Microwave Mechanisms – The Energy/Heat Dichotomy

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The current use of microwave technology in science creates a dichotomy. Is it the heat or is it the energy? One entire branch of science, chemistry, uses microwave energy to apply heat to a broad range of chemical processes, under pressure, to produce the desired end-products quickly and efficiently (1). The biological sciences, surgical pathology in particular, have tried to adapt the microwave oven to speed up a broad range of processes: fixation, decalcification, antigen retrieval, tissue processing for paraffin and plastic embedding, and histological staining, including special stains, immunolabeling, and *in situ* hybridization (2). The biologists have assumed that they are also applying heat to speed processing. However, recent improvements in the microwave suggest that the energy is the critical variable (9). We have designed fixation experiments to test the two views.

Overnight fixation schedules have been used for decades in surgical pathology (3,4). As turnaround times were reduced by automation, fixation times were not reduced. In general, tissue fixation is accomplished by passively "soaking" tissues in ample volumes of 10% neutral buffered formalin (NBF) or paraformaldehyde. In solution, formaldehyde exists in a temperature-dependent equilibrium with methylene glycol (methylene glycol ↔ formaldehyde + H2O). At normal room temperatures the actual concentration of formaldehyde in solution is ve[illegible] the order of $4x10^{-4}$ (3). This low concentration of formaldehyde, along with tissue composition and thickness, is what dictates the minimum times required for adequate tissue fixation. In practice, the minimum time for fixation at room temperature is approximately 24 hours. However, as stated above, this figure varies with tissue thickness and composition (5, 6).

Microwave fixation methods seemed to be ideal for reducing the fixation time. For the last 50 years, it has been known that higher temperatures (*e.g.* 40-50°C) increase the concentration of aqueous formaldehyde (7).The natural assumption was that microwave heating would also influence the dissociation constant of methylene glycol. Unfortunately, placing fresh tissue in NBF and then simply heating in the microwave did not improve the level of fixation (8). However, tissue fixation was improved when the tissue was allowed to sit in NBF for >4 hours and then heated in the microwave (55°C over a 1.5 to 4 minute period) (8), suggesting that diffusion of the glycol was necessary before the heat was applied.

The older experiments, however, frequently did not have appropriate control of the microwave environment and did not address the appropriate variables. Irradiation times and final fixative temperatures were the most commonly reported variables. Given the number of variables now known to be involved, real experimental control of microwave variables requires more than simply time, and temperature. Variables such as microwave power settings, magnetron power output, and temperature control also need to be considered. Table 1 has an accurate, up-to-date list of microwave processing variables and their control or mitigation.

Table 1: Control of the Microwave Environment

Type of Control	Method of Control	Comments
Temperature Probes Types: Fiber Optic Infrared Thermistor	Feedback loop - a temperature maximum is reached and the magnetron is turned off until the solution temperature cools below the set point. % Power loop - a temperature maximum is reached and the magnetron reverts to operation on a % power basis.	Controls a temperature maximum but limits the amount of microwave irradiation if the top limit is reached. Does not ensure maintaining a precise temperature maximum due to the irradiation continuing at a preset % power cycle (see below).
% Power Settings	Delivers the maximum wattage for a % of the time period chosen (e.g. an 800W microwave at a 40% power setting delivers 800W for 24 sec. during a 1 min. time period). This power output is frequently referred to as 320W (.40 x 800 = 320)	The cycle time of the magnetron will determine the length of each exposure. Example: If the cycle time were 20 sec. the magnetron would be on for 8 sec. of each 20 sec. cycle and 24 sec. total for the 1minute period. % power flattens the heating curve and limits the amount of microwave irradiation.
Variable Magnetron Power Output	Will deliver continuous microwave energy over a range of magnetron power outputs. Lower power outputs reduce cavity and sample heating during processing.	Heating rates can be controlled and changed depending on experimental requirements.
External Sample Temperature Control	Sample temperatures are controlled and/or maintained external to the microwave environment.	1) Continuous microwave irradiation 2) Static processing temperatures 3) Static processing environment
Reduction of Electric Field Strength Differences in the Microwave Cavity	1) Water loads 2) Large reagent volume ≥1000ml for 600-700W microwave[1] 3) Apparatus for dampening standing wave pattern (US Patent 6329645) 4) None other than sample placement.	The better the reduction the more uniform the processing environment.

[1]Neas, E.D. and Collins, M.J. (1988) Microwave heating. Theoretical concepts and equipment design. In: Introduction to microwave Sample Preparation. Theory and Practice. H.M. Kingston and L.B. Jassie, eds. American Chem. Soc., Washington, DC, pp. 7-32.

