Impact of Pre-Analytic Variation on Tissue Analysis: Issues & Practical Applications

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Development of Standard Operating Procedures for Inter-SPORE Prostate Biomarker Study

- **Handling of fluid specimens**
  - Blood, serum, plasma, etc
- **Handling of tissues**
  - Prostate needle biopsies
  - Radical prostatectomies

SPECIMEN HANDLING PRIOR TO PROCESSING

- **SPECIMEN PRE-FIXATION**
  - Time of anoxia prior to placement in fixative or freezing tissue for frozen biopsies (should be immediate by person taking the biopsies)
  - Marking of biopsies with dye, such as safranin

- **SPECIMEN FIXATION**
  - Type of Fixative: unless snap freezing specimens, usually 10% neutral buffered formalin, which is actually 4% formaldehyde
  - Temperature of Fixation: usually room temperature
  - pH and Osmolality of Fixation: controlled using neutral buffered formalin
  - Volume of fixative

TISSUE PROCESSING

- **POSTFIXATION**
  - Time of post-fixation (if any)
  - Chemical makeup of post-fixation
  - Temperature of post-fixation
  - Vendor of fixative

- **DEHYDRATION**
  - Time and number of steps
  - Chemical makeup of dehydration (i.e. 70 %, 80%, 95% ethanol)
  - Other non-ethanol chemicals (i.e. eosin, "pen-fix")
  - Temperature of each step
  - Vendor of chemicals

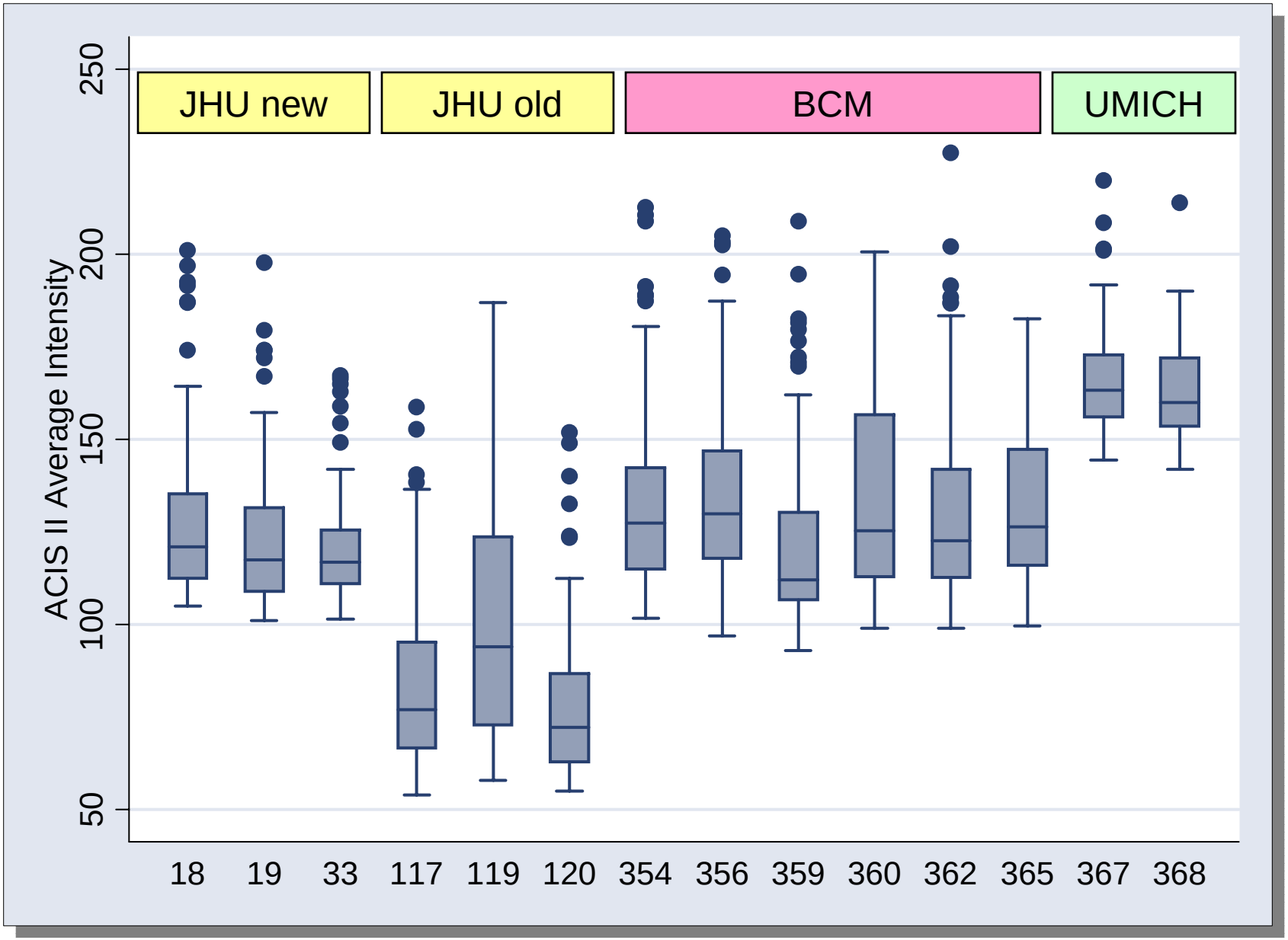
- **CLEARING**
  - Time and number of steps
  - Chemical makeup of clearing agent (most use xylene, but some use)
  - Other non-ethanol chemicals (i.e. eosin)
  - Temperature of each step
  - Vendor of chemicals

- **INFILTRATION**
  - Type of paraffin (there are several types)
  - Time, number of steps in paraffin
  - Temperature of paraffin

SPECIMEN HANDLING AFTER PROCESSING

- **TISSUE SECTIONING**
  - Thickness of sections
  - Water bath temperature
  - Presence of chemical in water bath (i.e. ammonia)
  - Time and temperature of slide drying
  - Time and temperature of baking slides (if done at this point)
- **SLIDE STORAGE**
  - Temperature of slide storage
  - Humidity of slide storage
  - Oxygen levels
  - Duration of slide storage under given conditions

PTEN INTENSITY



Rationale for Study

- Inter-SPORE Prostate Biomarker Study (IPBS)
- Issues regarding standard processing

- Prior studies in prostatic tissue have suggested that variability of tissue fixation and/or processing may affect biomarker interpretation

- **Quantify these variations and their potential impact on biomarker testing and analysis**

Study Design

- **Prostate needle biopsies**
  - Immediate fixation
  - Primary tissue to be used in IPBS Prospective arm

