# Microwave Processing Techniques for Microscopy

*From Energy Beam Sciences, Inc., Agawam, MA*



**How does a microwave oven work?**

Microwaves are electromagnetic waves. Electromagnetic waves can be classified by their frequencies, and include radio waves, television signals, radar beams, infrared waves, visible and ultraviolet light, X-rays and gamma rays. Electromagnetic waves which have a frequency between 300 MHz and 300 GHz are classified as microwaves. These two frequencies correspond to wavelengths of 1 m and 1 mm, respectively. All domestic microwave ovens and laboratory microwave processors operate at 2.45 GHz (corresponding to a wavelength of 12.2 cm, or just over 4-3/4").

Microwave technology evolved out of the development of radar (Radio Detection And Ranging). Because microwave pulses can be very short, they can be used for distance and time measurement. The simplest form of radar measures the time for an echo to return from a certain direction. Microwaves penetrate fog and clouds, travel in straight lines, and give distinct shadows and reflections.

The original magnetron was invented by Albert Hull at the GE Research Laboratory in 1916. The microwave oven was invented in 1945 by Percy Spencer of Raytheon, who received a U.S. patent in 1950. The first commercial microwave oven appeared on the market in 1947. In the early 1950's, 50-100 units were sold per month, at a price of about $4000.00 each. In 1967, Amana introduced a counter-top model with a retail price of less than $500.00. Ten years later, in 1977, 3% of U.S. households owned a microwave oven. In 1987, 12.8 million microwave ovens were sold. According to some sources, in 1992, 90% of U.S. households owned a microwave oven, and the number worldwide now exceeds 100 million.

Microwave ovens provide an effective way of heating many nonconductive materials. Microwaves penetrate the material; whether or not heat is generated is determined by the specific dielectric properties of the material itself. In most materials, the microwave-power absorption is proportional to the water content of the material. The frequency of commercial microwave ovens (2.45 GHz) was selected so that a standard portion of food would be heated uniformly. Because the heat does not have to be conducted thermally through the food, but is generated inside the materials, microwaving reduces the time needed for heating the food to a uniform temperature.

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Microwaves cause heating within a material by exciting molecules to rotate. This rotation produces energy in the form of heat. Unlike conventional heating, this effect occurs simultaneously throughout the whole material being microwaved. This has important implications for microscopy, because the basis of much specimen preparation is the effective diffusion of fluids in and out of tissue blocks or sections. Heat increases the rate of diffusion, and microwave (internal) heating can enhance it even more effectively.

As an example, two 2 x 2 x 2 cm3 cubes of beef (striated muscle) were dehydrated. One cube was heated externally at 70 C in 100% ethyl alcohol for 5 minutes, the other kept at that temperature by microwave exposure. In the case of external heating, only the outer part of the cube was slightly dehydrated (hard and grey), but the microwaved cube was completely dehydrated (hard and grey all the way through), illustrating the more effective diffusion of alcohol into the interior of the material.

These same properties of microwave heating will dictate the choice of which processing fluids to use. Different substances subjected to the same amount of microwave energy heat up at different rates. For example, 100 ml of water needs 2.2 times more heat to warm up than 100 ml of alcohol. The materials that heat up fastest are comprised of non-symmetrical polar molecules, which are easily rotated by microwave energy. This can have important implications for the microscopist. For example, xylene has been the clearing agent of choice for most conventional histology because of its fast diffusion rate, despite the fact that it is flammable, causes dermatitis and can shrink tissue. With microwave processing, however, isopropanol penetrates faster than xylene; and isopropanol is much less harmful, and causes less shrinkage of specimens.

