Ultrasound Facilitated Formalin-fixed and Paraffin-embedded Tissue Specimen Preparation Technology

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ABSTRACT

Ultrasound (US) facilitated formalin-fixed and paraffin-embedded (FFPE) tissue specimen preparation technology (US-FFPE) is developed from the widely accepted routine FFPE method with ultrasound applied to tissue fixation and processing. It does not change the "gold standard" of FFPE tissue morphology. However, it greatly reduces fixation and processing time, making it possible to standardize the procedure. The whole procedure of tissue specimen processing is reduced from 24-48 hr to less than 1 hr depending on tissue thickness. The US-FFPE procedure includes 2 to 10 min formalin fixation, 5 to 30 min graded alcohol dehydrations, 3 to 10 min xylene treatment, and 4 to 12 min paraffin embedding. US-FFPE technology changes the mechanism of biomolecule modification and crosslinking caused by formalin fixation. Protein antigens are much more accessible for detection and extraction. RNA degradation during fixation is also greatly reduced. The fact that tissue specimens are "frozen in time" at the point of US-FFPE fixation can greatly improve the quality and availability of biomolecules and facilitate molecular characterization.

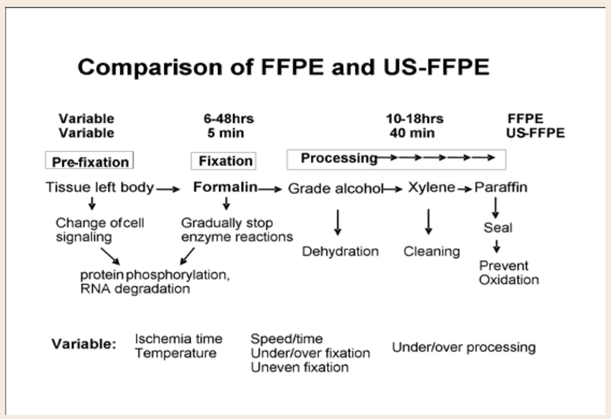
INTRODUCTION

Expanded knowledge of the molecular basis of cancer has shown that significant differences in gene expression patterns can guide therapy for a variety of solid tumors and hematologic malignant neoplasms. Based on these findings, personalized medicine is on the horizon. To realize the vision of personalized medicine, patient- and disease-specific molecular data must be derived from tissue specimens in an accurate and reproducible manner. This in turn requires that the tissue specimens themselves be prepared and handled according to standards that safeguard their quality. It makes perfect sense that we start standardization at the first step of tissue of preservation, before investing huge amount of time and resources for controlling the downstream assays.

Although many tissue preservation methods were developed using alternative chemical fixatives, formalin-fixing and paraffin-embedding (FFPE) method remains the most commonly used tissue preservation approach for more than a century since its development. FFPE method is extremely simple to perform and low in cost. It produces superior preservation of morphological details, and maintains high consistency under various conditions. Modern histology is largely based on tissue specimens produced by FFPE method.

With fast development of molecular assays in medical care in general and cancer care in specific and increasing dependence of treatment decisions on the assay results, standardization of tissue specimen preparation is highly anticipated. It is obvious that a rapid fixation protocol which can reproduce all the golden standards of FFPE tissue specimens while at the same time produce high integrity and availability of important biomolecules is of critical significance.

In an effort to find a solution that can significantly accelerate the FFPE process, we turned our attention to ultrasound (US). There have been a few attempts to apply US to tissue preservation without satisfactory results, mainly because of inconsistancy and tissue damage. We found that the critical factors for consistent US facilitated FFPE processing (US-FFPE) without tissue damage were maintaining US at a high frequency (>0.1 MHz) and high intensity (1-5 W/cm^2) and controlling the total energy received by the tissue.



