## History of microwave sample preparation

Specimen preparation for microscopy has changed little over the last 100 years. By 1900 formalin fixation followed by paraffin embedding, microtome sectioning, and staining with aniline dyes and hematoxylin had become accepted steps in tissue processing. At that time, specimen preparation was catching up with the light microscope, which been around for a few hundred years. The intervening period (1900-2000) saw the development of electron, confocal, and atomic force microscopy as new tools to examine ultrastructure, 3-D cellular arrangement, and atomic level structure.

Tissue processing techniques have always lagged behind the technological developments in microscopy. In the last century mechanical improvements have increased microscopy resolution, and have taken it from microns to nanometers. Apart from the introduction of automation, however, there have been few improvements in specimen processing techniques. New fixatives, stains and embedding media were developed to provide better image contrast and tissue preservation for transmission electron microscopy (TEM). Atomic force microscopy is unique in that specimen preparation is minimal and resolution of the instrument is free of the wavelength restrictions inherent to light and electron microscopy.

## High resolution microscopy

As resolution in the microscope has improved our knowledge of the damage caused by specimen processing has expanded. Classic TEM processing using a sequential process of chemical fixation, dehydration at ambient temperature, resin infiltration and polymerization, is notorious for producing structural reorganization within cells and the reader may want to look up a series of papers by Kellenberger to illustrate this point (Kellenberger et al., 1992. J. Microsc., 168:181-201; Kellenberger, 1991. J. Microsc., 161:183-203). Rapid freezing using high pressure freezing, and dehydration of frozen specimens by freeze substitution has reduced frequency of specimen artifacts and thus enabled a better understanding of cell structure. The technique reduced fixation times from hours to a matter of milliseconds but the subsequent steps of freeze substitution, resin embedding and polymerization extended the overall processing time. TEM processing steps require processing times that can vary from days to more than a week to complete.

## Light microscopy

Paraffin processing (histology) is uniquely different from the methods described above. Sample sizes are massive in comparison to the TEM techniques or atomic force microscopy. Tissues are cut 2-4mm thick and can have x-y dimensions measured in millimeters or centimeters. Fixation begins at some point after surgical removal of the tissue sample. Formaldehyde in the form of 10% neutral buffered formalin (NBF) has been the fixative of choice in histology for most of the last 100 years. It is inexpensive, hardens tissue uniformly and is quick to penetrate tissue but slow to cross-link it. NBF requires at least 24 hours at room temperature to achieve adequate tissue preservation. The slow fixation times result in numerous ultrastructural artifacts. Fixation is a bench procedure and processing (dehydration through paraffin infiltration) is usually automated and routinely performed overnight. Needle biopsies, due to their uniform small size, can be processed in 2-4 hours. NBF fixation times are not reduced significantly for small tissue pieces. Quality fixation and processing would require 1½ days to complete to achieve consistent processing results for all size tissues.

Reducing fixation times to facilitate rapid turnaround was not a major problem until immunohistochemistry (IHC) began to replace TEM in the early 1990's as an important diagnostic tool. There is an abundance of chatter on Histonet (histonet-bounces@lists.utsouthwestern.edu) that confirms the requirement for an adequate fixation with NBF in the interpretation of IHC results. The electron microscopist recognized well before the medical world the importance of tissue fixation and processing to antigen immobilization and masking (See: "Application of Cryoultramictomy to Immuocytochemistry" (J. Microsc. 143:139-149, 1986). Epitope retrieval techniques were introduced in the early 1990's to cope with the lack of quality control in NBF fixation times. Without the advent of antigen retrieval the role of IHC to pathology would probably be quite different today.

