Preservation of Molecular Information in Tissue and Blood at Room Temperature

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Introduction

Instability of tissue and blood biomarkers is a critical roadblock to the clinical application of novel diagnostic analytes. We describe two classes of novel technologies that can be applied at the time of tissue or blood collection and **seamlessly integrated into the clinical diagnostic workflow without requiring additional steps or equipment**. (A) A biomarker and histology preservative (BHP) which maintains full diagnostic histomorphology, while preserving labile tissue biomarkers, such as phosphoproteins. (B) Biomarker harvesting nanoparticles which sequester, concentrate and preserve blood biomarkers in one step.

Biomarker and Histology Preservative (BHP)

• BHP fixation preserves histomorphology and protein antigenicity

Histology of BHP preserved colon tissue was equivalent to formalin fixed paraffin embedded tissue. Cells showed minimal shrinkage, with full cytoplasmic and membrane detail, and retention of nuclear membrane, chromatin and nucleoli structure. To test protein antigenicity we selected several proteins representing different sub-cellular locations (see table below). Full immunoreactivity was retained after BHP fixation.

Positive Immunoreactivity	
EGFR (membrane)	Cytokeratin 20 (membrane)
Ki-67 (nucleus)	Smooth Muscle Actin (cytoplasm)
Estrogen Receptor (nucleus)	Progesterone Receptor (nucleus)
Periodic Acid Schiff (polysaccharide/mucin)	

Figure 1: Antigenicity is retained in colon mucosa fixed in BHP and processed in ethanol with paraffin embedding. Hematoxylin and eosin stain shows membrane, cytoplasmic and nuclear detail comparable to formalin fixed tissue. Proliferating cells at the base of the crypts are clearly visible with Ki-67 immunohistochemical stain (DAB, brown staining). Cytokeratin 20 staining shows differentiated cells at the surface and at the outermost edge of the crypt. Periodic Acid Schiff (PAS) stain shows intact acidic mucin (blue) and polysaccharides (magenta) within the crypt's goblet cell vacuoles (5μm sections, 20X and 100X magnification).

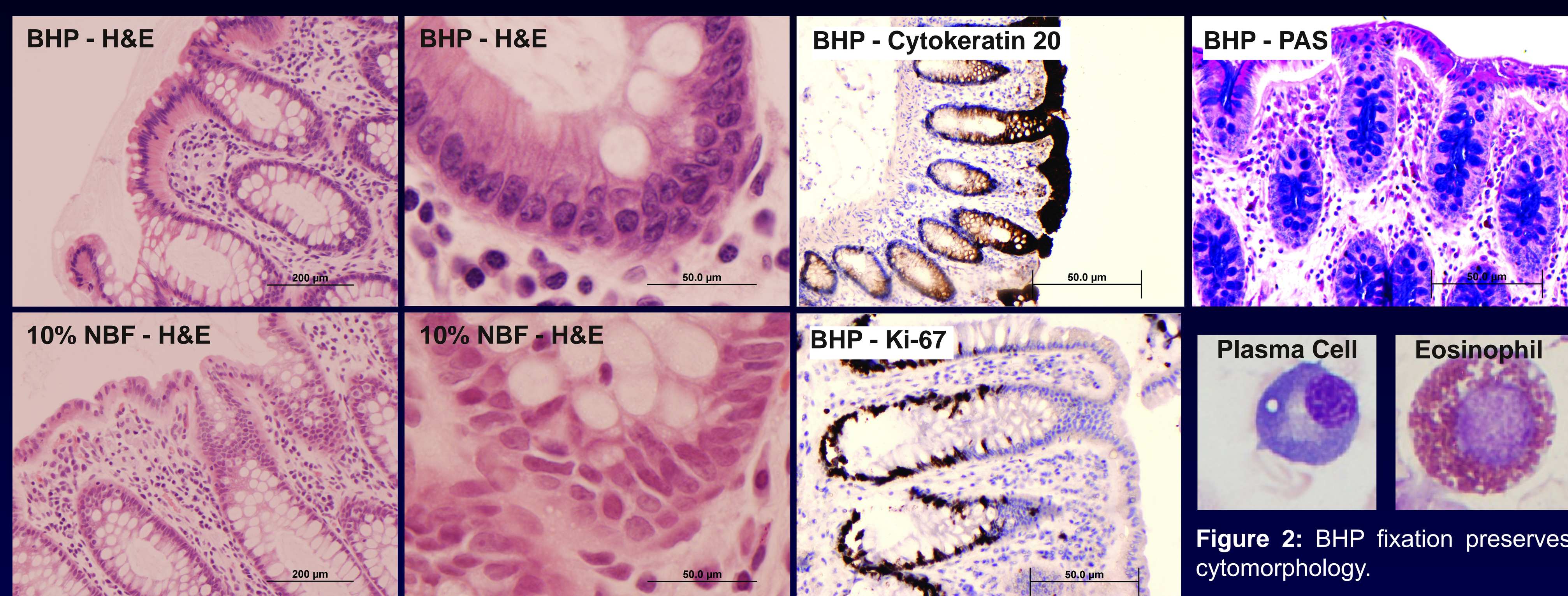
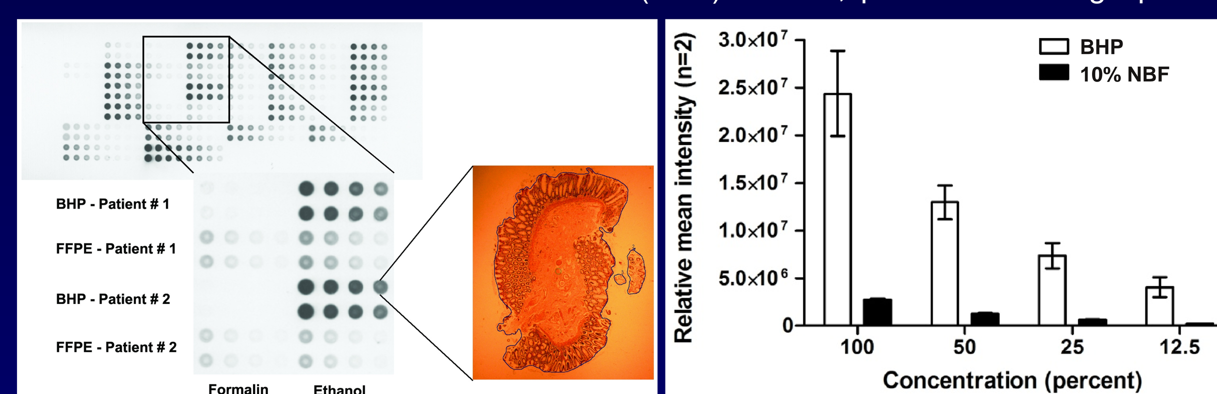


