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Tissue is alive: New technologies are needed to address the problems of protein biomarker pre-analytical variability

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Abstract

Instability of tissue protein biomarkers is a critical issue for molecular profiling. Pre-analytical variables during tissue procurement, such as time delays during which the tissue remains stored at room temperature, can cause significant variability and bias in downstream molecular analysis. Living tissue, *ex vivo*, goes through a defined stage of reactive changes that begin with oxidative, hypoxic and metabolic stress, and culminate in apoptosis. Depending on the delay time *ex vivo*, and reactive stage, protein biomarkers, such as signal pathway phosphoproteins will be elevated or suppressed in a manner which does not represent the biomarker levels at the time of excision. Proteomic data documenting reactive tissue protein changes post collection indicate the need to recognize and address tissue stability, preservation of post-translational modifications, and preservation of morphologic features for molecular analysis. Based on the analysis of phosphoproteins, one of the most labile tissue protein biomarkers, we set forth tissue procurement guidelines for clinical research. We propose technical solutions for (i) assessing the state of protein analyte preservation and specimen quality *via* identification of a panel of natural proteins (surrogate stability markers), and (ii) using multi-purpose fixative solution designed to stabilize, preserve and maintain proteins, nucleic acids, and tissue architecture.

Keywords

Biomarker; Fixation; Histology; Phosphoprotein; Preservation

1 Tissue protein biomarkers: Emerging opportunities for diagnosis and prognosis

Protein tissue biomarkers offer great promise to provide clinical diagnostic and prognostic information that cannot be obtained from genomics or serum biomarkers. The phosphorylation, or activation state, of kinase-driven signal networks contains important information concerning both disease pathogenesis and the ongoing state of kinase-associated therapeutic targets [1-3]. Modulation of ongoing cellular kinase activity represents one of the most rapidly growing arenas in new drug discovery. Identification of specific phosphoprotein signaling aberrations

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is leading to the development of targeted therapies for patients with lung, breast and colon cancer [4-8]. Profiling the tumor phosphoproteome and the transcriptome, using human tumor biopsy specimens, is a critical component of the perceived upcoming revolution of individualized cancer therapy [9]. Thus tissue protein biomarkers offer a means for individualizing therapy by revealing how an activated or amplified proteomic signal pathway drives an individual patient's tumor cells. If the revealed pathway contains the drug target of a specific therapeutic, then this information could be used by the physician to select the optimal therapy for this patient.

In the past, the discovery and clinical implementation of tissue biomarkers has been delayed by several hurdles including tissue heterogeneity and the lack of sensitive technology to identify and measure proteins in small volumes of human biopsy tissue. These hurdles have been largely overcome by the wide availability of laser microdissection technology, protein microarrays, and advances in mass spectroscopy [10-12]. Nevertheless, the problem of protein biomarker instability and perishability within the tissue aspirate or biopsy is a very serious issue that has not been adequately studied or solved. If this problem is not resolved in the real world of clinical practice, then the future promise of tissue biomarkers will never be realized. The present opinion piece sets forth the need for collecting data concerning the stability of tissue protein biomarkers and proposes goals for novel technologic solutions to address the problem of biomarker stability in tissue. An emphasis is placed on tissue phosphorylation, since this highly labile class of tissue biomarkers contains information about the state of kinase drug targets, and the vast majority of emerging therapeutics target kinases and kinase pathways.

2 Protein biomarker stability in tissue: A critical unmet need

There is a critical need to develop standardized protocols and novel technologies that can be used in the routine clinical setting for seamless collection and immediate preservation of tissue biomarker proteins, particularly those that have been post-translationaly modified such as phosphoproteins (Fig. 1). This vital need transcends the large research hospital environment and extends most acutely to the private practice, where most patients receive therapy. Although molecular profiling offers tremendous promise to change the practice of oncology, the fidelity of the data obtained from a diagnostic assay applied to tissue must be monitored and quality controlled; otherwise, a clinical decision may be based on incorrect molecular data. To date, clinical preservation practices routinely rely on protocols that are decades old, such as formalin fixation, and are designed to preserve specimens for histologic examination. Under the current standard of care, tissue is procured for pathologic examination in three main settings: (i) surgery in a hospital-based operating room, (ii) biopsy conducted in an outpatient clinic, and (iii) image-directed needle biopsies or needle aspirates conducted in a radiologic suite. In a typical tissue procurement research protocol, tissue is often frozen in dry ice or liquid nitrogen to preserve the tissue proteome. Unfortunately, however, in a busy clinical setting, it will be impossible to immediately preserve procured tissue in liquid nitrogen. Moreover even if the tissue is preserved by freezing, it may remain at room temperature awaiting pathologic gross examination, for an indefinite period prior to freezing. The time delay from patient excision to pathologic examination and molecular analysis is often not recorded and may vary from 30 min to many hours depending on the time of day, the length of the procedure and the number of concurrent cases. During this variable waiting period the tissue remains living and reactive. For this reason preanalytical variables can have a major impact on tissue biomarker fidelity and bias [13-15].