Time Required for US-FFPE Tissue Preservation

US-FFPE technology reduces the total fixing and processing time from 24-48 hours to within 1 hour. Like its routine counterpart, US-FFPE has a relaxed window of operating parameters making it easy to handle. Depending on the thickness of tissue samples, US-FFPE procedure requires varying time for both fixation and succeeding processing steps. The whole procedure lasts from roughly 20 min for tissues of 1-mm thickness to about 60 min for tissue of 4-mm thickness.

Table 1. Time required for US-FFPE tissue preservation

Tissue thickness	Formalin	Alcohol 1	Alcohol	Xylene	Paraffin
1 mm	2-3 min	2-4 min	2-4 min	2-4 min	3-5 min
2 mm	2-4 min	3-7 min	3-7 min	3-6 min	4-7 min
3 mm	3-7 min	6-12 min	6-12 min	6-8 min	6-10 min
4 mm	5-10 min	8-17 min	8-17 min	7-12 min	8-12 min

US-FFPE Method Produces Superior FFPE Tissue Morphology And Increases Antigen Availability

US-FFPE method produces superior FFPE tissue morphology, comparable to that produced by the routine FFPE procedure. US-FFPE reduces the masking of protein antigenicity. Antigen retrieval (AR) is not required in IHC staining for many antibodies that otherwise require AR on routine FFPE tissue sections. For those antibodies requiring AR, IHC assays on US-FFPE tissue sections showed much stronger signals.

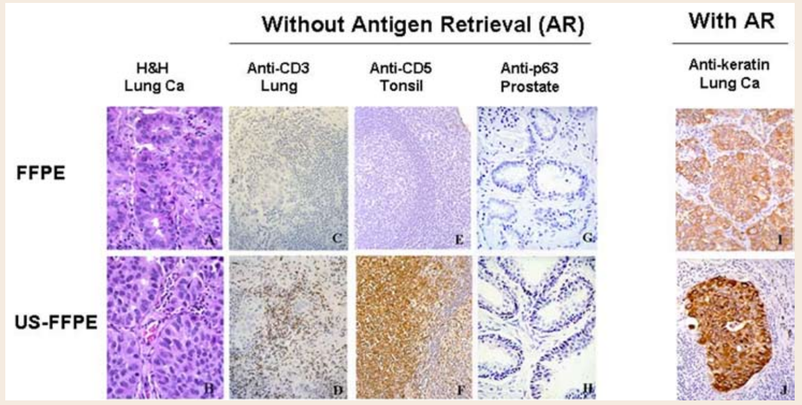


Figure 1. Comparison of morphology and IHC staining with and without antigen retrieval (AR) on routine FFPE and US-FFPE tissue sections.

US-FFPE Tissue Specimens Showed Better Preservation of mRNAs

Fast formalin penetration and cross-linking reduced mRNA degradation during the fixing process.

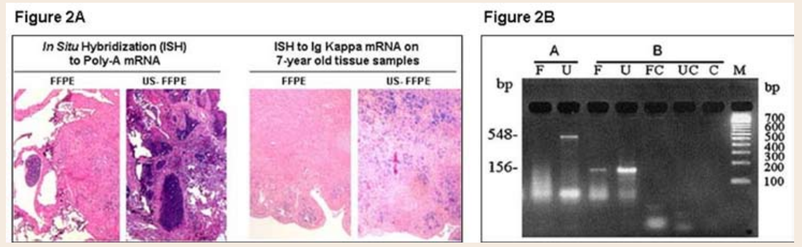


Figure 2. A) In situ hybridization to total poly-A mRNA (left) and immunoglobulin kappa chain mRNA (right) on routine FFPE and US-FFPE tissue samples. Tonsil tissue was dissected into two equal portions, one for routine FFPE the other for US-FFPE procedure. ISH to poly-A mRNA was performed shortly after both fixing procedures. ISH to Ig kappa mRNA was performed on the same tissue blocks 7 years later. These results strongly indicate that US-FFPE method is capable of rapidly fixing deep tissue regions, inactivating RNases, thereby protecting mRNA integrity. B) RT-PCR of beta-actin mRNA from pulmonary carcinoma with primer sets A (for amplicons of 548-bp) and B (for amplicons of 156-bp). F: routine FFPE tissue; U: US-FFPE tissue; FC: F without reverse transcription; UC: U without reverse transcription; C: water only; M: DNA marker.

