

Microdissection Technology and Challenges

Part 1: Laser Microdissection and the LCM Core Facility
Jaime Rodriguez-Canales, MD

Part 2: Challenges: Downstream Analysis from LCM
Samples
Jeffrey Hanson, MS

Center for Cancer Research
Laboratory of Pathology



Microdissection Core Facility,
Laboratory of Pathology, NCI

NATIONAL
CANCER
INSTITUTE

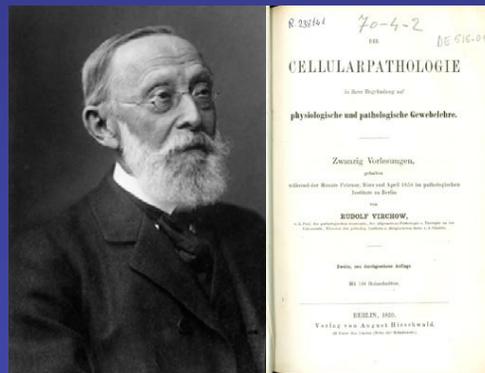
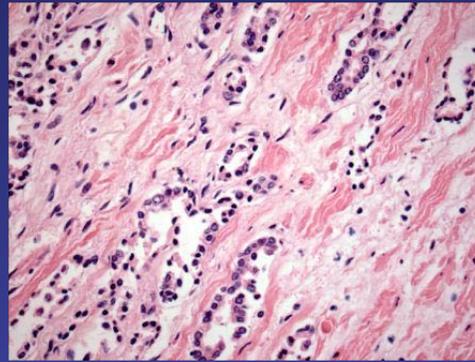
A New Biomedical Revolution

Anatomic level



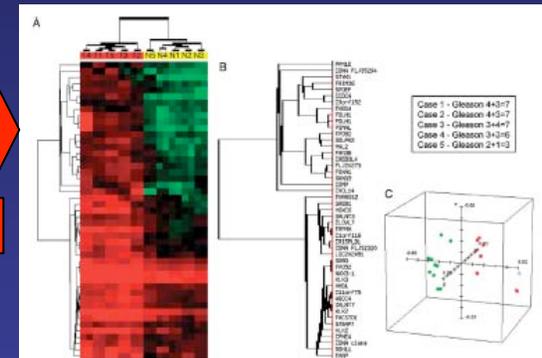
Morgagni
(1761)

Histologic and Cellular level



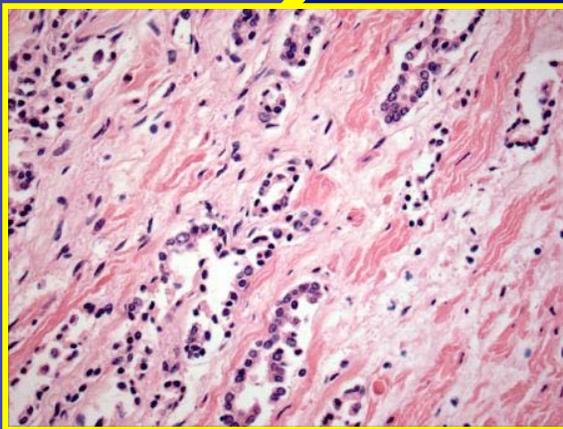
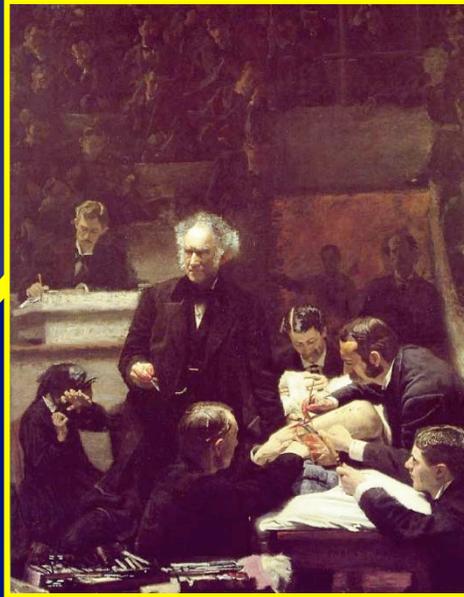
Virchow
(1858)

Molecular level

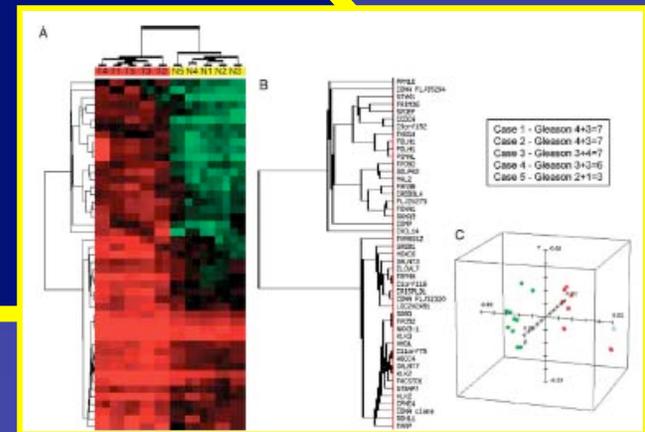


Multidisciplinary Team
Effort

The Challenge for a New Pathology: Integrating the Clinical, Morphologic and Molecular Dimensions of Disease



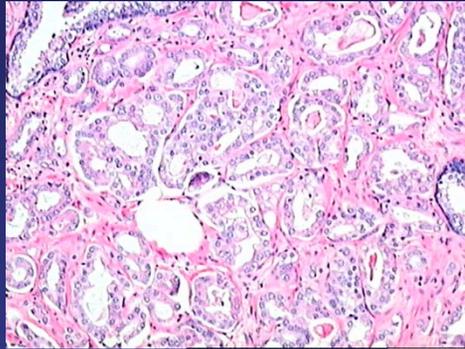
Pathology:
• Clinical Diagnosis
• Pathogenesis