Two categories of variable time periods define the stability intervals for human tissue procurement. A primary starting time, time point A, is defined as the moment that tissue is excised from the patient and becomes available *ex vivo* for analysis and processing. The post excision delay time, is the time from time point A to the time that the specimen is placed in a

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stabilized state, time point B, *e.g.* immersed in fixative or snap-frozen in liquid nitrogen. Given the complexity of patient-care settings, during the excision delay time the tissue may reside at room temperature in the operating room or on the pathologist's cutting board, or it may be refrigerated in a specimen container. The second variable time period is the processing delay time. At the beginning of this interval the tissue is immersed in a preservative solution or stored in a freezer. At the end of this interval, the tissue is subject to processing for molecular analysis. In addition to the uncertainty about the length of these two time intervals, a multitude of known and unknown variables can influence the stability of tissue molecules (Table 1). These include (i) temperature and pH fluctuations, hypoxia and dehydration, prior to fixation or freezing, (ii) choice of preservative chemistry and rate of tissue penetration, (iii) size of the tissue specimen, (iv) extent of handling, cutting and crushing of the tissue, (v) fixation and staining prior to microdissection, and (vi) the introduction of phosphatases, RNAases or proteinases from the environment, or from dying cells, at any time. Given current practices, in the face of these uncertainties it would appear virtually impossible to develop a standardized procedure for routine clinical profiling. Even if a strict protocol is followed, there is no ultimate assurance that processing variables are free from compromise up to the time that the molecular profile data are collected.

3 Protein stability may be unrelated to RNA transcript stability

Several studies have been conducted concerning the stability of RNA in tissue *ex vivo* [16-20]. These studies indicate that refrigeration is superior to room temperature and the addition of RNAase inhibitors may be useful as RNA preservatives. Although this information is applicable to gene array profiling, it may have little bearing on protein stability in general or phosphoprotein stability specifically. Chemical conditions favoring protein stability may be completely different from those for RNA stability.

4 Phosphoprotein stability: The balance between kinases and phosphatases

Phosphoproteins offer a unique minute-by-minute record of ongoing signal pathway events of high-functional relevance to therapeutic target selection and the prediction of toxicity. At any point in time within the tissue cellular microenvironment, the phosphorylated state of a protein is a function of the local stoichiometry of associated kinases and phosphatases specific for the phosphorylated residue. Protein phosphatases have been classified into three distinct categories: (i) serine/threonine (Ser/Thr)-specific [21], (ii) tyrosine-specific [22], and (iii) dual specificity phosphatases. Protein tyrosine phosphatases remove phosphate groups from phosphorylated tyrosine residues of proteins. A variety of chemical- and protein-based inhibitors of phosphatases exist [23,24]. An important long-term need for the clinical implementation of phosphoprotein biomarkers will be the design of stabilizers for the preservation of phosphoproteins without the need for freezing. Optimally the stabilizing chemistry should arrest both sides of the kinase/phosphatase balance, in order to prevent positive or negative fluctuations in phosphorylation events as the living-excised tissue reacts to the *ex vivo* conditions [13].

5 Recognition that tissue is alive and reactive following procurement

Although investigators have worried about the effects of vascular clamping and anesthesia, prior to excision, a much more significant and underappreciated issue is the fact that excised tissue is alive and reacting to *ex vivo* stress [13]. The instant a tissue biopsy is removed from a patient, the cells within the tissue react and adapt to the absence of vascular perfusion, ischemia, hypoxia, acidosis, accumulation of cellular waste, absence of electrolytes, and temperature changes [13]. In as little as 30 min post excision drastic changes can occur in the protein signaling pathways of the biopsy tissue as the tissue remains in the operating room suite or on the pathologist's cutting board. In response to wounding cytokines, vascular

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hypotensive stress, hypoxia, and metabolic acidosis, it would be expected that a large surge of stress related, hypoxia related, and wound repair-related protein signal pathway proteins and transcription factors will be induced in the tissue immediately following procurement [25-28].