Figure 2: BHP fixation preserves cytomorphology.

• BHP fixation facilitates high protein extraction yield

Yield of protein extraction is a critical component for molecular analysis of fixed tissue. Fixation with BHP leads to a 10-fold increase in extractable protein compared to fixation with formalin.

Figure 3: Normal colon mucosa was freshly procured and fixed in 10% neutral buffered formalin (NBF) or BHP, processed through paraffin embedding, and analyzed in serial two-fold dilutions by reverse phase protein microarray. H&E stained colon mucosa sections for each sample/fixative were outlined to calculate the total tissue area per extraction buffer volume. Total protein yield from whole slide colon lysates: BHP => white bar, ethanol processed, n=2, mean ± SEM, r2=0.9996; 10% NBF => black bar, formalin processed, n=2, mean ± SEM, r2=0.9988.



• BHP fixation preserves phosphoproteins

During "cold ischemia time" cells react to the absence of vascular perfusion, ischemia, hypoxia, acidosis, absence of electrolytes, and temperature changes [1]. Fixation with BHP protects highly labile post-translational protein modifications [1, 2].

Figure 4: Many phosphoproteins are not preserved after formalin fixation. Recovery of total and phospho-proteins prepared from whole tissue slide lysates for BHP (white bar, mean ± SEM) versus 10% NBF (black bar, mean ± SEM) fixed samples. Data is normalized relative to the amount of β-actin extracted from the tissue (amount close to zero if black bar not visible).

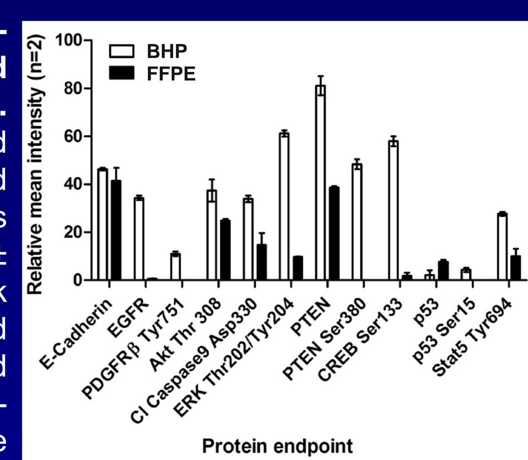
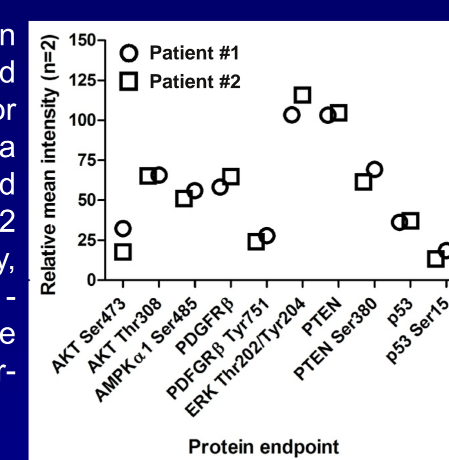


Figure 5: Reverse phase protein microarray analysis of the indicated phosphoprotein signatures for microdissected colon mucosa preserved in the histology and biomarker preservative from 2 different patients (mean intensity, n=2; circle - patient 1; square - patient 2). No significant difference was noted (p=0.52), verifying inter-patient reproducibility.