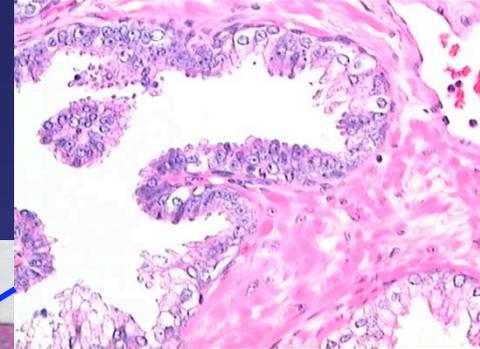
Translational Research, From Molecular Biology to Pathology & Medicine: Value of Tissue Specimens

- Tissues represent morphological basis of disease
- Discovery & characterization of molecular mechanisms of disease
- Validation of new potential biomarkers (from *in-vitro* to *in-vivo*)
- Valuable source stored in Pathology archives and bio-repositories; clinical records
- Challenges: Collection of samples, ethical & legal issues, processing (integrity of biomolecules), complexity

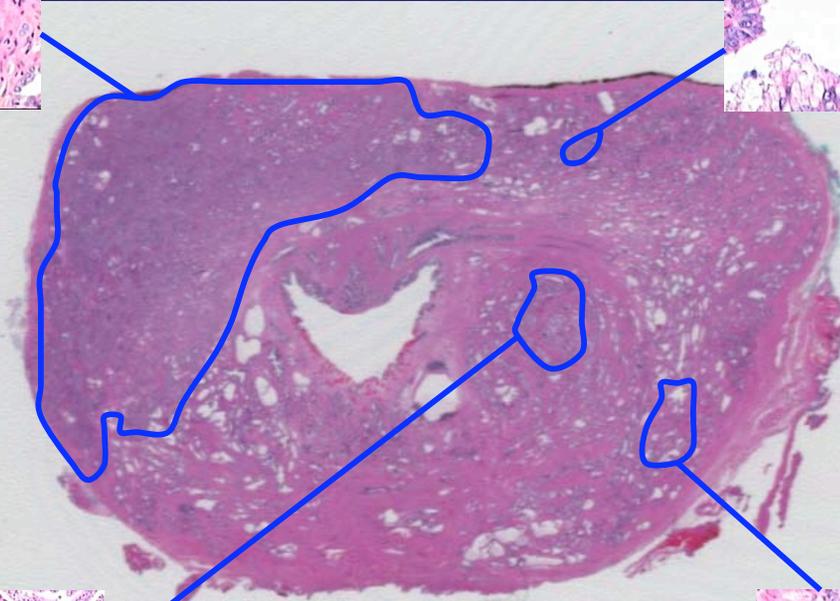
...tissues are complex



Carcinoma

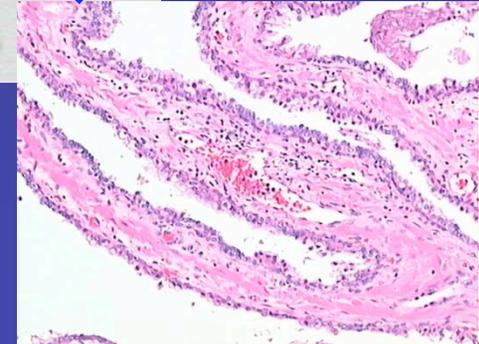
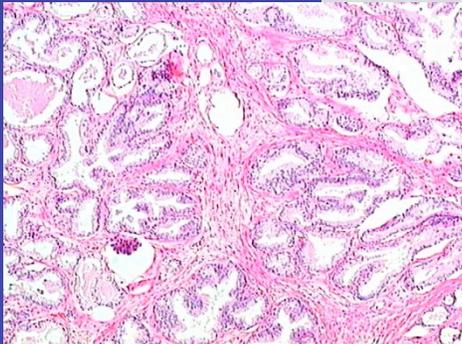


Prostatic
Intraepithelial
Neoplasia (PIN)