During the *ex vivo* time period, as the tissue cells are alive and reactive, phosphorylation of certain kinase substrates may transiently increase due to the persistence of functional signaling, activation by hypoxia, or some other stress–response signal [13,29-31]. The excised tissue is hypotensive and wounded, triggering a cascade of further reactive changes, including the activation of kinase pathways, which may predominate close to the cut surfaces. Forensic wound evaluation and wound vitality studies further support the persistence of functional protein signaling. Immunohistochemical analysis of cytokines (TGFα, TGFβ, IL1-β, IL-6, and TNFα) from human skin wounds demonstrated increases in cytokine levels at wound sites in as little as 10 min post wounding, with additional increases noted over 30–60 min [29-31]. Although these reactive changes would be expected to increase protein phosphorylation, the availability of ubiquitous cellular phosphatases would be expected to ultimately destroy phosphosphorylation sites, given enough time [13,25,26]. This will significantly distort the molecular signature of the tissue compared with the state of the markers *in vivo*. The manner of distortion will be dependent on the time delay following procurement, with reactive increases in phosphorylation at early times, and loss of phosphorylation at later times. Moreover the degree of *ex vivo* protein fluctuation could be quite different between tissue types and influenced by the pathologic microenvironment. This physiologic fact must be taken into consideration as we plan to implement tissue protein biomarker analysis in the real world of the clinic, where the living, reacting tissue may remain in the collection basin for hours.

6 Timecourse of post-translationally modified proteins post excision

We present the following example of supravital tissue fluctuations post excision. Uterine tissue (leiomyoma (myometrium)) was obtained under informed consent following an IRB-approved protocol in a community hospital (Inova Fairfax Hospital, Falls Church, VA, USA). Tissue was excised in the surgical suite following standard of care guidelines. Tissue was transported at room temperature to the surgical frozen section room. A board-certified pathologist performed gross examination of each tissue sample and provided non-diseased tissue that was not required for diagnosis. The tissue was cut with a scalpel into eight relatively homogeneous pieces. One piece of tissue, designated as time zero (10 min post excision), was immediately processed by embedding in a cryomold and freezing on dry ice. The remaining tissue pieces were incubated at room temperature for 21, 40, 70, 100, 130, 160, and 190 min post excision and processed as described above. Cryosections (8 μm) were cut from the tissue block at a depth of 1.0–2.0 mm from the surface to mimic conditions found at the center of a core needle biopsy specimen. The cryosections were fixed, stained, and analyzed by reverse phase protein microarray (RPPA) as described previously [27,28].

The time zero sample data were designated as 100%, assuming that any changes during incubation would be reflected in deviations from the time zero sample. Room temperature incubation of tissue revealed significant (±20% from the time zero sample) augmentation, as well as decreases, of phosphoprotein levels over time, independent of post-translational modification and protein sub-cellular location (Fig. 2A). Although some individual proteins participating in stress/inflammation, hypoxia, proliferation/survival, and cell cycle signaling pathways showed increases over time *ex vivo*, other protein levels remained stable over time. This is in keeping with the physiologic concept that excised tissue is alive and reactive prior to processing/fixation [13].

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Based on the emerging published data [13,29-33] revealing that excised tissue is reactive, and showing how this state can introduce sources of variability for diagnostic molecular endpoints, we can propose guidelines for the reduction of pre-analytical variables.