**How does a laboratory microwave processor differ from a kitchen microwave oven?**

The problem in establishing routine laboratory procedures using microwave technology has been the inadequacy of kitchen microwave ovens for laboratory use. Kitchen microwave ovens are rated by their maximum output power levels (e.g. 700 watts), and the only way of varying the amount of microwave energy entering the oven cavity is by switching the magnetron on and off over a period of time. All kitchen microwave ovens have preset cycle times, usually between 15 and 30 seconds. Therefore, if an oven with an output of 700 W and a cycle time of 30 seconds is required to operate at half its power (350 W), the magnetron is on for the first 15 seconds and off for the subsequent 15 seconds. In laboratory use, this often results in a cycle of heating and cooling that produces suboptimal and inconsistent results. At even lower power levels, the problem is exacerbated by the fact that the magnetron needs a second or so to warm up and begin emitting microwaves. For example, if 150 W of power is required, the magnetron should only emit microwaves for the first 6 seconds of each 30 second cycle. But, in this case, it makes no difference whether total time has been set for 6, 15 or 30 seconds. In all of these cases, the magnetron will actually emit microwaves for 6 seconds only. In the same example, time settings of 2:08 and 2:30 minutes give the same amount of exposure, whereas 2:36 minutes will give much more.

Further control of the temperature of microwaved materials can be achieved through use of a temperature probe which is connected to power-level control. After a cycle of exposure, temperature is checked against a preset value. When this value is reached, the exposure pattern is adapted to maintain this temperature. However, this control is still very imprecise with kitchen microwave ovens. Often, temperatures can only be set in multiples of 5 C or over a limited range of temperatures. More important, when after a cycle, the desired temperature has almost, but not quite, been reached, the

next cycle may overshoot the preset temperature. The lack of fine control becomes especially dramatic in the case of small laboratory samples, which can easily overheat and become damaged. Conversely, in the case of relatively microwave-transparent materials (like paraffin), this pattern does not usually suffice to maintain the desired temperature.

A further way of controlling temperature is through the use of a "dummy load"- a vessel of tap water placed in the back of the oven which functions as a heat sink, and thereby reduces the power absorbed by other specimens in the oven. In general, the rate of temperature rise slows in proportion to the size of the dummy load, but the shape of the container, its location, and the initial temperature of the water, all have an effect.

**Safety Features and Temperature Control of the Energy Beam Sciences H2800 Microwave Processor**

The H2800 is a microwave processor designed specifically for laboratory use. It differs from kitchen microwave ovens in respect to its safety features, and in the degree of user control it provides.

In a microscopy laboratory, solvents and toxins are heated, producing fumes. In any kitchen microwave oven, there is a risk that these fumes could be inhaled, or that they could enter the electrical control system, where high voltage switching occurs, with subsequent risk of ignition. The Energy Beam Sciences H2800 Microwave Processor is outfitted with a high-powered extraction system which removes air from the cavity at a rate exceeding 100 cfm (cubic feet per minute), which is then vented into a fume hood or other exhaust system.

Moreover, this extractor is interlocked with the microwave control system to prevent operation of the instrument should the fan fail, or the venting become obstructed. A range of specially-designed plasticware is available to avoid the use of metal and glass containers in the H2800.

The H2800 incorporates a unique, custom-made and very sensitive temperature probe, which is accurate to +/-1/2 C. Other features include a rotator and a sophisticated wave stirrer, both designed to minimize temperature variations within the cavity. An adjustable-speed air pump agitation system is provided to produce even distribution of temperature within a container of stain or other reagent. Cycle time can be selected by the user. Since a shorter cycle time results in more precise temperature control, a cycle time of 2 seconds is recommended. The key component of the instrument is a built-in microprocessor which allows almost perfect realization of an ideal temperature curve. Two modes of timer control are available: one, "total time", in which the total process time is selected, and the timer begins counting down as soon as the "run" control is activated, and a second, "time at temperature", in which the timer is activated only after a preset desired temperature is reached.

With the H2800, either an actual temperature, or a power setting can be selected by the user, depending on the microwave procedure being followed. Reliable temperature control within 1/2 C can be achieved. In the power control mode, relative settings from 1% to 100% can be chosen, and both the percentage power and the temperature are continuously displayed during operation.

**What are some applications where a microwave processor would be useful?**

Fixation: Microwave Stabilization of Unfixed Tissue:
Fixation of Tardigrades (Echiniscus viridissimus)

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**Fixation: Microwave Stabilization of Unfixed Tissue:**

The great advantage of microwave stabilization is that there are no chemicals involved which would extract important components from the tissue. Researchers have found that up to 40% of protein can be lost after formaldehyde fixation. However, other researchers have found significant disadvantages in this method (shrinkage, sponginess of tissue, and breakdown of red blood cells). Kok and Boon recommend a "hybrid" method, in which chemical postfixation is done after the initial microwave stabilization. In this case, the "poaching" effect of microwave stabilization seems to create channels through the tissue, permitting subsequent enhanced diffusion of fixatives into the cell.