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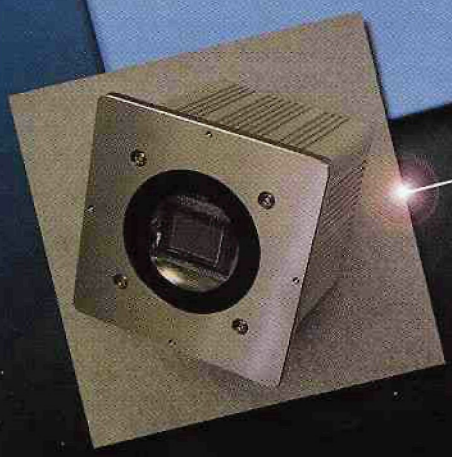

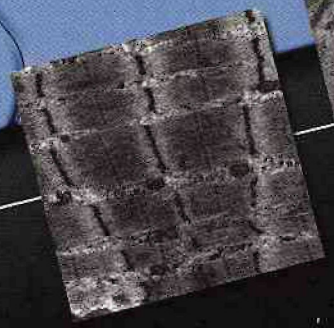

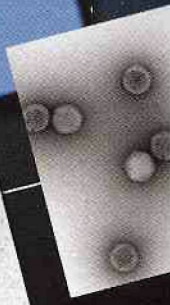

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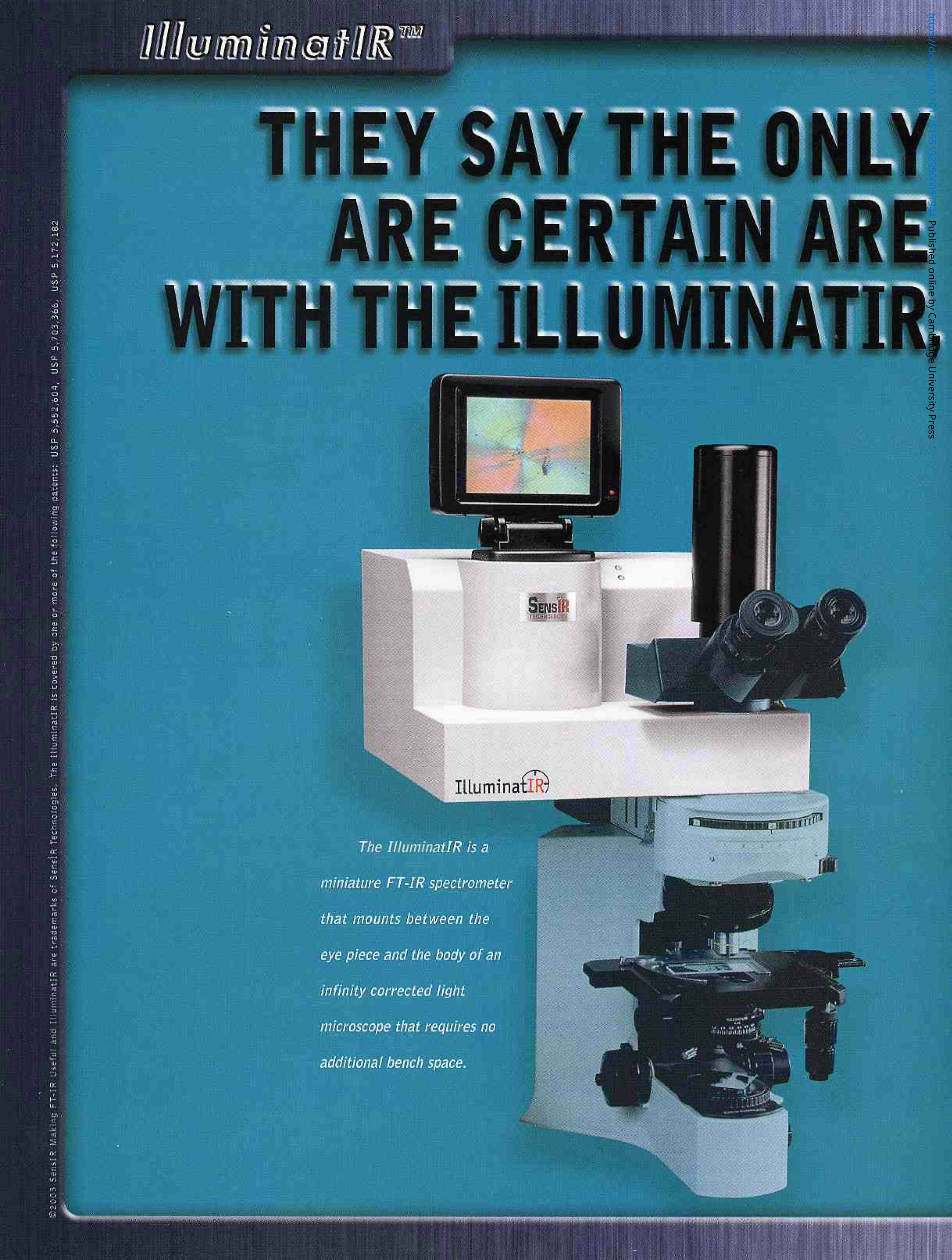