Tissue procurement guidelines for molecular analysis:

- **i.** Tissue procurement protocols must recognize the fact that excised tissue is alive and reactive to *ex vivo* stress. Kinase pathways are active and reactive until the tissue cells are stabilized.
- **ii.** Reactive changes occurring in tissue post excision can generate false elevation as well as false declination in protein and nucleic acid analytes. This may be a significant source of bias in the analysis of protein or nucleic acid as potential biomarkers.
- **iii.** Kinase pathway stabilization methods should block both sides of the kinase/phosphatase kinetic reaction. Blocking only phosphatases can cause false elevation of an analyte's phosphorylation level.
- **iv.** Tissue should be stabilized as soon as possible after excision. Taking into consideration the average time for procurement in a community hospital, the recommended maximum elapsed time is 20 min from excision to stabilization (*e.g.* flash freezing, thermal denaturation, or chemical stabilization).
- **v.** Tissue stabilization and preservation methods should be compatible with the intended downstream analysis. Preservation of tissue histology and morphology is essential for the verification of tissue type and cellular content.
- **vi.** Documentation of the sample excision/collection time, elapsed time to preservation/stabilization, and length of fixation time are critical data elements for sample quality assessments.

7 Technologies for the future: Surrogate markers of stability and multi-purpose fixative

Surrogates of tissue protein biomarker stability

An immediate technologic approach to the preservation of the tissue proteome is a mean to qualify the state of protein analyte preservation in a tissue, prior to molecular analysis. In order to address the uncertainty of protein and phosphoprotein analyte quality within an individual patient's biopsy, the authors propose the concept of endogenous protein surrogate markers for tissue preservation: the identification of a panel of natural proteins and phosphorylated amino acids that are highly labile and constitute an early warning of a compromised preservation state [34]. Such surrogates would be selected from a variety of classes of phosphoproteins, including those from specific pathways; classes of residues (*e.g.* tyrosine, serine, or threonine); and nuclear, cytoplasmic, and cell membrane compartments [13,34]. For a tissue sample of unknown or questionable state of preservation, the panel of endogenous surrogate markers would be measured before the tissue could be cleared for molecular diagnostic analysis. If values of the surrogate markers fall within cut points defining tissue that has been preserved adequately (*e.g.* frozen in less than 30 min), then the tissue would pass initial quality control assessments for further analysis. The choice of the surrogates must of course be different than the diagnostic protein analyte that would be measured in the same piece of tissue. Developing a list of tissue protein stability surrogate markers is an important goal for molecular profiling research.

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8 Non-formalin fixation chemistries for molecular analysis

Formalin fixation may be unsuitable for quantitative protein biomarker analysis in tissue

Although it is now possible to extract proteins from formalin-fixed tissue [35], formalin penetrates tissue at a variable rate, reported to be within the range of millimeters *per* hour [36-38]. During this time delay period the portion of the living tissue deeper than several millimeters would be expected to undergo significant fluctuations in regards to phosphoprotein analytes. When one considers the volume of a typical 16-gauge core needle biopsy (7 mm × 1.6 mm (volume = 17.9 mm^3)) it is clear that the cellular molecules in the depth of the tissue will have significantly degraded by the time formalin permeates the tissue [36,39]. In addition, penetration rate is not synonymous with fixation. In aqueous solution formaldehyde becomes hydrated, forming methylene glycol [36,38]. The small percentage of formaldehyde in solution forms the actual covalent cross-links with proteins and nucleic acids. Methylene glycol penetrates the tissue, yet it is the carbonyl formaldehyde component that causes tissue fixation [36,38]. Formalin cross-linking, the formation of methylene bridges between amide groups of protein, blocks analyte epitopes as well as decreases the yield of proteins extracted from the tissue. Since the dimensions of the tissue and the depth of the block from which samples are prepared are unknown variables, formalin fixation would be expected to cause significant variability in protein and phosphoprotein stability for molecular diagnostics [36,40,41].

Although new fixatives have been developed for preservation and/or extraction of RNA from formalin-fixed tissue, there is an awakening recognition that new chemistries are needed for preserving proteins and post-translationally modified proteins [20,42,43]. Rapid fixation chemistries and formalin alternatives are being developed but as yet have not been thoroughly evaluated as a timecourse analysis of phosphoproteins or other post-translationally modified proteins [39,42-47]. Thermal/pressure inactivation of protein kinases and phosphatases has been developed as an effective, rapid protein stabilization/inactivation method [48]. Rapid thermal inactivation of enzymes ensures stabilization of kinetic reactions but fails to maintain the tissue morphology. Although this technique has shown utility for mass spectrometric analysis it is not compatible with histomorphology including paraffin embedding, cryosectioning, or laser capture microdissection, which are critical components of the clinical/translational research tool kit. Ultra-sound rapid fixation [39,44,45,47,49] and non-formalin-based fixatives [42,46] processed with or without microwave assistance, are technologies that were developed with the goal of preserving diagnostic macromolecules during tissue processing for subsequent histopathologic analysis. Nevertheless, the contribution of pre-analytical variables (prior to immersion in the non-formalin fixative or delay in processing) and the preservation of phosphoproteins, must still be addressed before these technologies gain widespread clinical utility. Moreover, microwave, infrared heating, and ultrasound technologies proposed for tissue preservation may be expensive and may not be available to the community hospital or out-patient clinic.

