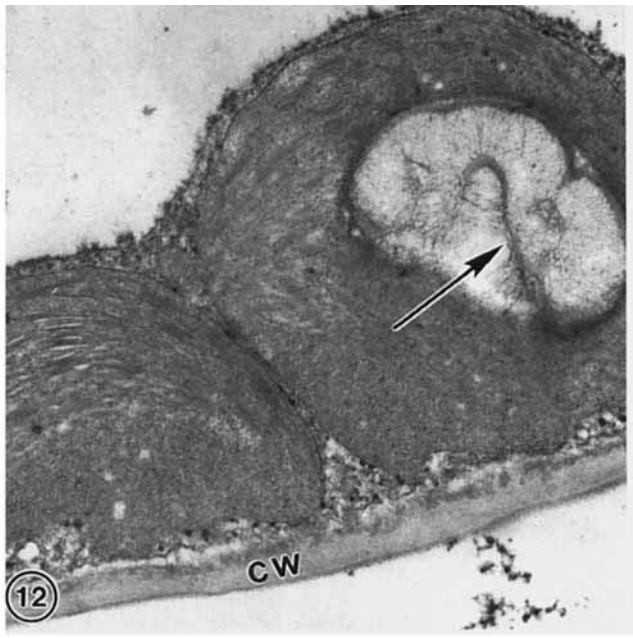
Fig. 7. Microwave fixation of yew needle for 8 seconds at 100% power in a hot spot. Cytoplasmic extraction (*) is severe, and the cell wall (cw) structure is poorly defined. There are bubbles (arrows) evident in the chloroplasts. Overall membrane preservation is poor, and the cell membrane is not evident. ×14,700.

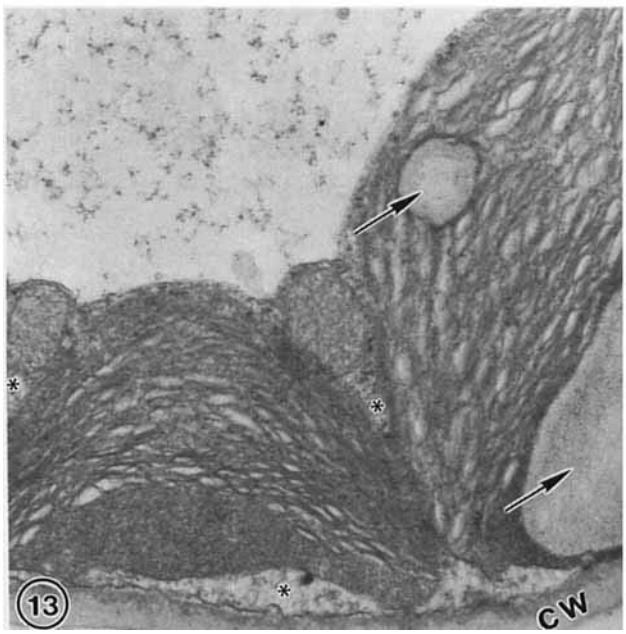
Fig. 8. Microwave fixation of yew needle for 12 seconds at 100% power in a hot spot. Cytoplasmic extraction (*) is extensive, and the tonoplast membrane is apparent in some areas (arrow). Cell wall (cw) structure is evident but poorly defined. Heat artifacts (arrowheads) are present in the chloroplasts. ×8,400.

Fig. 9. Microwave fixation of yew needle for 16 seconds at 100% power in a hot spot. Cytoplasmic extraction (*) is evident, but cell wall (cw) structure is poorly defined. Heat artifacts (arrows) are present in the chloroplasts. ×21,000.

Fig. 10. Microwave fixation of yew needle for 20 seconds at 100% power in a hot spot. Cell wall (cw) structure is satisfactory, and the plasma membrane (small arrow) is evident. The characteristic stacked grana (large arrow) in the chloroplast are evident but poorly defined. Cytoplasmic extraction is apparent, and heat artifacts (arrowheads) are present in the chloroplasts. ×14,700.







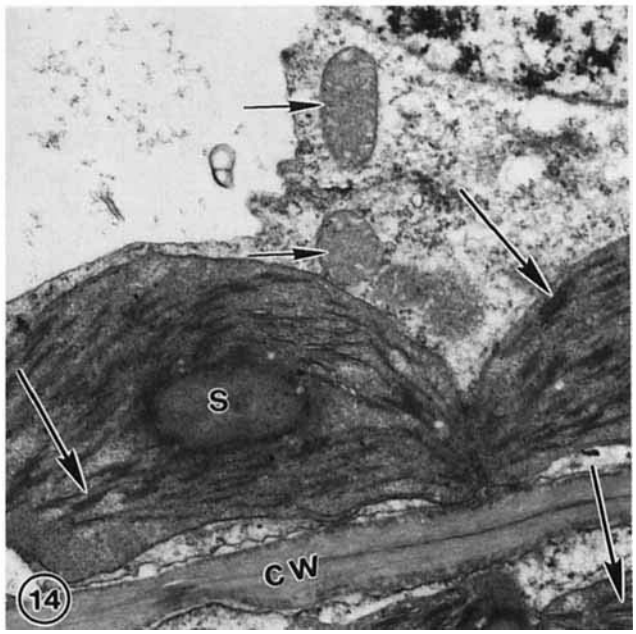


Fig. 11. Microwave fixation of yew needle employing an 8-16-8 time interval in a hot spot. Cell wall (cw) structure is poor, but the tonoplast membrane (arrows) is well defined and cytoplasmic density (*) good. Immature chloroplast structure is satisfactory, but heat artifacts (arrowheads) are apparent. ×15,000.