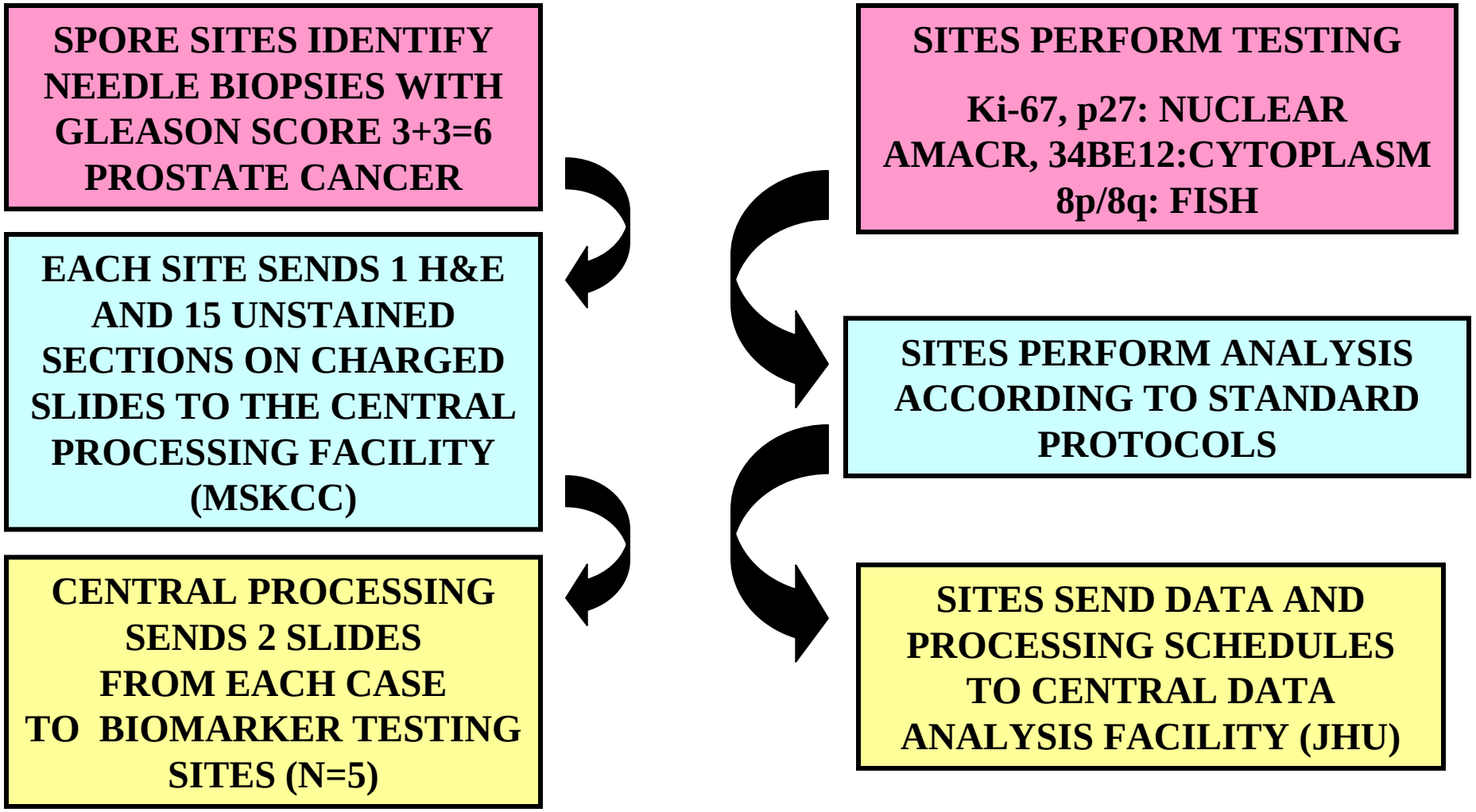
- **Biomarkers**
  - p27
  - AMACR
  - Ki-67
  - 34βE12

  } **IMMUNOHISTOCHEMISTRY**

  - Loss/gain in chromosome 8

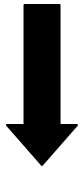
  } **FISH**

Effects of Tissue Processing Techniques on Biomarker Analysis for Prostate Cancer Specimens: An Inter-SPORE Study



Effects of Tissue Processing Techniques on Biomarker Analysis for Prostate Cancer Specimens: An Inter-SPORE Study

DATA FOR EACH MARKER IS ANALYZED PER SITE



IF SIGNIFICANT DIFFERENCES ARE DETECTED MARKERS ARE ANALYZED BY PROCESSING SCHEDULE VARIABLES

Study Participants

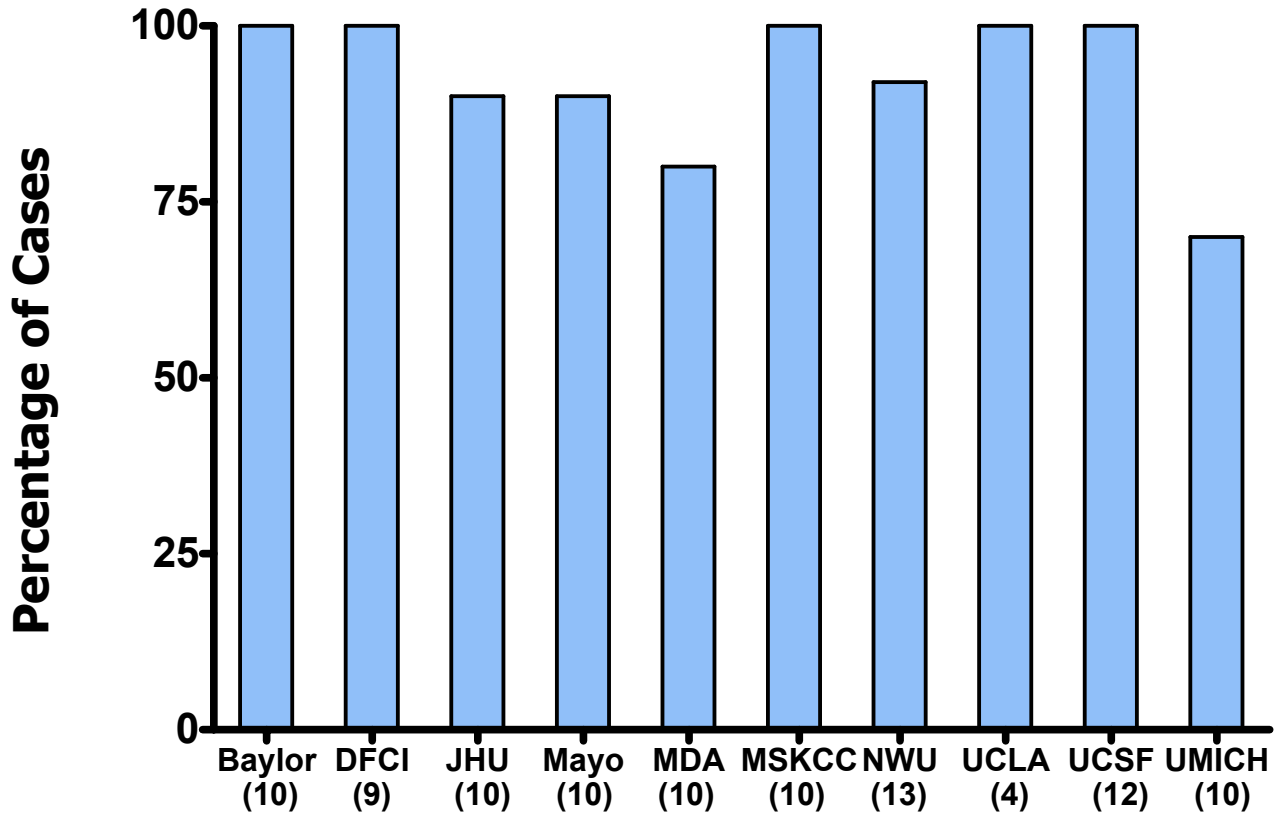
- BCM - Gustavo Ayala
- DFCI – Mark Rubin (*)
- FHCRC – Larry True
- JHU – Angelo DeMarzo; Bruce Trock (*)
- MAYO – Robert Jenkins; John Cheville (*)
- MDACC – Patricia Troncoso
- MSKCC - Samson Fine; Victor Reuter (*)
- NWU – Ximing Yang
- UCLA - Jonathan Said
- UCSF – Jeff Simko
- UMICH - Rajal Shah (*)