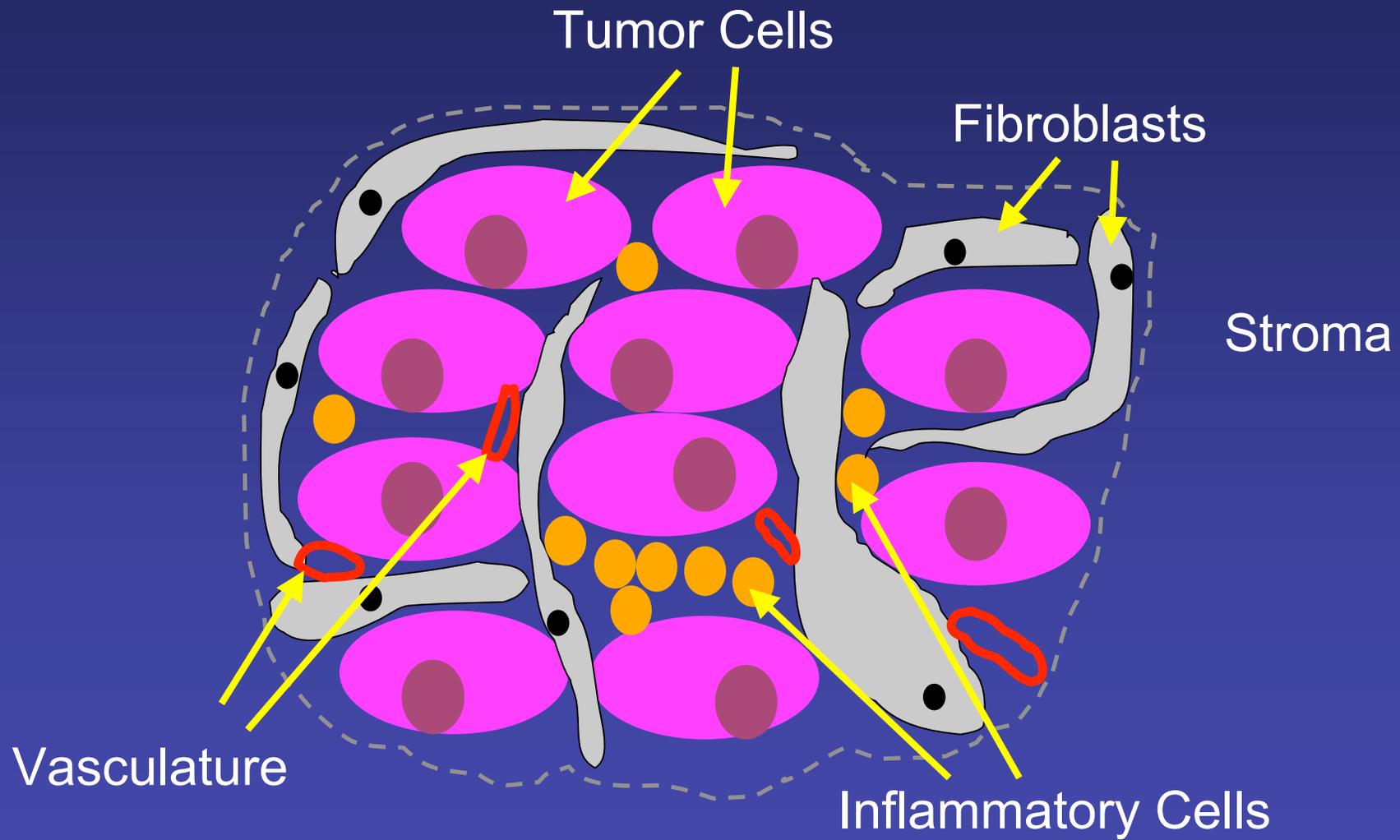


Hyperplasia

Normal



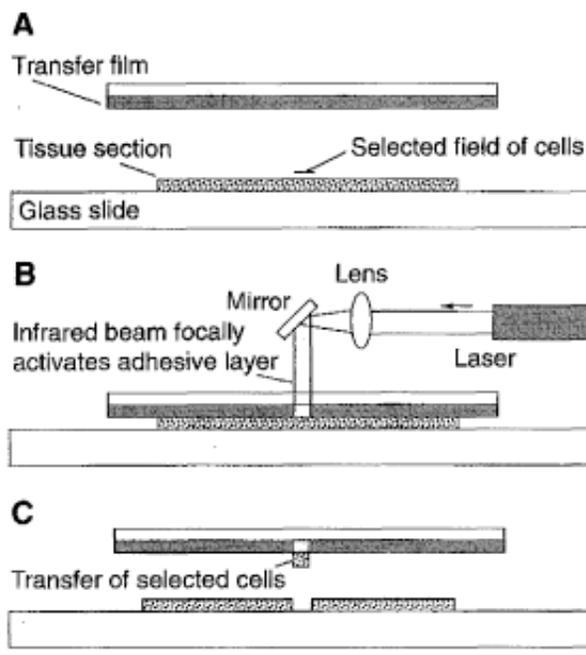
Tumor microenvironment contains diverse cell subpopulations



Laser Capture Microdissection

Michael R. Emmert-Buck, Robert F. Bonner, Paul D. Smith,
Rodrigo F. Chuaqui, Zhengping Zhuang, Seth R. Goldstein,
Rhonda A. Weiss, Lance A. Liotta*

Laser capture microdissection (LCM) under direct microscopic visualization permits rapid one-step procurement of selected human cell populations from a section of complex, heterogeneous tissue. In this technique, a transparent thermoplastic film (ethylene vinyl acetate polymer) is applied to the surface of the tissue section on a standard glass histopathology slide; a carbon dioxide laser pulse then specifically activates the film above the cells of interest. Strong focal adhesion allows selective procurement of the targeted cells. Multiple examples of LCM transfer and tissue analysis, including polymerase chain reaction amplification of DNA and RNA, and enzyme recovery from transferred tissue are demonstrated.



Whole tissue analysis versus pure cell populations: Impact on Molecular Profiling Studies

RESEARCH PAPER

Contaminating cells alter gene signatures in whole organ versus laser capture microdissected tumors: a comparison of experimental breast cancers and their lymph node metastases

Joshua Chuck Harrell · Wendy W. Dye ·
Djuana M. E. Harvell · Carol A. Sartorius ·
Kathryn B. Horwitz