Fig. 12. Microwave fixation of yew needle employing an 8-20-8 time interval, ice-encased MC, and magnetic stirrer in a cold spot. Cell wall (cw) and immature chloroplast structures are good. The starch granule (arrow) in the chloroplast has partly pulled away. ×16,100.

Fig. 13. Microwave fixation of yew needle employing a 10-20-10 time interval, ice-encased MC, and magnetic stirrer in a cold spot. Cell wall (cw), immature chloroplasts, and cytoplasmic density (*) are well preserved. Starch granules (arrows) show no shrinkage. ×17,000.

Fig. 14. Microwave fixation of yew needle employing a 10-20-10 time interval, ice-encased MC, and magnetic stirrer in a cold spot. Cell walls (cw) and mitochondria (small arrows) are well preserved. Mature chloroplasts exhibit the characteristic stacked grana (large arrows), and the starch granule (S) shows no shrinkage. ×16,100.

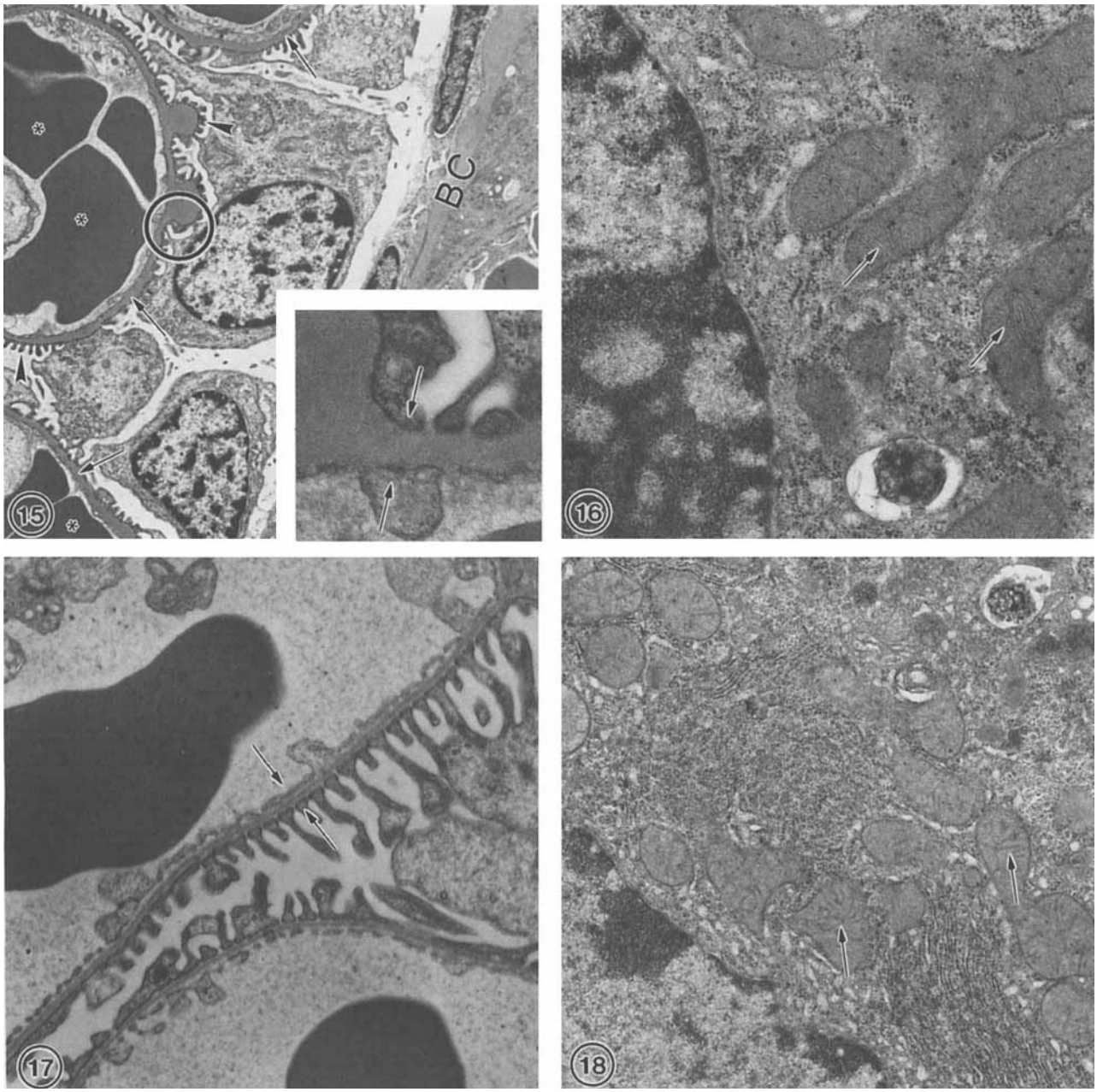


Fig. 15. Microwave fixation of mouse kidney employing an 8-20-8 time interval, ice-encased MC, and magnetic stirrer in a cold spot. Fine structure in the glomerulus as well as red blood cells (*), basement membranes (arrows), and foot processes (arrowheads) are well preserved. Bowman's capsule (BC) is present. Circle denotes area of inset. ×6,900. **Inset:** The trilaminar appearance of the basal lamina (opposing arrows) is not apparent. ×30,800.

Fig. 16. Microwave fixation of mouse liver employing a 10-20-10 time interval, ice-encased MC, and magnetic stirrer in a cold spot. Mitochondrial cristae are evident (arrows), and overall preservation is good. ×33,000.

Fig. 17. Immersion fixation of mouse kidney. Characteristic trilaminar appearance of the basal lamina is present (opposing arrows). Overall preservation is good. ×16,000.

Fig. 18. Immersion fixation of mouse liver. Mitochondrial cristae are evident (arrows), and overall preservation is good. ×16,100.

and ice) have low dissipation factors (~10 or less) and do not heat. Reflective materials are conductors (metals) which reflect microwave energy and do not heat.

Many authors (Kok and Boon, 1992; Login and Dvorak, 1993; Login et al., 1986, 1990) describe or give recommendations on the type of containers to use for microwave fixation. The shape, size, composition, and ease of sample handling all enter into the type of MC to use. An important consideration is to choose an MC which will not directly interact with the electromagnetic radiation being produced in the microwave oven. The use of an ice-encased MC, therefore, should not affect the amount of microwave energy reaching the sample. It appeared to promote more rapid cooling during the 0% power cycle. This, in turn, kept the final fixative temperature lower (see Table 1, trials 6-10-6 and 8-16-8 (no ice) vs. 8-16-8 (ice) and 10-16-10). Based on our results (Figs. 13, 14 vs. Fig. 11) preservation was improved with the addition of an ice-encased MC.