Needle Biopsy
Tissue Processing Protocols

		Harvard/DFCI			FHCRC/U Wash			JHU			Umich			MDACC			Mayo Clinic			MSKCC	
	Condition	Time	Temp		Time	Temp		Time	Temp		Time	Temp		Time	Temp		Time	Temp		Time	Temp
Post-Fixation	10% Formalin	-	-		-	-		2	40		20	37		-	-		20	?		30	40
	10% Formalin	-	-		-	-		2	40		20	37		30	RT		-	-		30	40
	Penfix	-	-		10	37		-	-		-	-		-	-		-	-		-	-
	10% formalin in 95% eto	-	-		-	-		-	-		-	-		30	RT		-	-		-	-
Dehydration	70% ethanol	5	RT		-	-		2	40		20	37		30	RT		-	-		20	40
	70% ethanol	-	-		-	-		-	-		-	-		-	-		-	-		-	-
	80% ethanol	15	RT		-	-		-	-		20	37		-	-		2	?		-	-
	80% ethanol	15	RT		-	-		-	-		-	-		-	-		5	?		-	-
	95% ethanol	15	RT		10	37		2	40		30	37		30	RT		-	-		10	40
	95% ethanol	15	RT		10	37		2	40		30	37		-	-		5	?		20	40
	100% ethanol	15	RT		10	37		2	40		35	37		30	RT		5	?		10	40
	100% ethanol	15	RT		10	37		-	-		35	37		30	RT		15	?		10	40
	100% ethanol	-	-		-	-		-	-		-	-		30	RT		-	-		20	40
	100% etoh/ 17%eosin	-	-		-	-		2	40		-	-		-	-		-	-		-	-
	50% etohl/ 50% Xylene	-	-		-	-		5	40		-	-		-	-		-	-		-	-
Clearing	Xylene	15	RT		10	37		2	40		35	37		30	RT		5	?		15	40
	Xylene	15	RT		10	37		2	40		40	37		30	RT		5	?		15	40
	Xylene	20	RT		10	37		-	-		-	-		-	-		-	-		-	-
Infiltration	Paraffin	20	RT		10	58		2	60		15	60		30	60		30	?		15	60
	Paraffin	20	RT		10	58		2	60		25	60		30	60		2	?		15	60
	Paraffin	-	-		10	58		5	60		25	60		30	60		-	-		20	60
	Paraffin	-	-		-	-		5	60		15	60		-	-		-	-		-	-
Total Time (minutes)		185			110			37			365			360			94			230	

1. p27 is a cell cycle inhibitor expressed in the secretory cells of normal prostatic glands
2. Reduced p27 staining has been proposed as a prognostic biomarker in prostate cancer
3. Shown by DeMarzo and Rubin to be artifactually decreased in RP specimens with brief fixation times

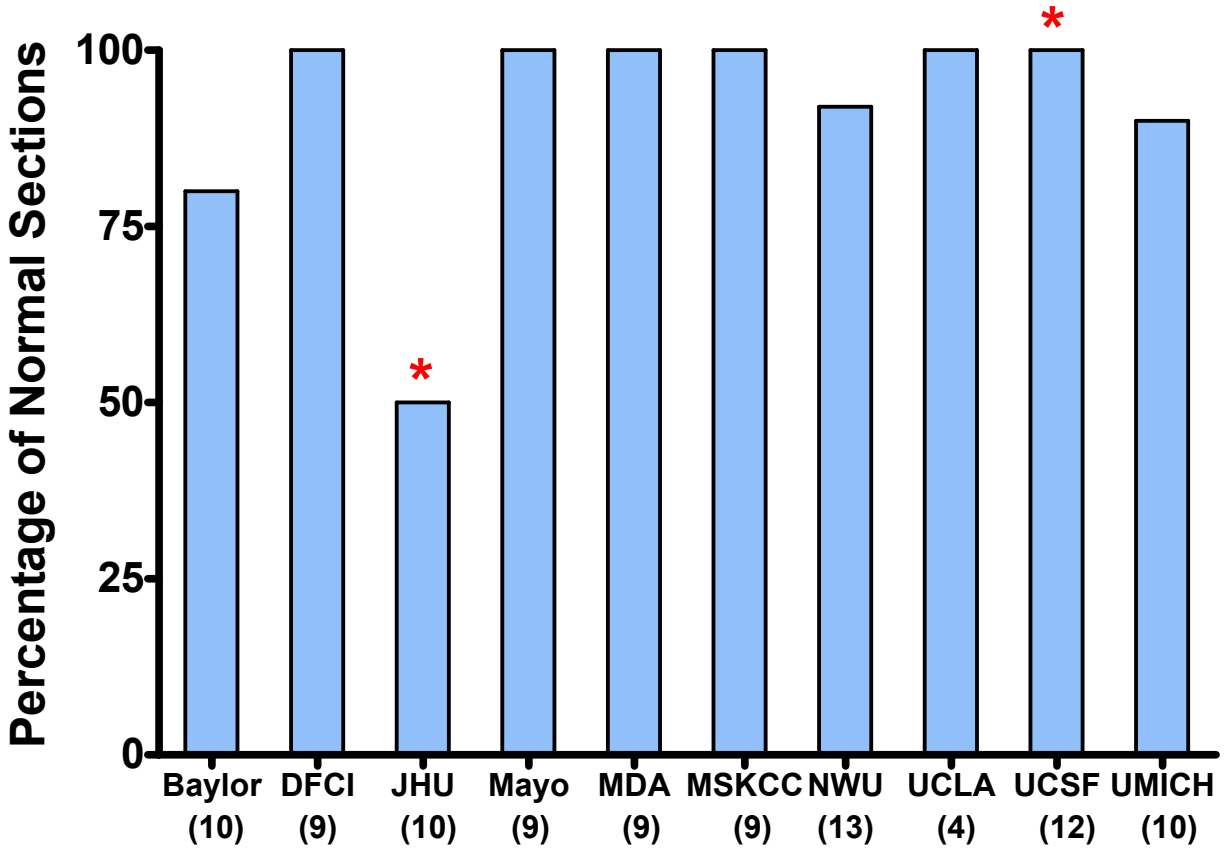
Percentage of cases with p27 staining intensity = 3 to 4