Dr. Anthony S.-Y. Leong has published a method of processing 30,000 surgical biopsies per year, incorporating a microwave stabilization step. This method involves microwaving 20 blocks of tissue in cassettes, placed in a beaker of 500 ml normal saline on a rotator for 5 minutes at 68 C. The stabilized blocks are then transferred to a tissue processor for dehydration, clearing and paraffin embedding. According to Leong, the absence of noxious formalin fumes in the processing room is an important advantage of the procedure, and the elimination of formalin in both the fixation process and in the processing has improved the quality of antigen preservation in the tissue sections.

**Fixation of Tardigrades (Echiniscus viridissimus)**

From Ruth and Bill Dewel, Appalachian State University, College of Arts and Sciences, Department of Biology, Boone, North Carolina, 28608.

Tardigrades (Echiniscus viridissimus) were placed in 2ml of preheated prefix (see below) and immediately exposed to microwaves. The instrument (an Energy Beam Sciences H2500 Microwave Processor) was set for maximum power with a 40 degree C endpoint measured by the temperature probe set in water in a beaker. The specimens were then rinsed in 0.2M s-Collidine buffer for 20 minutes and postfixed (see below) using the same method as above.

Prefix:

4% paraformaldehyde

1.5% glutaraldehyde

0.1M cacodylate buffer

0.025% Calcium chloride

Postfix:

2% Osmium tetroxide

0.2M s-Collidine buffer

0.07% Calcium chlorid

**Microwave-Stimulated Fixation with Fixatives:**

Microwave exposure can be used to enhance diffusion of fixation reagents into the tissue, and to accelerate the chemical process by which the fixative cross-links with the protein of the tissue. The most common histological fixative, formalin, is a solution containing methylene glycol and a little fomaldehyde. Normal formalin fixation takes place in three steps: first, the methylene glycol quickly penetrates the tissue (formalin will penetrate a 5 mm block in 4 hours); second, some methylene glycol is slowly converted to formaldehyde by dehydration; third, formaldehyde binds very slowly to the proteins in the tissue by cross-linking. All three of these steps can be accelerated by microwave exposure.

However, simply microwaving tissue in formalin produces disappointing results, because the outside of the tissue fixes so rapidly and well that it effectively prevents further diffusion of fixative into the central part of the biopsy. For that reason, a "hybrid" procedure is recommended. First, tissue blocks are soaked in formalin for 4 hours at room temperature (longer, if blocks thicker than 5 mm are used); next, the blocks are microwaved for 1.5 minutes at 55 C. Some researchers use shorter soaking times in diluted formalin solutions. Excellent immunostaining has been achieved using this hybrid method of fixation.

However, excellent fume extraction (such as the system supplied with the H2800) is a necessity when using formalin, fumes are still highly unpleasant, and great care must be taken when handling the heated formalin. For these reasons, we recommend the use of Leong's method, or the substitution of an ethyl alcohol-based fixative, Kryofix..

The absence of formalin is clearly a great advantage for the clinical personnel and laboratory technicians. However, the microscopist will be confronted with a slightly different morphology than that of formalin-treated tissue. There are some disadvantages (more pronounced shrinkage, for example) and some advantages, particularly in the field of immunopathology, where Kok and Boon have found superior positive staining. As with formalin, better results are achieved by soaking tissue in Kryofix. prior to microwaving; however, the soaking times are much shorter than for formalin.

**Histoprocessing: Microwave Histoprocessing Using Paraffin**

Paraffin wax has been used as an embedding medium in histoprocessing for over 100 years. It is a good embedding medium for routine histology because it can thoroughly permeate the tissue in its liquid form (when warm), and it solidifies (when cooled) with little damage to the tissue. However, before tissue can be embedded, it must be subjected to the following procedures:

Completion of fixation;

Removal of formalin from the tissue (if fixed in formalin);

Gentle and complete dehydration;

Removal of the dehydration fluid with an intermedium (clearing agent) miscible both with the dehydration agent and paraffin wax;

Impregnation of the tissue with melted paraffin wax.