Mayers (1970) and Kok et al. (1993) recommend using water as a cooling device. Ice as a cooling device appears to us to be a more logical choice. Unlike water, ice is transparent to MWI and easy to work with, and it requires no special equipment or modifications to the microwave oven.

The wattage of a microwave oven will influence the time of fixation, from milliseconds (Login et al., 1991) to seconds (Leong, 1993; Login and Dvorak, 1993; Kok and Boon, 1992). Other factors affected are water load size/location and the intensity of the hot spots (Kok et al., 1993). Microwave manufacturers recommend that a load be present in the oven cavity during use. In a household microwave oven this load is normally the water to be warmed or the food to be cooked. In a laboratory microwave this load (the water load) is usually defined as a beaker or other container of water of known volume which is placed in a predetermined location. A 200 ml water load in a low wattage (<600 W) microwave oven is not comparable to the same water load in a high wattage (>650 W) oven based on neon light illumination patterns (unpublished data). Oven wattage will also influence the type of neon bulb to use. From our experience (unpublished data) a standard brightness bulb gives little usable information (location of hot and cold spots) in a high wattage (>650 W) microwave oven.

Increasing the water load number and volume and incorporating a zero power sequence between two 100% power sequences (time interval) vs. continuous power resulted in improved preservation and decreased extraction (Fig. 11; Table 1, microwave trial 8-16-8 [no ice]).

A number of methods have been proposed to identify hot spots within the microwave oven cavity (Kok and Boon, 1992; Kok et al., 1993; Login, personal communication; Login and Dvorak, 1993, 1994). Neon bulbs were an easy and reproducible way to identify hot spots (illuminated bulb). The spots not designated as hot spots are by default cold spots. The determination and subsequent mapping of these locations is important in determining sample location for fixation. Use of the ASG blocks was a quick method to cross-check a cold spot location.

Although the use of microwave ovens for the fixation of tissue dates back to the early 1970s, the ability to get satisfactory and consistent results has been difficult to achieve at the EM level. The major research efforts for the use of microwaves for the preservation of tissue for electron microscopy was done by Login and colleagues (see review by Login and Dvorak, 1994).

We have extended upon those studies to argue that time is a more important consideration than temperature as a determinant for adequate fixation when the temperature increase can be controlled. The introduction of an ice-encased MC in a cold spot during microwave fixation led to a decrease in final temperature and the disappearance of the bubbles within the Yew needle chloroplasts. This suggests that the bubbles were a heat-induced artifact.

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