The paramount requirement of any human tissue procurement is an accurate histopathologic diagnosis. The pathologic diagnosis is the determining factor for irrevocable clinical decisions about mode and extent of surgery, the successful attainment of clean surgical margins, and the administration of toxic therapies. Surgeons, pathologists, and IRB review boards, are frequently concerned that tissue procured for molecular diagnostics, or for exploratory research, will compromise the accuracy of the histopathologic diagnosis. Imagine the scenario that one peice of tissue from a patient's surgical specimen is procured for research, while an adjacent region of tissue is used to make the pathologic diagnosis. The research tissue sample is frozen and pulverized to extract proteins and RNA. Imagine further that the tissue that is removed for research purposes contains invasive cancer cells or reveals an aggressive neoplastic morphology, while the tissue used for diagnosis contains only benign tissue. The

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unfortunate outcome is a missed cancer diagnosis because the processing of the research specimen precludes an accurate histologic diagnosis.

Future tissue preservation technology must take into consideration the requirement for an accurate histopathologic diagnosis, and recognize limitations in the availability of specialized processing instruments, or liquid nitrogen, in the typical community hospital or clinic. In the face of the aforementioned limitations and requirements, the ideal future technology is a new class of multipurpose fixative chemistries (Fig. 1). At the time of tissue procurement in the OR or clinic the tissue would be immediately immersed in the stabilizing chemistry to arrest all reactive fluctuations in protein and nucleic acid macromolecules.

This proposed chemistry would also maintain tissue arcitecture and morphology, and be compatible with cryosectioning techniques and paraffin embedding for standard of care histopathologic diagnosis. Such a multipurpose chemistry would be the starting point for processing all pathologic specimens into a standard paraffin block. Multiple sections from the same paraffin block could be cut and distributed for all the following uses: (i) histopathologic diagnosis, (ii) immunohistochemistry, (iii) microdissection, (iv) proteomic analysis, and (v) nucleic acid analysis. Importantly, the tissue with its preserved macromolecules could be stored indefinitely at room temperature as a paraffin block in the standard fashion of an anatomic pathology archive.

Espina *et al*. [13] have described an ethanol-based fixative chemistry that contains phosphatase and kinase inhibitors to successfully arrest the reactive kinase pathways activated *ex vivo* following tissue procurement. This previously described chemical composition preserves phosphoproteins while maintaining tissue histomorphology for frozen sectioning and paraffin embedding (Fig. 2A). Human breast cancer tissue immersed in either the multi-purpose stabilization solution or the 5% formalin presented similar cellular morphology post processesing in formalin *via* UltraLight Histology, a method which provides nucelar detail at a resolution between electron microscopy and light microscopy (Fig. 2C and D).

In order to achieve the full purpose, one step chemistry proposed in Fig. 1, RNA preservation must be added as a necessary functionality. Since chemical solutions designed to preserve phosphoproteins may not be compatible with RNA preservation we tested this candidate chemistry for the ability to preserve RNA in a T47D human mammary adenocarcinoma cell line model (Fig. 2B). RNA integrity was maintained over a 72-h timecourse as compared with RNA*later*-(Qiagen) preserved cells. RNA integrity numbers (RIN) for the cells preserved in RNA*later* and our multi-purpose fixative were not statistically different over the 72-h timecourse, indicating that the multi-purpose chemical stabilization solution containing phosphatase and kinase inhibitors was indeed compatible with nucleic acid preservation.

9 Concluding remarks

Clinical standard of care guidelines and surgical techniques subject most tissue samples, prior to excision, to some degree of anesthesia exposure (local or systemic), potential ischemia due to clamping of blood vessels, wounding *via* incisions or cauterization, and exposure to dyes, stains, or contrast media. Although these sources of cellular stresses may affect potential biomarkers, the extent of tissue reactivity post excision has been greatly underappreciated by the biomarker community. Although it may be on a microscopic scale, there is a life and death struggle playing out in the living tissue cells that are reacting to hypoxia, nutrient deprivation, wounding, and metabolic acidosis [13]. Interpretation of biomarker data will require the acknowledgement of this struggle and the pre-analytical variables affecting molecular biomarkers.