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Most histopathology labs now use automated tissue processing machines which use 12 containers and require 6-20 hours for processing.

When microwaving is used in histoprocessing, these procedures are not only accelerated, but fundamentally changed:

Completion of fixation is achieved prior to histoprocessing in the microwave oven.

Dehydration is achieved in one step, instead of the 2-6 steps used in conventional procedures. The use of a graded series of alcohols is not necessary in the microwave method.

Isopropanol can be substituted for xylene as a clearing agent, and one bath is sufficient.

Higher temperatures are required for the impregnation of the paraffin wax.

Therefore, microwave histoprocessing becomes a three-step process:

One dehydration step

One clearing step

One paraffin wax step (at two temperatures)

Using the H2800, processing schedules for thin (>1 mm), medium-thin (1-2 mm) and thick (2-5 mm) blocks of tissue take a total time of 25 minutes, 60 minutes and 165 minutes, respectively. This technique allows the histology lab to routinely process tissue for same-day diagnosis. Using four special Teflon® histoprocessing racks, which each accommodate 24 standard cassettes, up to 96 cassettes can be processed at a time using this method. The cassettes themselves are never handled during this process. The racks holding the cassettes are transferred from a tray containing the ethyl alcohol to a tray containing isopropanol, then to a tray containing liquid paraffin. One laboratory which processes 300 biopsies a day saved more than $10,000 in reagents over the first year it employed this technique.

**Combining Microwave and Freezing Techniques:**

The principal advantage in freezing techniques in surgical pathology is the speed of preparation of a tissue block that can be cut. In addition, no fixative is required. The hardening of tissue, necessary for cutting thin sections, is a one-step process, and cutting can be carried out in minutes. The principal disadvantage of the cryostat technique is the relative poor quality of the ultimate light microscope images. Major improvement can be achieved by subsequent microwave-stimulated fixation.

In the method recommended by Kok and Boon, frozen sections are cut by a cryostat and mounted on slides. Next, the sections are quickly covered with a few drops of Kryofix.. The slide is then quickly transferred, in the horizontal position, to a polystyrene platform in the H2800, and microwaved for 20 seconds at 450 W. Finally, the slides are stained with Hematoxylin-Eosin. This procedure takes less time than conventional frozen section techniques, and produces substantially improved microscopic images.

**Microwave Staining:**

Staining of tissue is based on two factors: diffusion of the dye into the cells, and binding of the dye to the substrate. Diffusion is a physical process, and can be enormously accelerated by microwave exposure. Binding of stains to cell substrates is a physical-chemical process, and the role of microwave exposure depends on various factors. Generally, staining methods that normally take minutes can be

done in a microwave oven in seconds; those that take hours, in minutes; and those that take days or even weeks can be completed in a matter of hours using microwave techniques.

There are two basic methods for staining in the microwave oven. Whenever possible, we recommend that staining be performed in a plastic coplin jar or staining rack, with the temperature probe of the H2800 inserted into the fluid in the jar. This allows use of the probe for very accurate temperature control. The optimum temperature for most non-metallic stains is around 60 C, and for metallic stains approximately 95 C. Stirring the solution by air-bubble agitation in the H2800 is usually advantageous, facilitating more even staining from top to bottom of the slide. A second method involves covering the slide with a few drops of staining solution, placing the slide on a platform in the microwave oven, and microwaving for 20-30 seconds.

Kok and Boon present numerous methods for microwave staining of histologic sections in their Microwave Cookbook for Microscopists. These methods include techniques for both paraffin and plastic sections.

For more on-line information on staining, please see the complete listing of Technical Papers from Energy Beam Sciences, Inc.

**Antigen Retrieval:**

Microwave techniques for antigen retrieval have become increasingly popular in the years since Dr. Shi first pioneered this technique. Still, the use of kitchen microwaves for antigen retrieval has produced notoriously inconsistent results, with poor reproducibility. For optimum results, the slides should be placed in plastic racks, and a temperature probe used to measure and regulate the temperature. Proper fume extraction is necessary when heavy metals (lead or zinc) are present in the antigen retrieval solutions.