## Microwave processing

Microwave radiation found its way to microscopy through Mayer (1970). He reported that the direct microwave heating of tissue was a reliable means to achieve histological fixation with minimum tissue shrinkage in 90 seconds. That report was the first recipe for the microwave cooking of tissue for microscopy. What is worth noting is that Mayer had an instrument with variable continuous wattage and used a dummy load (water load) for his work. The significance of wattage and loads took many years to understand and take full advantage of for processing. Rapid microwave heating became the norm for the majority of the reports to follow. The problem from the outset had been control of the microwave processing environment. The presence of hot and cold spots inside all microwave ovens has created numerous problems during processing. Mapping and understanding the role of rapid heating due to these energy density differences were a nemesis for all who tried microwave fixation, staining or processing methods. For the first 30 years the only element of standardization in a microwave protocol was the time component. Wattage, heating rates, temperature control, hot and cold spots and the duration of actual microwave exposure remained difficult or impossible to standardize.

Early thoughts on why microwaves reduced processing times were greatly influenced by the following statement: "Whenever there is an effect of microwaves there is necessarily a temperature effect (the reverse is obviously not true)" (Kok and Boon. 1990. Physics of microwave technology in histochemistry. Histochem. J. 22:381-388). That statement is difficult to argue with and seems obvious to anyone who has placed something in a microwave. However, what appears obvious may not be. The discussion of a microwave effect independent of temperature was akin to heresy in the 1990's. In an editorial from The Microwave Newsletter No. 8 (Eur J Morphol. 1993. 31:141-144) entitled "A Nonthermal Microwave Effect Does Not Exist" the authors concluded that "Evidence, therefore, is accumulating that the microwave effect is purely a temperature effect." At the time there were no commercially available microwave devices available that would demonstrate otherwise. However, the following year Hans Smid (J Neurosci Methods. 1994. 55:151-161) published an article entitled "Microwave Applications on Small Insect Brain Tissue Irradiated in a Water-perfused Cooling-jacket for Temperature Control." The author noted: "It proved to be possible to reduce total fixation and washing time from overnight to 30 min, which simply means that we can do more experiments in the same time. The water jacket-based operation of our microwave oven has been used for some years now, and we have never noticed negative influences on morphology or on immunodetection of peptides and monoamines." It would take several more years to develop a commercial microwave processor with direct reagent cooling to test the heating dogma of the 90's.

In 2004 Tinling et al. published the following article: Microwave exposure increases bone demineralization rate independent of temperature. J. Microsc. 215:230-235. The availability of external temperature control had become a commercial reality. The technology Tinling et al. used made it possible to clearly examine the role of microwaves independent of temperature. A recently submitted paper to the Journal of Histochemistry and Cytochemistry by Galvez et al. entitled: "Controlled Microwave Energy Dramatically Accelerates Formaldehyde Tissue Fixation," details the mechanism of microwave-assisted formaldehyde fixation. The study used the same technology of Tinling et al. (2004) to demonstrate the role of microwave energy on the reduction of classical formaldehyde fixation times from 24 hours to 20 minutes.

## Conclusions

What microwaves bring to microscopy is an edge to those who understand and use the correct technology for a given process. Microwave methods have improved through better technology and that technology has provided the ability to control all of the processing variables inherent to a microwave environment.

The edge given to clinical or research laboratories in using the new technology and processing protocols can be measured in time saved, increased productivity and better control of processing artifacts. If a research laboratory can get more results for a given time period than its competitors, it stands to reason the potential for new funding improves. If a clinical laboratory can produce a quality slide faster than using current methods then hospital stays may be shortened, treatments started sooner, cost overhead reduced and general patient care improved.

The primary obstacle to an immediate implementation of microwave methods is a cultural bias towards change and a high incidence of bad technology and methodology from the past. A recent study evaluated bench versus microwave techniques in diagnostic electron microscopy (Gerrity RG and Forbes GW. Microwave processing in diagnostic electron microscopy. Microscopy Today, Nov/Dec 2003). The study was done from 1997 to 2002 and directly compared turnaround times for over 1600 cases. The competitive edge coming from this study is clear: Almost 60% of microwave-processed cases were turned around in 2 days compared to 24% for conventionally processed tissue. Further, no cases where tissues were processed conventionally were turned around in a day, whereas 15% of microwave-processed cases were.

Microwave radiation can benefit both the researcher and clinician in achieving quality results while reducing classical turnaround times. Controlling all the variables is the key to high quality results, a reduction in artifacts and reproducible processing.