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Aspects of the technologies and discoveries discussed in this manuscript are the subject of US Government and University assigned patents of which the authors are co-inventors. Under US Law they would be allowed to receive royalties on any licenses taken. VE, EFP and LAL are shareholders and/or consultants for Theranostics Health, LLC, which is a licensee for the technology described herein.

Abbreviation

RIN RNA integrity number

10 References

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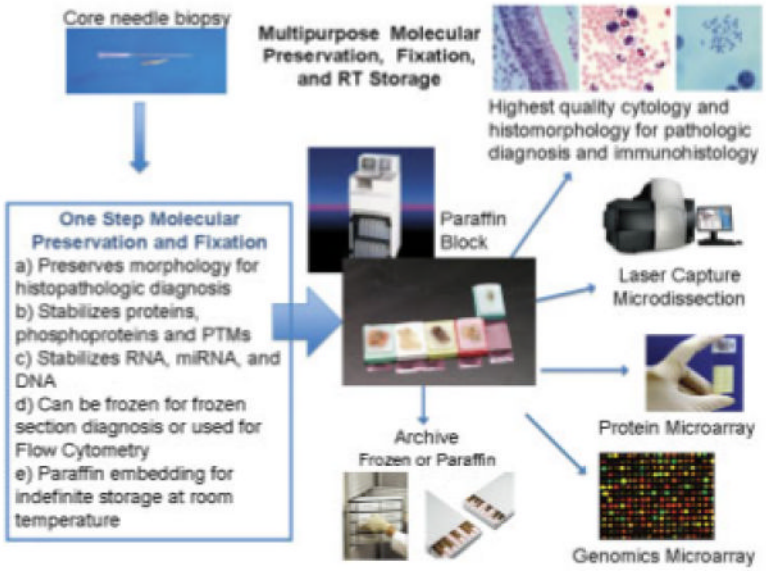

Figure 1.

Ideal tissue stabiliziation/preservation scheme for biomarker analysis. A core needle biospy is immediately placed in a multi-purpose stabilization/preservation solution at room temperature. The fixed biopsy specimen could subsequently undergo cryosectioning, processing in standard formalin or ethanol tissue processors, or flow cytometric analysis. The fixed samples could be archived as frozen sections/blocks or paraffin-embedded blocks. The samples would be compatible with standard protein/nucleic acid methodologies.

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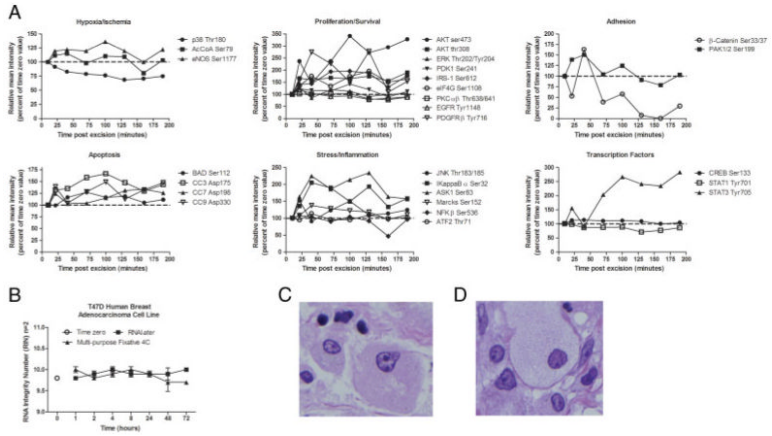

Figure 2.