Overall Staining Intensity: mean=3.5, median=4
No significant difference among SPOREs, p=0.105

1. 34βE12 is an monoclonal antibody against high molecular weight CK expressed in basal cells of normal prostatic glands
2. Although unlikely to have prognostic value, 34βE12 is an important diagnostic marker with its absence denoting loss of basal cells, a cardinal feature of prostate cancer

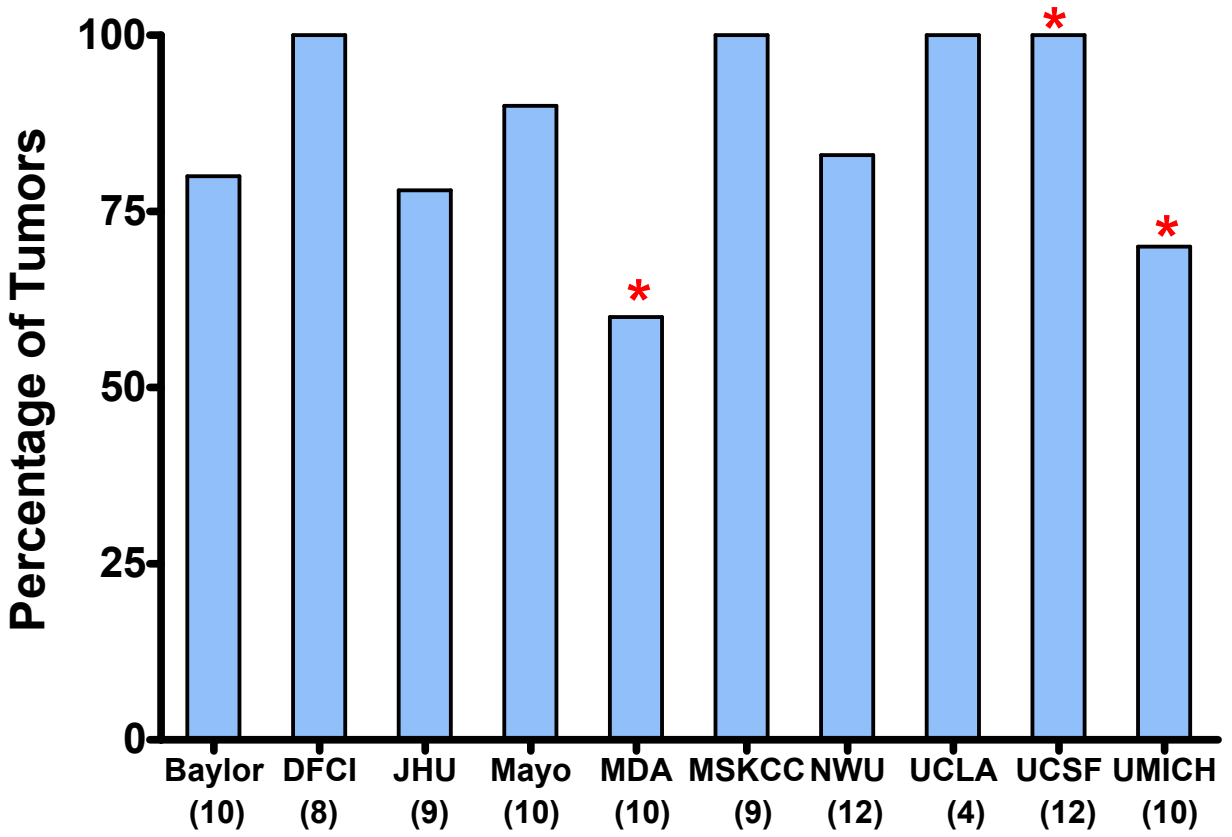
Percentage of cases with 34βE12 staining intensity = 3



* Significant difference in mean staining intensity (normals): UCSF=3.0, JHU=2.5, p=0.0009 (overall mean=2.91, median=3.0) (all normal sections had diffuse staining)

1. AMACR is an enzyme involved in β-oxidation of dietary branched-chain fatty acids which have been associated with an increased risk of prostate cancer
2. Elevated levels of AMACR RNA and protein have been implicated as biomarkers for prostate cancer and have been shown to have both diagnostic and prognostic value