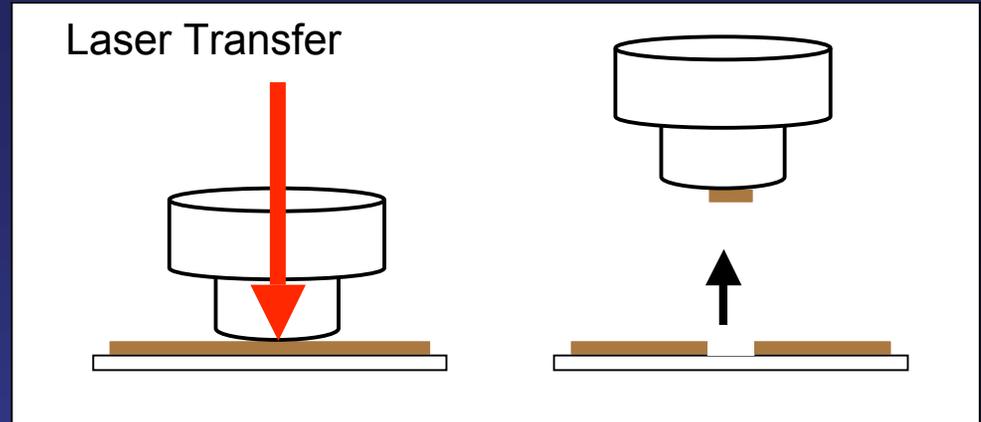
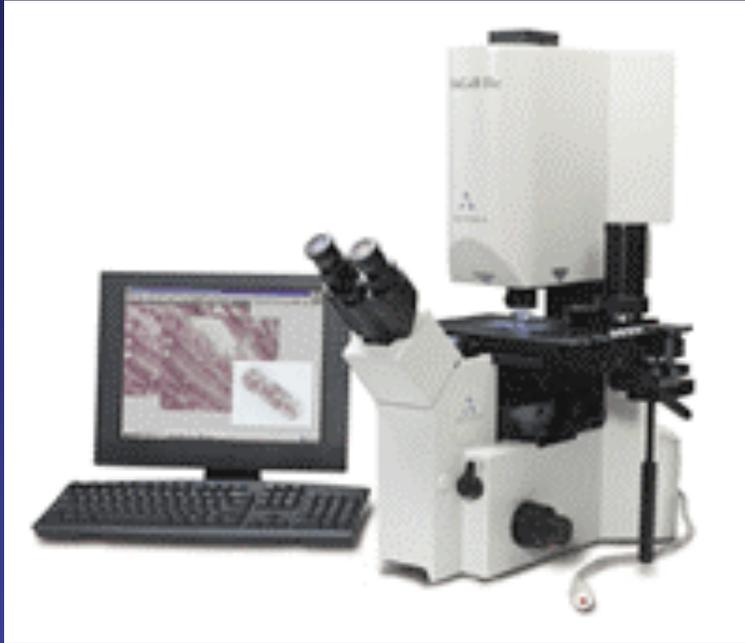
Clin Exp Metastasis. 2008;25(1):81-8

- Gene expression study of breast cancer metastasis in lymph nodes (Affymetrix, 47,000 transcripts)
- RNA recovered via LCM versus whole tissue
- Less than 1% of the gene expression changes were common to both methods in the comparison of primary tumor and metastasis
- Whole organ and LCM based gene profiling yield distinctly different lists of metastasis-promoting genes

Infrared and UV Laser Based Systems

- Infrared Laser:
 - Original LCM design
 - IR Laser = Capture (transfer cells to a thermoplastic polymer from which molecules are extracted)
 - IR laser does not damage tissues
- UV Laser Systems:
 - UV laser = cutting microdissection
 - Cells are extracted from a receptacle or membrane
 - Some systems allows closed and contamination free analysis
 - UV laser could damage molecules in the cut perimeter, particularly important in very small samples

Infrared-Laser Microdissection: LCM



Before LCM

After LCM

LCM Film



**Laser Capture Microdissection
(Arcturus/MDS, Inc):**

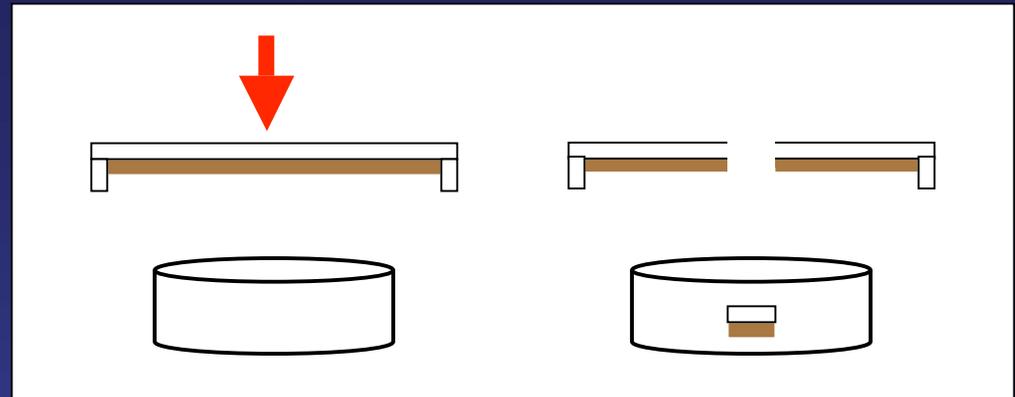
- PixCell II, AutoPix
- Veritas
- Arcturus XT