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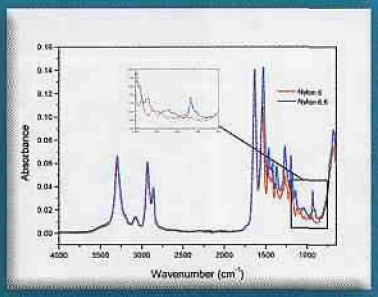

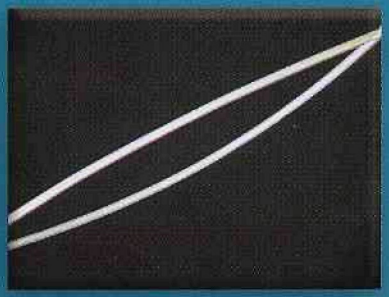

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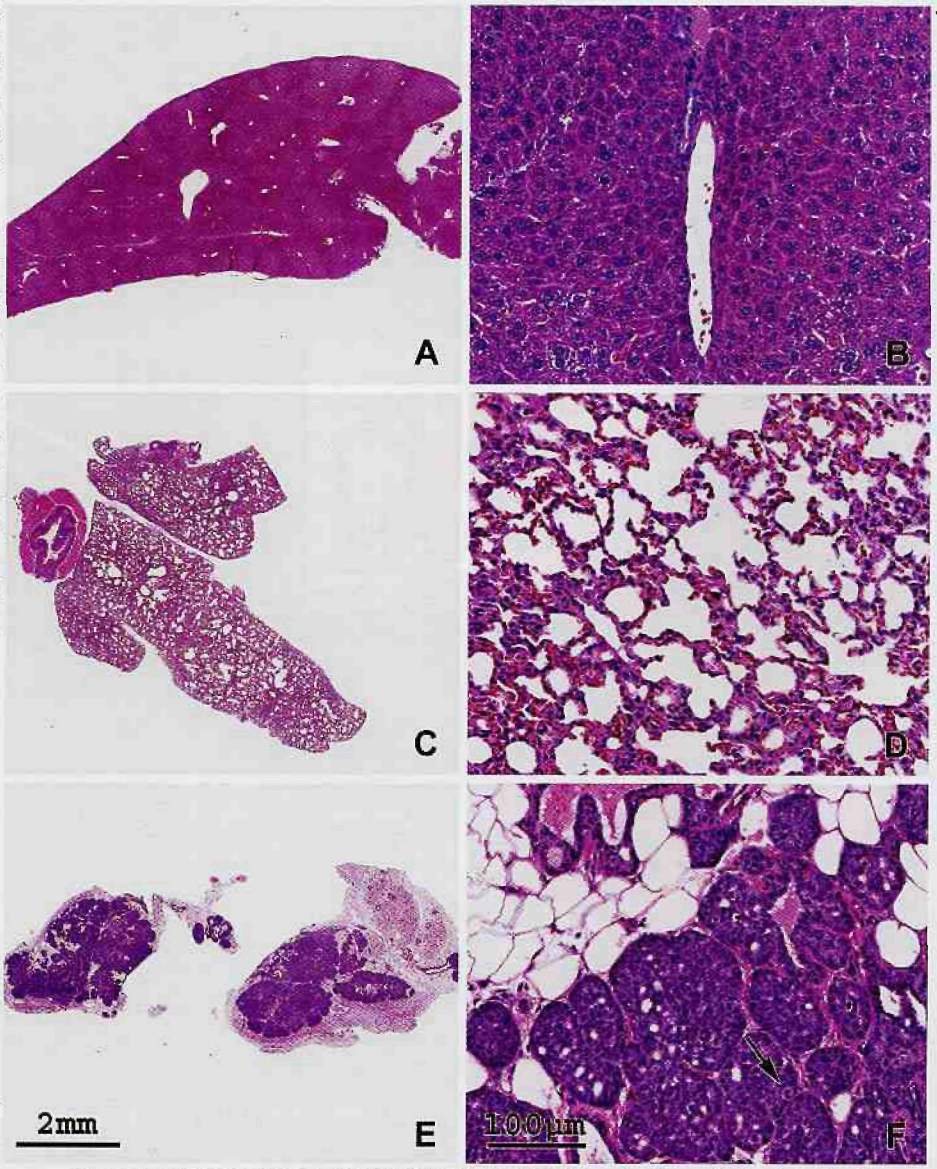
Over 30 years of microwave research (the entire body of literature began with Mayers' paper in 1970) has added little to our understanding of the fixation process beyond the concept of microwave heating (10). Low tech household microwaves were prevalent in most histology laboratories and were mainly used for silver stains to shorten the bench heating step. More sophisticated laboratory microwave models were first introduced in the middle 1980's (8). The primary differences between laboratory and household units were shortened magnetron cycle times, exhaust venting and better temperature control than the later instruments. Those using conventional microwave systems had generally accepted heating as the mechanism of action, especially for biological applications (8). The persistence of this assumption has been primarily due to the slow evolution of microwave technology from 1986 to 2001. During this time, microwave power output was never examined. Further, the technology was never commercially available that separated microwave heating of samples from microwave energy during the processing. In 2001 Giberson and Elliott reported that microwave power output was the important variable in fixing fresh tissue in formaldehyde (9). This report has resulted in a complete reexamination of the whole process.