Validation of US-FFPE

US-FFPE technology was validated by Ventana Medical Systems, Inc. by IHC and ISH.

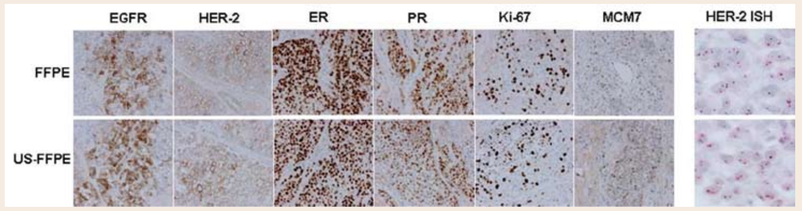


Figure 3. Left panel: IHC staining with antibodies against EGFR, HER-2, ER, PR, Ki-67, MCM7 and on routine FFPE and US-FFPE ovarian cancer tissue sections. Right panel: double staining ISH for HER2 gene. Performed by Dr. Grogan's group in Ventana Medical Systems, Inc.

IHC of Breast Cancer Tissue Specimens

In the ASCO/CAP guidelines, the breast tissue requires 6-48 hours fixation for HER2 and ER testing. Below we show a specific ER IHC staining of breast specimens fixed by 5-min ultrasound fixation and 24-h conventional formalin fixation.

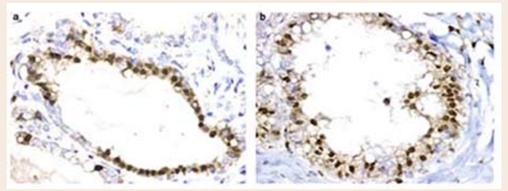


Figure 4. Breast cancer tissue fixed by 24 hr routine FFPE (a) and 5-min US-FFPE.

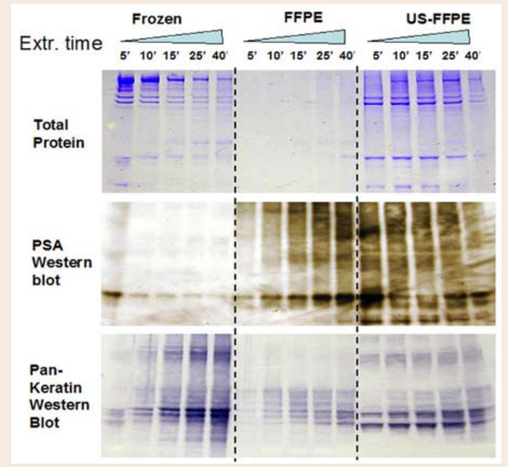


Figure 5. Time course for protein extraction. Profiles of extractable proteins in frozen and US-FFPE tissue sections are similar to each other: more proteins are extracted in earlier extractions. In contrast, routine FFPE require longer time to reverse cross-linking and get released. These results indicate that cross-linking in US-FFPE is more easily reversed than the routine FFPE.

SUMMARY

1. Traditionally, a tissue specimen is fixed and processed from 16 hours to 48 hours. With the assistance of Ultrasound, we have achieved within 1 hour superior FFPE tissue morphology, comparable to that produced by the routine FFPE procedure.
2. Although the exact mechanism is not clear, we have observed that antigen retrieval (AR) is not required in immunohistochemstry (IHC) staining for many antibodies that otherwise require AR on routine FFPE tissue sections. For the antibodies that do require AR, IHC assays on US-FFPE tissue sections showed much stronger signals.
3. Compared with traditional fixed tissue, US-FFPE method is capable of preserving mRNA integrity as demonstrated by ISH signal uniformity across tissue section and by increased RT-PCR signal and product length.
4. We are currently planning to validate this technology through breast cancer clinical assays.

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