UV Laser Microdissection Systems



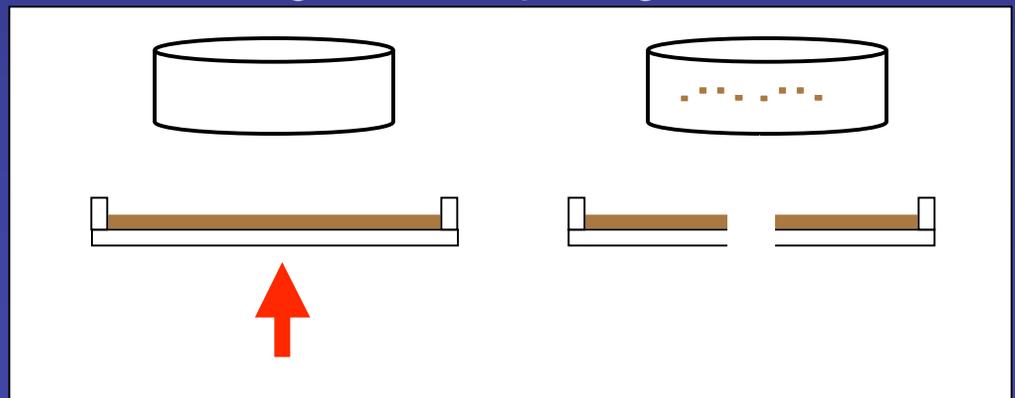
Laser Microdissection
Leica

Laser Cutting and Gravity Deposition

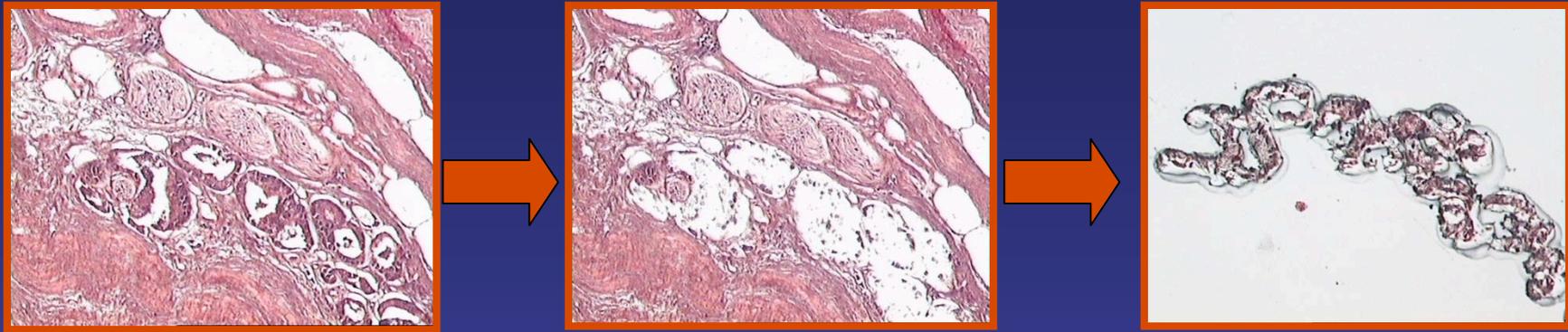


Laser Microdissection and Pressure
Catapulting (P.A.L.M.)

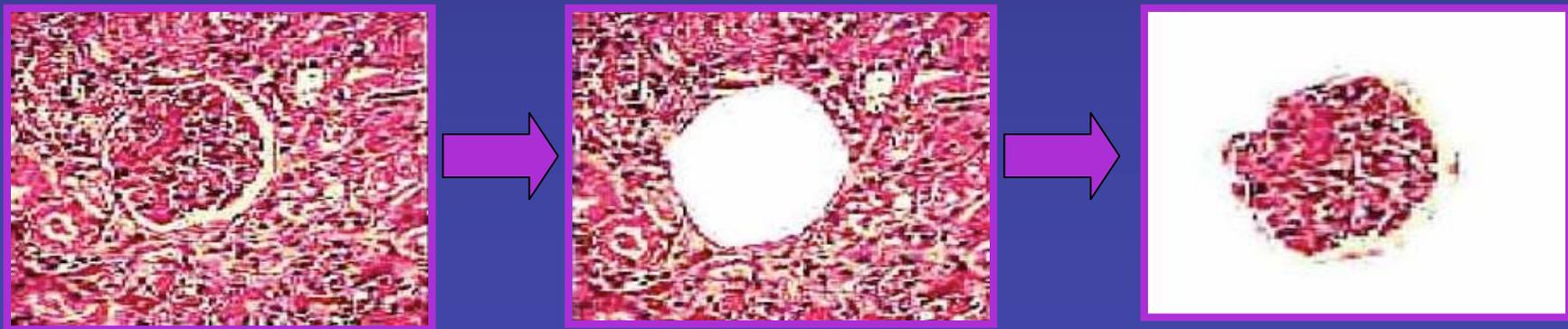
Laser Cutting and Catapulting



Capture & Cutting Microdissection



Infrared Laser captures the target cells into a film (cap) "shot by shot"



UV Laser cut around the target cells: good for dissect areas and around individual cells (carefully!)

Main types of Laser Microdissection:

- **Infrared-Laser based systems** (Laser Capture Microdissection, Arcturus/MDS Inc.)
 - PixCell II, AutoPix
 - Veritas (combined IR & UV laser)
 - Arcturus XT (combined IR & UV Laser)
- **Ultraviolet-Laser based systems:**
 - Laser Microdissection (LMD/Leica)
 - Cut and Catapulting System (PALM/Zeiss)
 - mmi-CellCut (MMI AG)
 - Veritas & Arcturus XT (combined IR & UV) (MDS Inc)
- **New types under development:**
 - Expression Microdissection (laser, molecular target based)

Type of dissection	Capture	Cut
Type of Laser	IR Laser	UV Laser
Slide Required	Plain glass slides	Membrane slides
Mechanism	IR laser transfer cells into a thermoplastic polymer	UV laser cut a membrane with tissue/cells, then collected into a cap by gravity, capture or catapulting
Main Advantages	<ul style="list-style-type: none"> •IR laser does not damage tissues. •Good for small and scattered targets (shot by shot) •Work with just plain glass slides 	<ul style="list-style-type: none"> •Fast dissections •Our choice for clusters of cells or big areas of target cells •Able to dissect thick sections (>20um) •Able to dissect large amount of cells •Our choice for proteomic applications
Main Disadvantages	<ul style="list-style-type: none"> •Slower than UV, requires more time for big areas •Require special caps from one source •Not good for dissections of sections thicker than 10um 	<ul style="list-style-type: none"> •Require tissues mounted on special slides •High energy laser could affect biomolecules in very small target dissections (?)