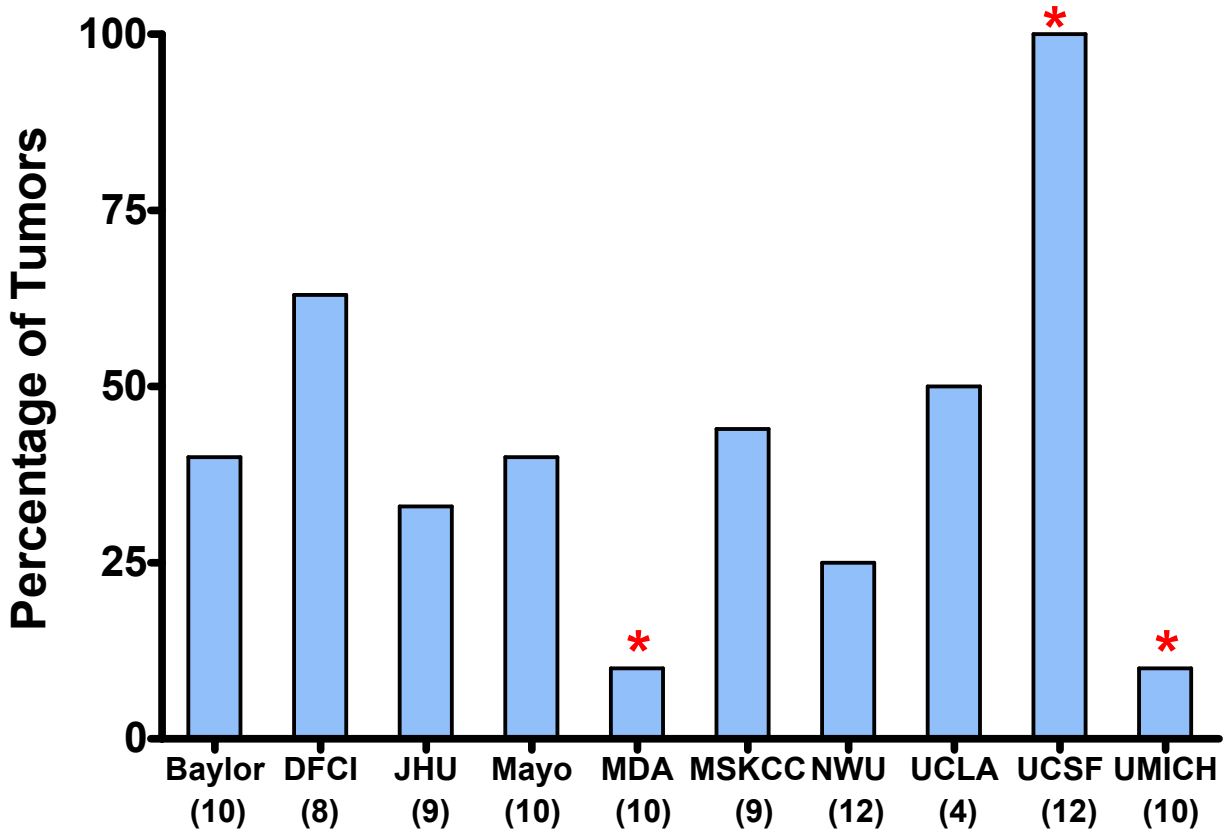
Percentage of tumors with AMACR staining intensity = 3 to 4



* Significant difference in mean staining intensity among SPOREs: MDA=2.7, UMICH=2.8, UCSF=4.0, p=0.0002 (overall mean=3.3, median=3.0)

1. Are these differences due to tissue processing variability or the underlying biologic potential of the tumors studied?
2. While nearly all (94/98) cases studied were Gleason score 3+3=6, volume/density of tumor, stage and grade post-therapy and biologic outcome were not controlled

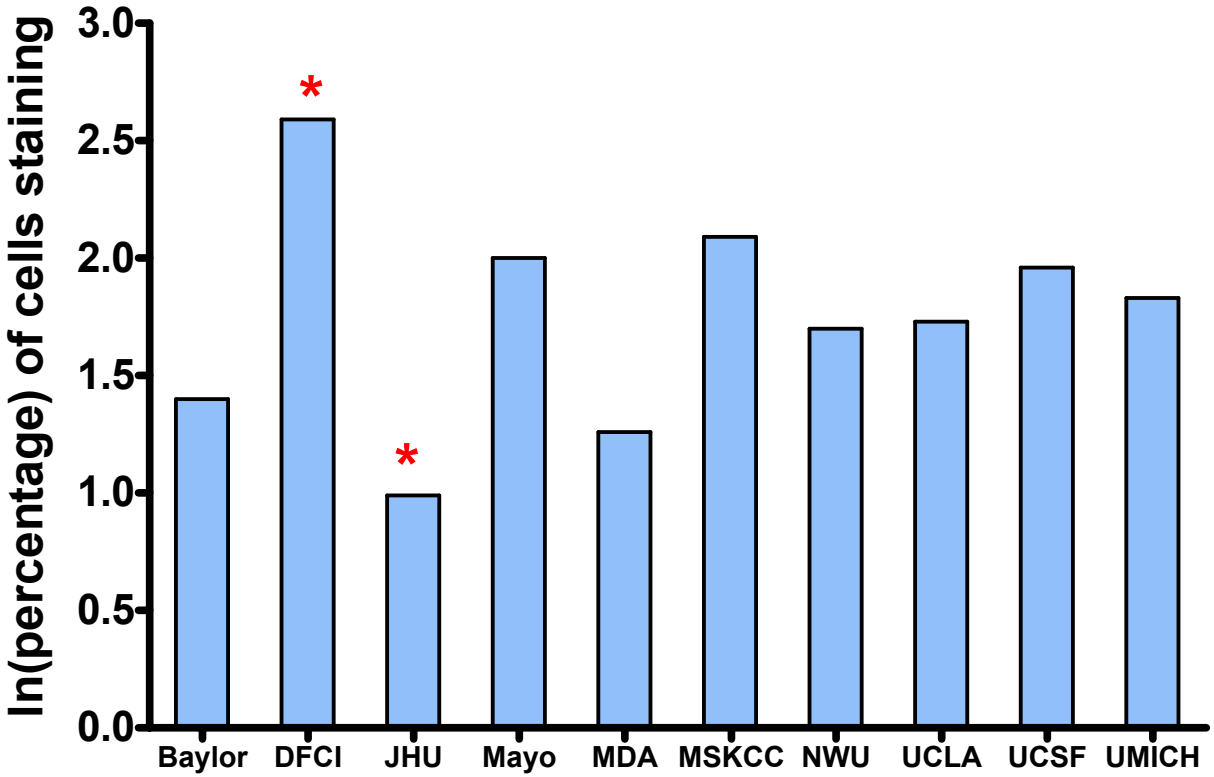
Percentage of tumors with AMACR staining intensity = 4



* Significant difference in mean staining intensity among SPOREs: MDA=2.7, UMICH=2.8, UCSF=4.0, p=0.0002 (overall mean=3.3, median=3.0)

1. Ki-67 is a nuclear proliferation antigen
2. Quantification of Ki-67 antigen using IHC has been shown to provide an estimation of growth fraction
3. Numerous studies have associated Ki-67 with tumor grade/stage, recurrence and metastasis post-therapy and cause specific death from prostate cancer