As a result, laboratory microwave equipment has become commercially available that can control and adjust magnetron power output. These newer systems can now control sample temperature external to the microwave environment without interrupting the magnetron power output (DFR-10, Ted Pella, Inc., Redding, CA). The experimental design becomes relatively straightforward when all of these microwave processing variables can be varied under controlled conditions. This equipment permits the separation of the variables. Microwaves can be delivered continuously throughout fixation, at a predetermined power, while reagent (NBF) temperatures can be maintained constant during irradiation.

A research project, in conjunction with the Center for Comparative Medicine at University of California, Davis was designed using a newer microwave system. It was designed to validate the earlier findings of Giberson and Elliott (2001). The microwave-assisted formaldehyde fixation of fresh tissue at 30°C was evaluated by electron microscopy, and by light microscopy using immunohistochemistry and hematoxylin and eosin (H&E) staining of paraffin sections. The level of fixation was evaluated by subjectively scoring the H&E and immunohistochemical slides using a three point scale, with one being poor fixation and 3 being good fixation. The electron micrographs were evaluated on the quality of ultrastructural preservation using the same three point scale. The quality of fixation was compared to a matching set of bench-fixed tissues, which had fixation times starting at 3 hours and doubling thereafter for up to 8 days, and evaluated in the same manner as the microwave-assisted formaldehyde fixed tissues. The results were very promising and the specific findings are currently being readied for submission.

The methodology used in the initial research project was put into practice to attain a 4-hour turnaround time from necropsy to paraffin section. Necropsy technique, microwave-assisted rapid throughput processing, and histologic scanning modalities were the basis for a pre-meeting workshop held in conjunction with The 24th Congress of the International Association for Breast Cancer Research (Sacramento Convention Center, November 1-5, 2003). Workshop trainees, supervised by experienced faculty, performed necropsies on various types of genetically engineered mice. The tissue samples were submitted



Figures A-F. Mouse Histology. Digital photomicrographs of hematoxylin and eosin stained sections of mouse tissue. Sections A,C and E are low magnification images of Liver, Lung and Mammary gland, with tumor respectively. Sections B,D and F represent high power magnification images of the same Liver, Lung and Mammary gland. Note the mitotic figure (arrow) in panel F. The mammary gland and tumor is that of a Polyoma virus middle T transgenic mouse.

for microwave fixation and rapid processing. The fixation time was 20 minutes, dehydration and paraffin perfusion was also performed in the DFR-10 using a ColdSpot™ (Ted Pella, Inc., Redding, CA). Paraffin sections were prepared, mounted and stained within 6 hours. The resulting slides from that workshop are shown below for 3 different mouse tissues (Figures A-F).

Our current observations strongly suggest the previous ideas about the action of microwaves are probably wrong. The review of Leong and Sormunen (1998) indicates similar conclusions by other groups. Microwave energy and heat can be used to achieve different goals. On the one hand, chemical synthesis, digestion and separation, which require heat, are enhanced by microwave heating. Microwave heating on the other hand appears to be of little benefit for tissue fixation. Tissue processing, done routinely at room temperature (*e.g.* fixation, immunolabeling and decalcification), appears to benefit less from microwave heating and more from the energy of the microwaves themselves (based on papers in press-immunolabeling, submitted-decalcification, current research-formaldehyde fixation). In fact, heating could be detrimental to some biological processing methods. We have been able to obtain rapid and high quality fixation at lower temperatures. This is consistent with the original energy hypothesis of Giberson and Elliott that the chemical equilibrium of formaldehyde is altered by the microwave power output and is not due to microwave-induced heating of the sample. Formaldehyde fixation of fresh tissue proceeds rapidly and reproducibly by varying the magnetron power output from a low to high wattage output. With the new and improved microwave methods, formaldehyde fixation can be reduced from 24 hours to 20 minutes for 2mm thick tissue samples. ■

Acknowledgments

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