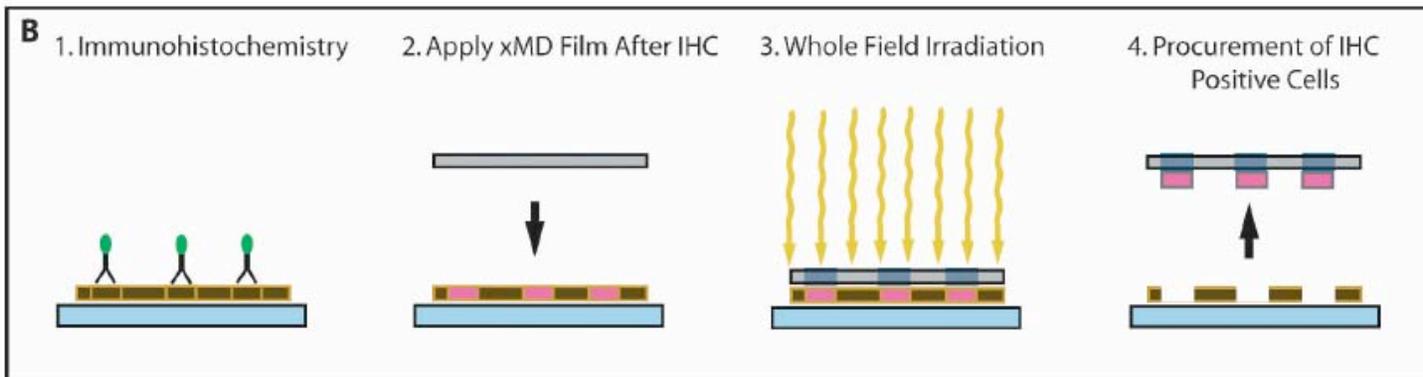
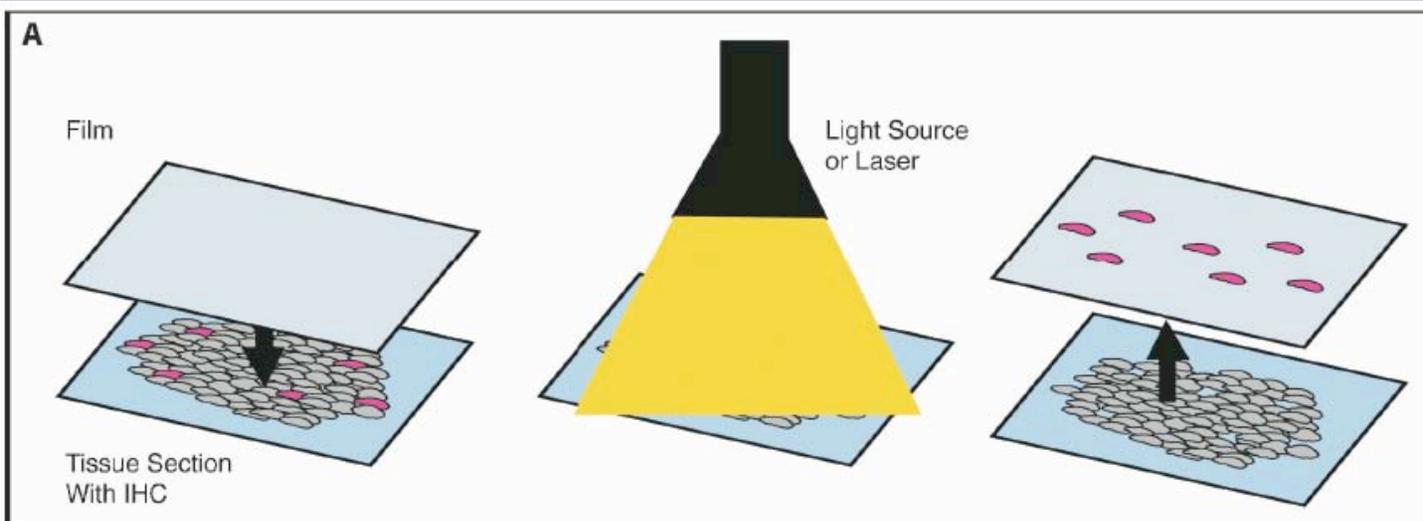
Challenges of Laser Microdissection systems

- All Laser Microdissection systems are microscope-based:
 - Operator-dependent.
 - Histopathology training needed in order to sample the right cells
- Throughput:
 - Procurements of large number of cells for array-based platforms, proteomic analysis is sometimes unfeasible
- Not always adequate for fine targets:
 - Isolated or scattered cells, subcellular microdissection

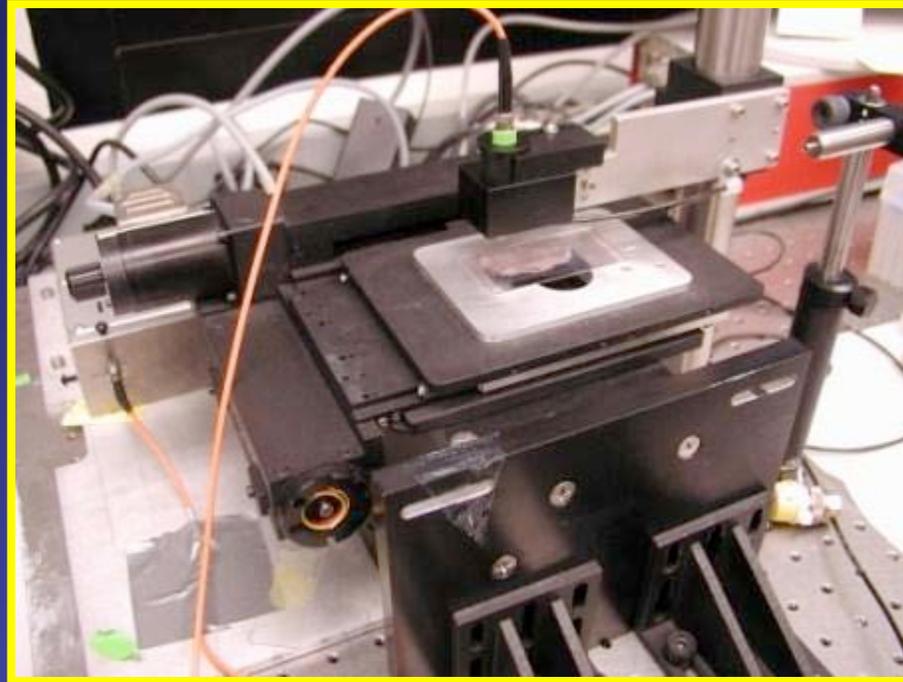
Expression Microdissection

Operator-Independent Retrieval of Cells for Molecular Profiling

Michael A. Tangrea, PhD,* Rodrigo F. Chuaqui, MD,* John W. Gillespie, MD,*†
Mamoun Ahram, PhD,* Gallya Gannot, DMD, PhD,* Benjamin S. Wallis, BA,*
Carolyn J. M. Best, PhD,* W. Marston Linehan, MD,‡ Lance A. Liotta, MD, PhD,§
Thomas J. Pohida, PhD,|| Robert F. Bonner, PhD,¶ and Michael R. Emmert-Buck, MD, PhD*