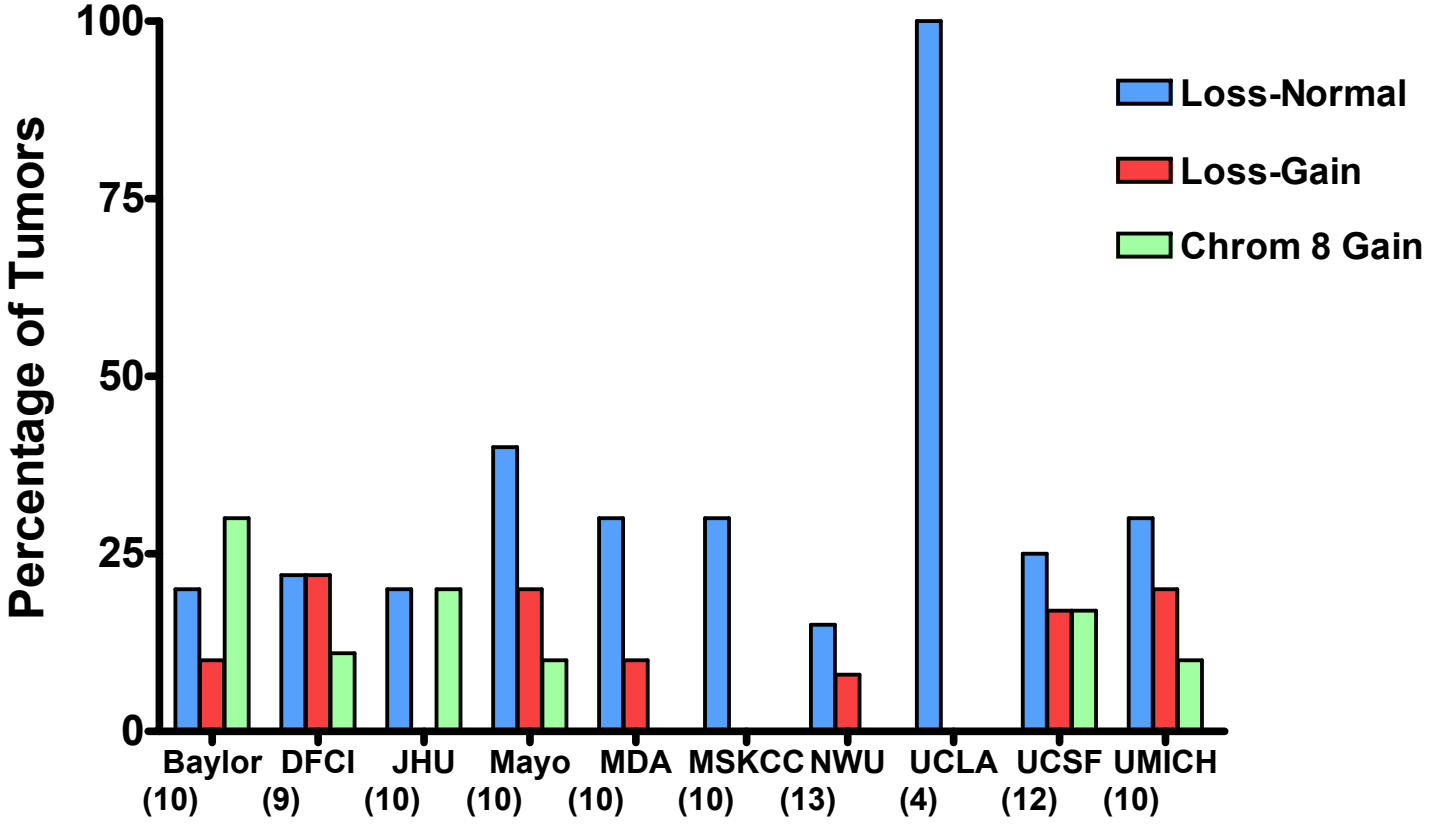
Mean ln(%) tumor cells staining for Ki-67



* Significant difference in ln(percentage) of cells staining among SPOREs: DFCI 2.6 (19.2%), JHU 1.0 (3.5%), p=0.0002 (overall mean=7.5, median=5.8)

1. 8p22 loss & 8q24 gain are common alterations in prostate CA
2. Losses/gains of chr. 8 are implicated in tumorigenesis, advanced, metastatic, and androgen-independent disease
3. Quantification of signal is dependent on having intact interphase nuclei

Frequency of 8p22 loss – 8q24 normal, 8p22 loss – 8q24 gain, or gain entire chromosome 8



Results

- Significant associations:
  - p27 staining intensity: ↓ with ↑ minimum fixation time (p=0.039); ↑ with ↑ dehydration time (p=0.011)
  - 34βE12 staining intensity: ↑ with ↑ maximum fixation time (p=0.015)
  - AMACR staining intensity : ↓ with ↑ minimum fixation time (p=0.001); ↑ with ↑ dehydration time (p=0.0002)
  - Ki-67 ln(%) cells staining: ↓ with ↑ infiltration temperature (p=0.035)

Conclusions

- As a predecessor to the IPBS, the current study demonstrates the collaborative potential of the Prostate SPORE sites to conduct biomarker studies

- Pilot data for p27 and 34βE12 in needle biopsies suggest that near-equivalent labeling of normal prostatic tissue is possible across SPORE sites

- Variability in results seen with biomarkers likely reflects both processing and tumor biology

Lessons Learned – Phase I

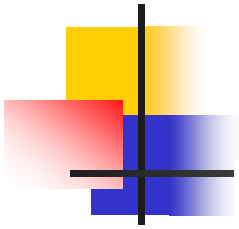
- Pathologists from the 11 Prostate SPORE sites can work together to accomplish projects of global importance
- Significant variability exists in processing schedules
- Good correlation may be achieved for some markers of nl prostate
- Interpretable FISH signals could be detected across SPORE sites
- Significant variation exists for tumor markers: processing v. tumor heterogeneity
- Associations between specific processing/sectioning steps and biomarker results may be identified

Next Steps (Phase II) – Control of Biological Variability

- Take multiple biopsies from human prostate cancer xenograft tumors, fix immediately under identical conditions and send specimens to various sites for processing; blocks sent to MSKCC, sections cut and sent out to labs for biomarker testing.
- Take multiple biopsies from a single RP case and fix immediately under identical conditions and do the same.

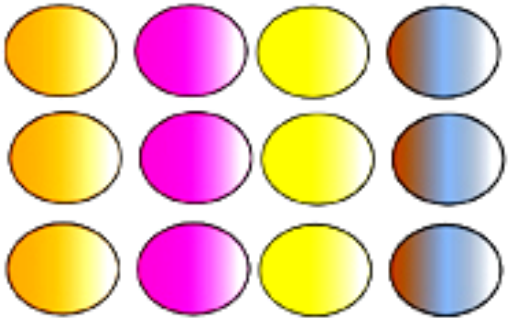
Next Steps (Phase II) – Control of Biological Variability

- N = 3 xenograft tumors already biopsied, fixed, sent to 11 institutions for their processing, processed and awaiting cutting at MSKCC.
- N=15 RRPs already biopsied at JHU, sent and processed and blocks being sent to MSKCC.
- Creation of a “processing array.