Hydrogel Nanoparticles

We have created N-isopropylacrylamide (NIPAm) hydrogel nanoparticles containing an internal affinity bait [3, 4]. The nanoparticles perform three independent functions within minutes, in one step, in solution (serum, plasma, or urine):

a) molecular size sieving,

b) affinity capture of all solution phase low abundance target analyte molecules,

c) complete protection of harvested proteins from enzymatic degradation.

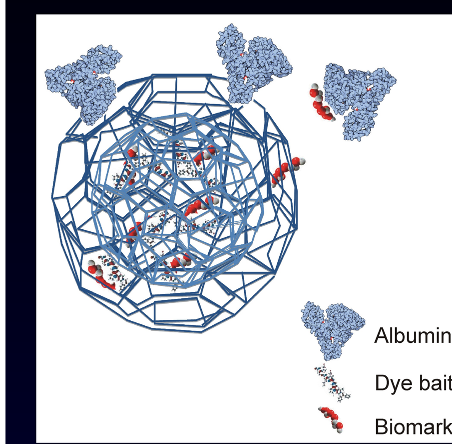


Figure 6: The core shell particle is an open porous polymer network with a porosity determined by the percent of cross links. The core of the particle is decorated with a covalently bound, high affinity chemical (dye) bait. Low molecular weight biomarkers can pass through the pores to enter the particle and bind to the bait, while high affinity and high molecular weight proteins such as albumin are excluded.

• Biomarkers are protected from degradation

The "smart" nanoparticles perform affinity capture of all solution phase target molecules within minutes. The porous structure performs molecular size sieving with complete separation from high abundance proteins such as albumin and immunoglobulin. When captured, proteins are completely protected from enzymatic degradation even when the enzyme penetrates the nanoparticles [3, 4]. We incubated a panel of highly labile biomarkers with hydrogel nanoparticles (see table below). In all cases they were completely protected from degradation.

Table 2: A panel of highly labile biomarkers were incubated with our hydrogel nanoparticles. In all cases, these labile biomarkers were completely protected from degradation when sequestered in the nanoparticles.

Labile Biomarker Preserved	
Interleukin 2	MEC/CCL28
Interleukin 4	SDF-1beta/CXCL12b
Interleukin 6	Eotaxin-2/CCL24
Interleukin 8	PDGF
Interleukin 10	Lysozyme
Interleukin 18	Carbonic anhydrase II
TNF alpha	hGH

Figure 7: NIPAm/AB48 nanoparticles protect human growth hormone (hGH) from enzymatic degradation by high trypsin concentration. Lane 1: "Protein" = hGH solution. 2: "Sup" = nanoparticle supernatant. 3: "Protein + Tryp + NP" = content of nanoparticles incubated with hGH and trypsin. 4: "Protein + Tryp" = hGH incubated with trypsin.

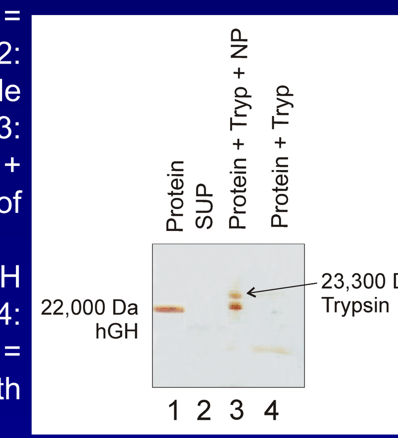
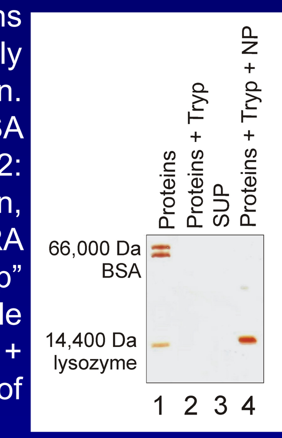


Figure 8: NIPAm/CB nanoparticles protect reduced and alkylated (RA) lysozyme from tryptic degradation. Reducing disulfide bonds and alkylating proteins makes them extremely sensitive to trypsin digestion. Lane 1: "Proteins" = RA BSA and RA lysozyme. 2: "Proteins + Tryp" = trypsin, RA albumin, and RA lysozyme. 3: "Sup" =NIPAm/CB particle supernatant. 4: "Proteins + Tryp + NP" = content of nanoparticles.



Conclusions

The technologies evaluated in the present study increase the yield of biomarkers, and thereby reduce the required volume of sample, for both tissue and body fluids (e.g. blood), to a level acceptable for routine individualized molecular profiling. Both technologies protect labile protein biomarkers from degradation in tissue and blood. The BHP fixative preserves tissue biomarkers in a paraffin block, obviating the need for frozen storage. Thus one paraffin block can be used for diagnosis and molecular profiling.

The harvesting nanoparticles can be pre-introduced into a vacutainer tube for immediate preservation and sequestration of all target analytes. Based on these data, the candidate fixative/preservative technologies warrant large-scale comparisons with other technologies for potential routine use in clinical molecular profiling and biobanking.

References & Acknowledgements

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