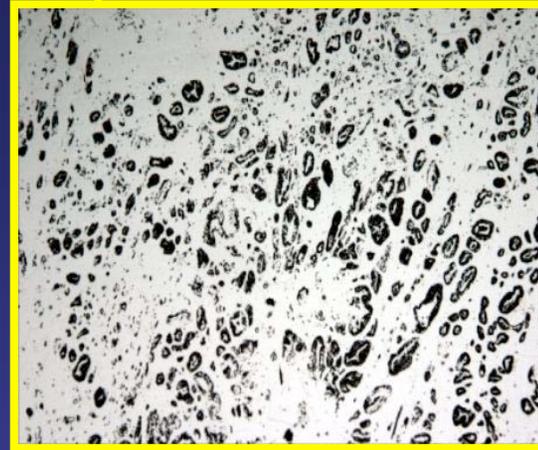
Expression Microdissection (xMD) Prototype



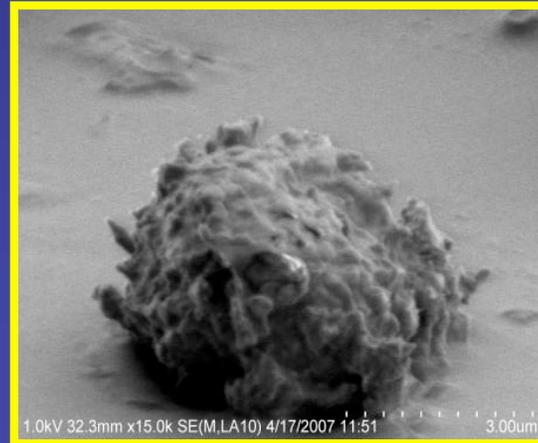
GOALS:

- Automation (High-Throughput Microdissection, ~50,000 cells/sec, prostate)
- Procurement of fine histological targets (~1mm Diameter)
- Removal of variance among users
- Elimination of targeting difficulties due to poor image quality

xMD: High Throughput and Fine Target Examples



Images taken from the xMD film after dissection of normal and tumor prostate immunolabeled with cytokeratins (AE1/AE3): ~50,000 cells per second



Nuclear dissections (histone antibody), image from xMD film under light microscope (left) and SEM (right)

xMD future development

- IHC procedures affects DNA & RNA yield:
 - Optimize IHC protocols for molecular retrieval
 - Increase amount of dissected cells
- Optimization of automatic high-throughput capabilities
 - Improving hardware and software for imaging, target recognition and dissection
- Use of other molecular targets (in-situ hybridization, etc)

Laser Microdissection Applications

- DNA (LOH, SNP, mtDNA, etc)
- Epigenetics (DNA methylation, Histones analysis by reverse phase arrays)
- RNA: RT-PCR, gene expression microarrays (Affymetrix)
- Proteomics: Western Blot, 2D-PAGE, zymograms, reverse phase arrays (current work) Mass Spec (current)

Review Article

Use of laser capture microdissection, cDNA microarrays, and tissue microarrays in advancing our understanding of prostate cancer

Mark A. Rubin*

Departments of Pathology, Urology, and the Comprehensive Cancer and Geriatrics Center of the University of Michigan, Ann Arbor, MI 48109, USA