A multi-purpose chemical solution for stabilization and preservation of proteins and RNA, and maintenance of histomorphology. (A) Frozen uterine leiomyoma tissue reveals on-going phosphoproteomic changes post excision. A reverse phase protein microarray analysis of tissue analyzed from a deep area of the tissue block (1.0–2.0 mm from surface) showed reactive protein changes as compared with the time zero sample (100% value, 10 min post excision, outermost tissue surface). Reactive proteins constituted a variety of molecular cascades: apoptotic pathway, stress/inflammation pathway, hypoxia/ischemia pathway, transcription factors, proliferation/survival pathway, and adhesion/cytoskeleton proteins. (B) RNA stability time course of T47D-cultured cells. T47D human mammary adenocarcinoma cell lines were cultured at 37°C, 5% CO_2. Medium was removed and cells washed three times with cold phosphate-buffered saline. The cells were scraped from the flasks, combined and aliquots were incubated with RNA*later* (Qiagen) (black square) or our multi-purpose fixative (black triangle) at 4°C for 1, 2, 4, 8, 24, 48, and 72 h. An untreated cell culture aliquot was used as the time zero control (open circle). RNA was extracted using the RNA Mini Kit (Qiagen) and RIN were determined using a Bioanalyzer 2100 (Agilent) for duplicate samples at each time point. (C and D) Tissue stabilized at room temperature in a multi-purpose chemical solution yields histomorphology similar to formalin fixed tissue. (C) Human breast tumor epithelium fixed in a multi-purpose stabilization solution containing phosphatase and kinase inhibitors, alcohol, and a permeation enhancer, was processed *via* a standard histology technique. H&E staining showed well-delineated nuclear membranes and chromatin clumping comparable to an adjacent tissue sample fixed in 5% formalin (D). *(UltraLight Histology*™ processing courtesy of Dr. Thomas Donndelinger, Bi-Biomics).

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Table 1

Tissue preservation/stabilization variables associated with biomarker analysis

Variables	Possible reasons	Potential solutions
Pre-analytical		
Tissue evaluation time (grossing)	Delay in transport	Transport and process tissues as soon as possible post excision
Time delay to preservation	Heavy work load or back-log of cases	Communicate tissue processing requirements with clinical and laboratory staff
Phosphatase, kinase and proteinase activity in tissue	Type of preservative/fixation	Use rapid preservative method compatible with downstream analysis
	Lack of inhibitors/stabilization in transport medium	Use preservative/fixative containing phosphatase, kinase, proteinase inhibitors or with inhibitor activity
	Delay in transport/preservation/ stabilization	Transport, preserve and process tissues as soon as possible post excision
	Storage temperature	Store tissue at appropriate temperature for preservation solution
	Introduction of phosphatases/ proteinases from the environment	Maintain clean tissue processing/grossing areas
Degree of fixative cross-linking	Preservative/fixative containing formalin, gluteraldehyde or other cross-linker	Use minimal amount of cross-linking agent to achieve adequate fixation
	Excessive tissue size in relation to fixative volume	Reduce tissue size. Increase volume of fixative
	Penetration time of preservative solution	Reduce tissue size. Use preservative with permeabilization reagents
	Elevated storage temperature	Store tissue at appropriate temperature for preservation solution. Reduce storage temperature
Tissue hydration state	Low ambient humidity. Open specimen container	Transport tissue in a closed container
	Delay in transport/preservation	
Post-analytical		
Time delay to analysis	Lack of proper equipment, reagents, storage conditions	Prepare reagents in advance. Plan procedure prior to retrieving samples
	Malfunctioning equipment	Develop a plan to use an alternate source of equipment
Poor/variable quality protein and/or nucleic acids results	Delay in stabilizing tissue sample	Transport/preserve/fix tissue as soon as possible post excision
	Elevated ambient temperature during tissue transport/processing	Place samples on wet ice or in chilled container
	Inadequate fixation/preservation	Follow directions for type of fixation/tissue

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Variables	Possible reasons	Potential solutions
	Use of post-mortem tissue samples	Limit ischemic conditions. Procure samples as soon as possible post-mortem
	Differences in sample handling and processing	Stabilize/preserve tissue as soon as possible post excision (time <20 min)
		Obtain highly pure cell populations (>75%)
		Freeze tissue in such a method as to ensure rapid, uniform cooling (<5 min).
		Use RNAse-free precautions: wear gloves, use nucleic acid free plastic ware, clean work area with RNAse decontamination solutions
		Add protease inhibitors to appropriate reagents
	Extensive cross-linking due to fixation	Determine type of fixation, volume or size of tissue sample, depth of tissue within the tissue block
	Inadequate removal of paraffin from formalin-fixed paraffin embedded tissue	De-paraffinize in two changes of xylene for 5–15 min each