The TMAJ Software Project

http://tmaj.pathology.jhmi.edu



What is *TMAJ?*

- *TMA-J* is a set of open source software tools and backend database structure to facilitate management and analysis of tissue microarrays and associated pathology and image data

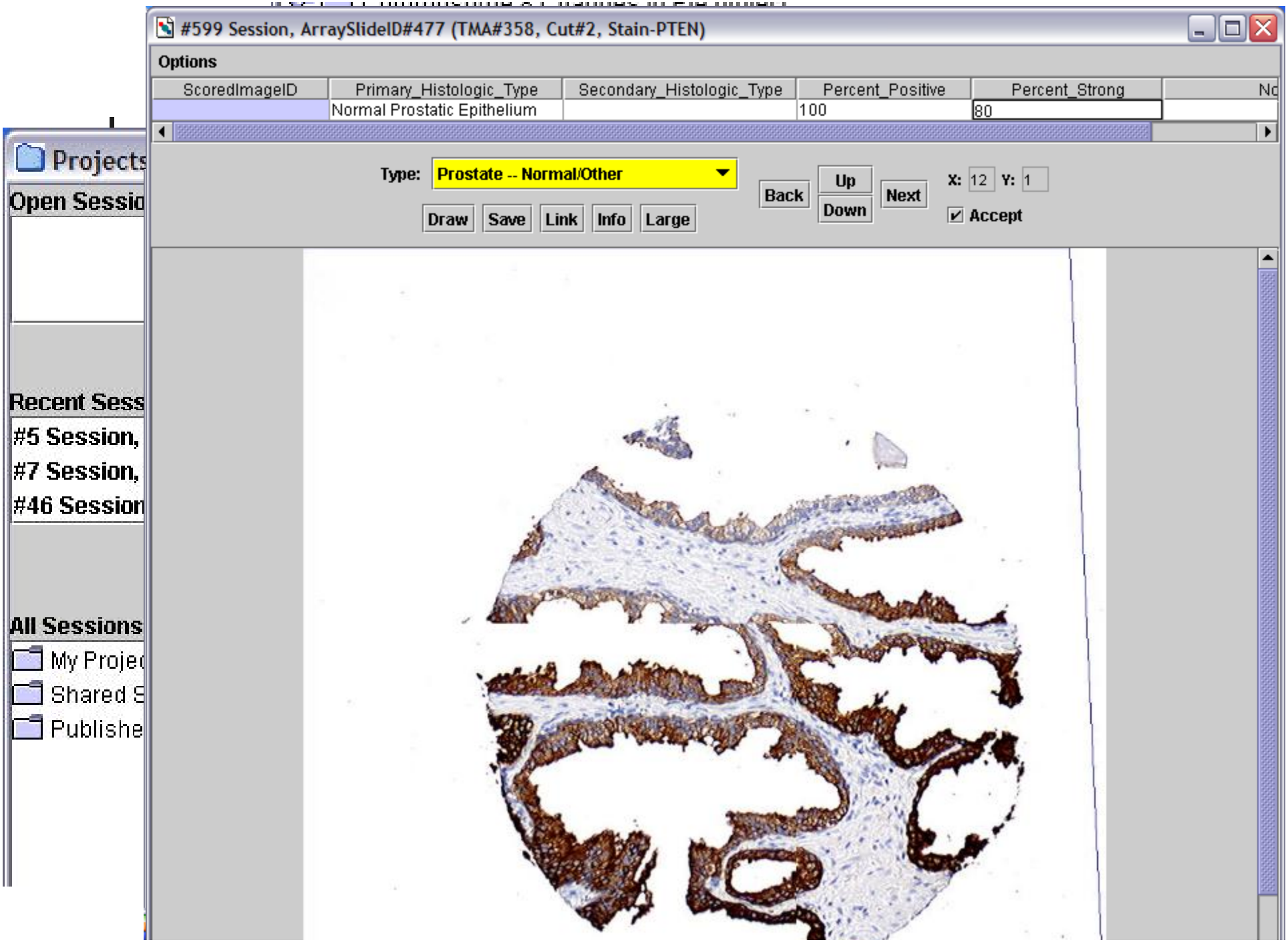

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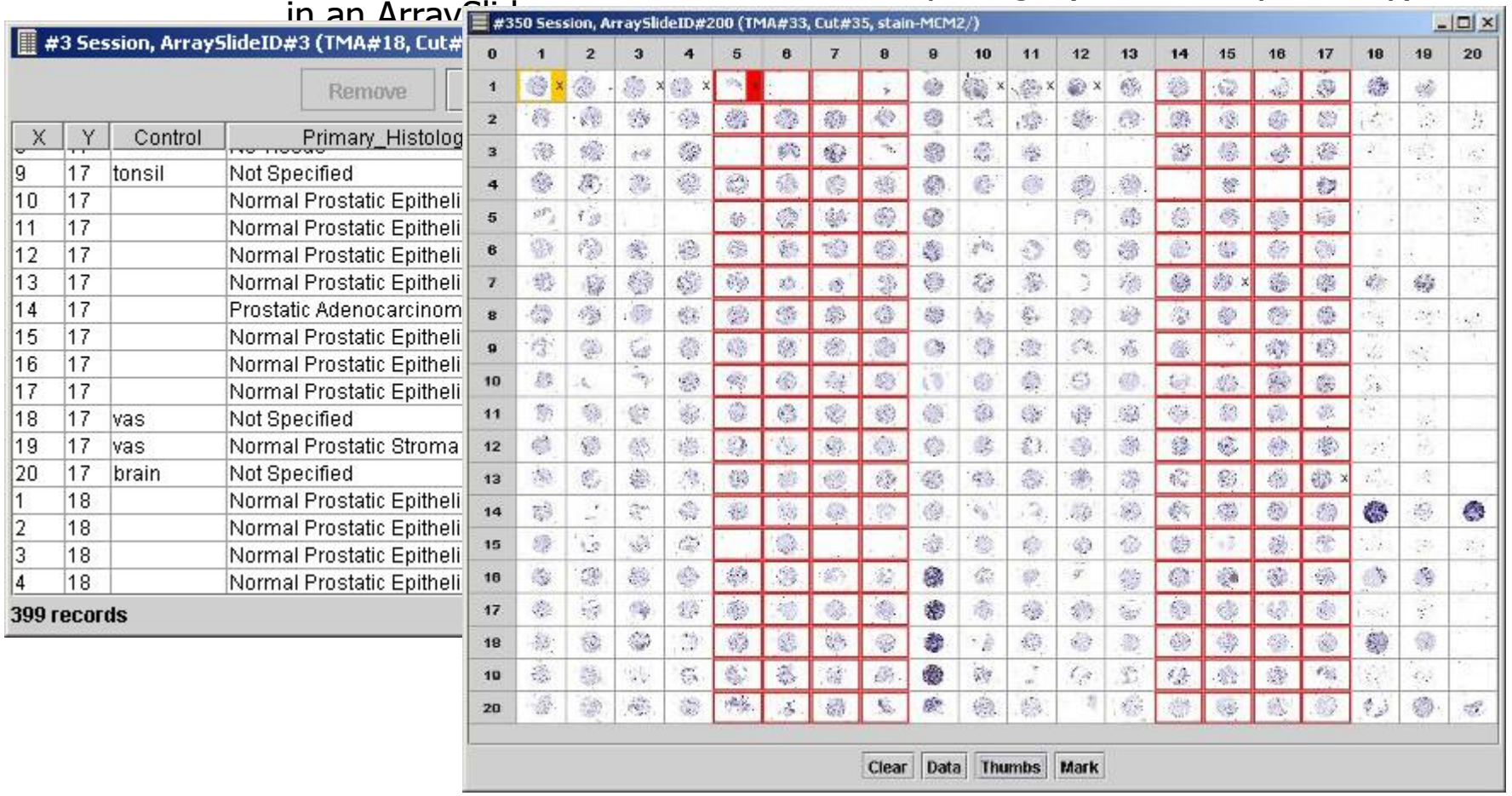
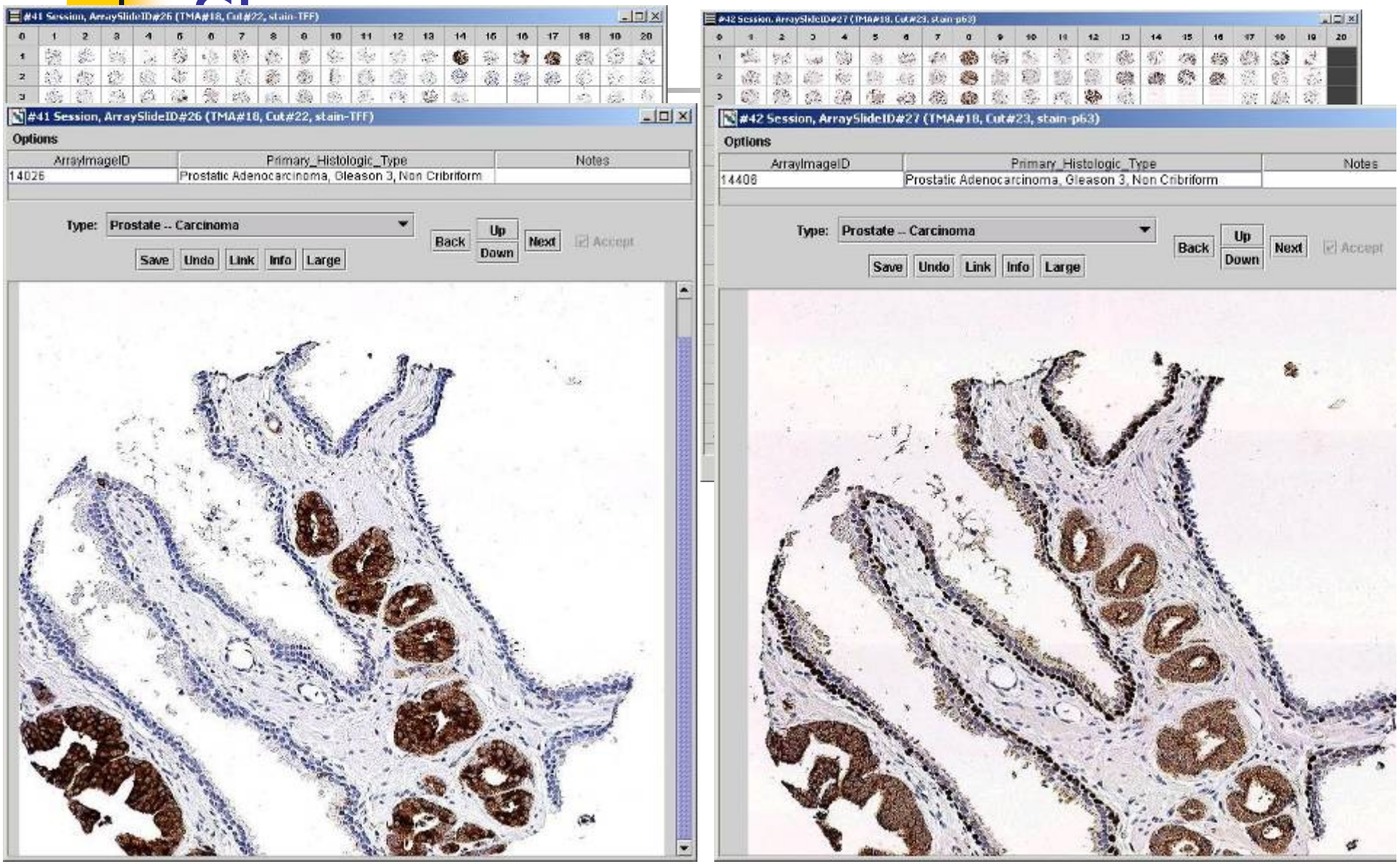
Image Application: Filtering

- The table shows information about every image (identified by x and y) in an ArraySlide



Images Application: Viewing 2 Stains



Publishing TMA Images and Scoring Data Over the Internet

- Roughly modeled after Stanford Microarray Database
- Concept:
  - Once a study is published by a journal, all TMA diagnoses, image, scoring and non-protected clinical data can be "published" as supplemental data to the Internet for public online viewing or down loading
  - TMAJ Images now linked to "Proteinpedia" database

  (http://humanproteinpedia.org) by Akhilesh Pandy, MD PhD.

For More Information

- http://tmaj.pathology.jhmi.edu
- To see published images
  - login to tmaj as a guest and then click the Images button.
    - Username: guest
    - Password: guest

Institutions Using TMAJ

- Johns Hopkins University
- Harvard Dana Farber Cancer Institute
- Cleveland Clinic
- University of Texas Southwestern
- Vanderbilt University