**How is a laboratory microwave oven useful in electron microscopy?**

**Microwave Tissue Fixation for EM:**

The pioneering work in this area has been done by Gary Login and Ann Dvorak at Beth Israel Hospital, in Boston, MA, U.S.A.. They present, in series of publications, a comprehensive approach to microwaving for electron microscopy, comprised of five aspects:

A standardization method for testing microwave oven performance.

Postfixation and processing of microwave-fixed specimens for EM.

Ultrafast microwave fixation.

Evaluation of specimen morphology following microwave exposure in different solutions.

Postembedding immunoelectron microscopy of microwave-fixed specimens.

Detailed procedures for each are provided in Chapter 19 of the Microwave Cookbook for Microscopists.

**Microwave Exposure in Immunoelectron Microscopy:**

This seems to us to be one of the most promising areas for further exploration. Several studies indicate that excellent results can be obtained when gold-labelling is performed in the microwave oven under

controlled conditions. For successful labelling, accurate temperature control such as that provided by a laboratory microwave like the H2800, is essential (it is desirable to keep the temperature in the antigen-antibody system at around physiologic temperature).

In one technique cited by Kok and Boon, free floating 405 Vibratome. sections are used for preembedding imummunoelectron microscopy. This procedure was performed in an H2800.

**Microwave Exposure and Epoxy-Resin Embedding for Transmission EM:**

Thin sectioning of epoxy-resin embedded material is the most widely used method for TEM investigations of biological material. As in histoprocessing techniques, the cells or tissues are first fixed, then dehydrated and embedded. (The epoxy resins are miscible with alcohol, so no clearing agent is required). Again as with histoprocessing, conventional methods are very time-consuming, requiring up to three days. This is particularly true in cases where the resin has to penetrate barriers such as thick cell walls. Such long processing times give rise to embedding artifacts and a low overall contrast (mainly a result of extraction of subcellular compounds). Therefore, it seems worthwhile to try to shorten these steps through the use of microwave-stimulated diffusion of the reagents through the thick barriers.

Microwave techniques have successfully been demonstrated for dehydration for epoxy-resin embedding through a series of graded ethyl alcohols. Microwaving can shorten dehydration times, with no noticeable disadvantages.

For the embedding in plastic itself, the processing time can also be shortened, but the quality of the resulting blocks using kitchen microwaves has often proved unpredictable. Beverly Giammara and Jacob Hanker have been studying these techniques, with good results using EPON/Araldite mixtures and silicone rubber molds. A dummy load may be used to absorb excess microwave energy. Giammara has been able to achieve consistent polymerization in approximately 30 minutes when blocks of the same size and fixation were placed carefully in the same locations within the microwave cavity. Using the H2800 Microwave Processor, rather than a kitchen microwave oven, the procedures that Giammara has developed can be further improved through careful temperature control. Preliminary experiments have been encouraging, even with LR White resin.

**Rapid Processing of Tissues for Transmission Electron Microscopy**

Technique courtesy of: Ed Calomeni Dept. of Pathology Medical College of Ohio Toledo, Ohio 43699

Note: Set the microwave at 60% power, and use a 300ml water load for all microwave steps.

Fix in 3% glutaraldehyde for 5 minutes. Microwave for 30 seconds at 50°C. Note: Tissue should be trimmed to 2mm or smaller. If the tissue was not received already in formalin, increase the glutaraldehyde fixation time to 15 minutes.

Rinse 2X with sodium cacodylate for 30 seconds each.

Post-fix in 1% OsO4 for 14-1/2 minutes. Microwave for 30 seconds.

Rinse in s-collidine 2X for 30 seconds each.

Tertiary fix in saturated Uranyl Acetate for 15 minutes. Microwave for 30 seconds.

Dehydrate in a graded series of ethanols (30, 50, 70, 90, 95, 32X 100%) for 30 seconds each in the microwave.

Clear with 100% acetone 4X for 30 seconds each in the microwave.

Infiltrate with a 1:1 mixture of acetone: Spurr's resin (rapid cure) for 1 hour, microwaving every 10 minutes.

Place tissue in filled BEEM capsules, and place in convection oven at 70°C.

Increase temperature 2-1/2°C every 10 minutes. At the end of 1-1/2 hours, remove blocks and section. Do not exceed 100°C.

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