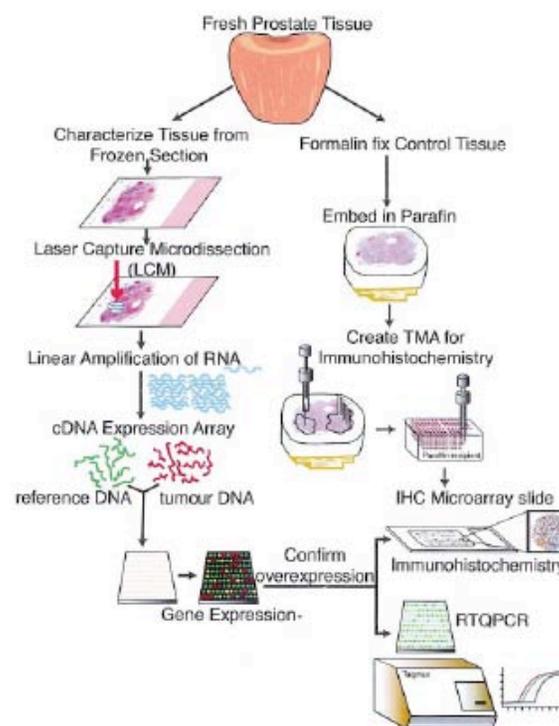
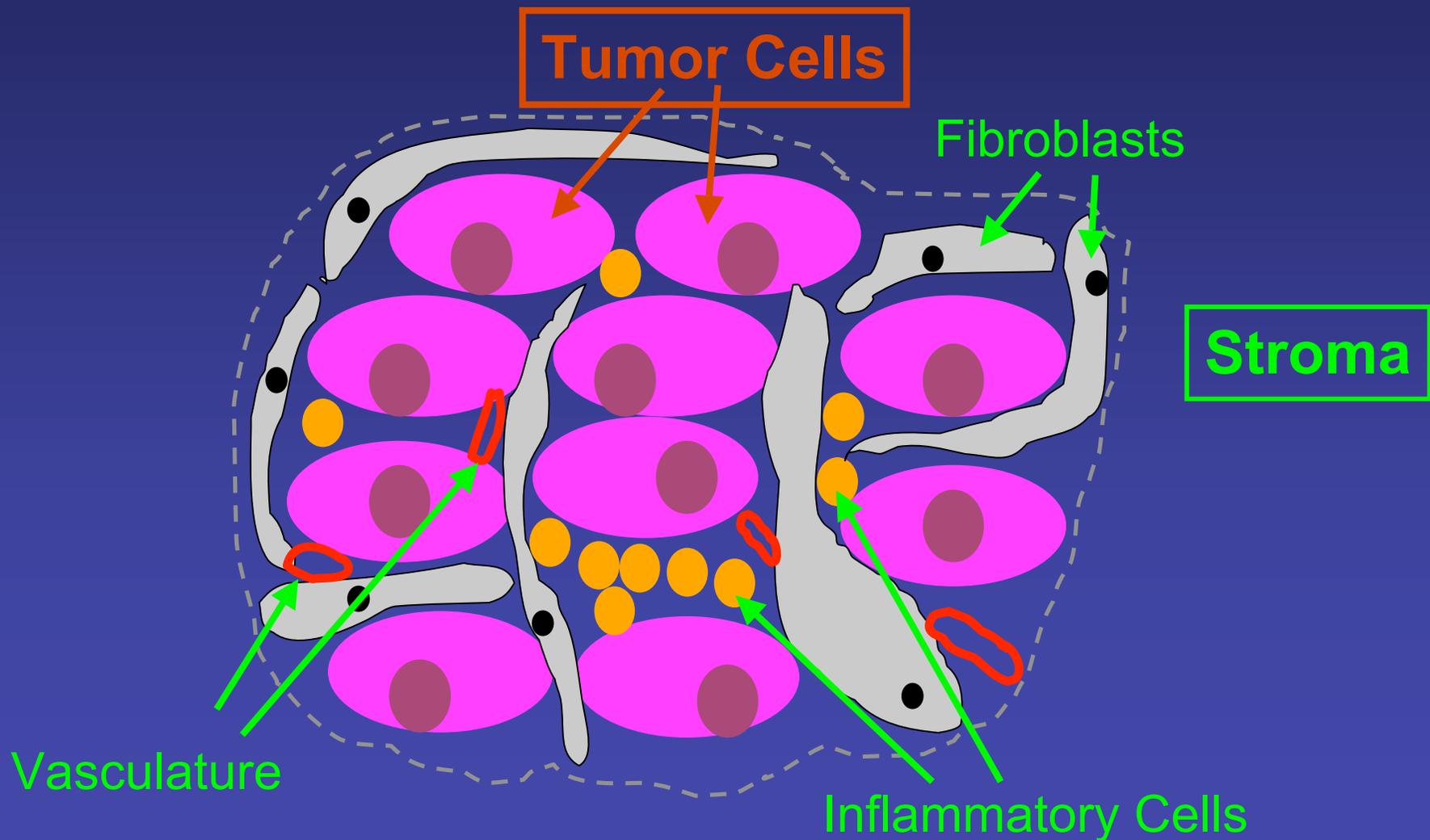


Figure 4. Schematic representation of a high-throughput approach performing precise molecular profiling of prostate cancer. The prostate gland is immediately retrieved from the operating room. After appropriate prostate tissue is taken for pathological tumour staging, frozen tissues samples are acquired for research. Laser capture microdissection is performed and total RNA is extracted and after a linear amplification, is labelled and hybridized on a cDNA microarray. The results are evaluated and important candidate genes are confirmed using quantitative RT-PCR or high-density tissue microarrays with various stages of prostate tumours

Examples of Application of LCM technologies to the study of Tumor Microenvironment in tissue samples

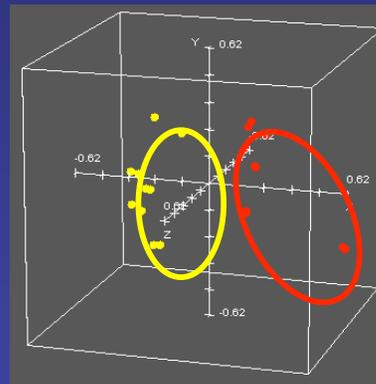


Global Expression Analysis of Prostate Cancer-associated Stroma and Epithelia

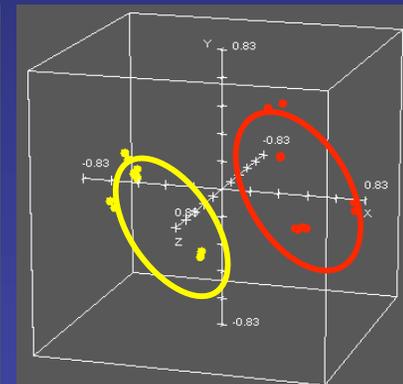
Annely M. Richardson, BS, † Karen Woodson, PhD, ‡ Yonghong Wang, PhD, §
Jaime Rodriguez-Canales, MD,* Heidi S. Erickson, PhD,* Michael A. Tangrea, PhD,*
Kristian Novakovic, MD, || Sergio Gonzalez, MD, ¶ Alfredo Velasco, MD, #
Ernest S. Kawasaki, PhD,** Michael R. Emmert-Buck, MD, PhD,*
Rodrigo F. Chuaqui, MD,* and Audrey Player, PhD***

- 5 frozen prostates specimens with cancer
- LCM Samples:
 - Epithelium & Stroma
 - Normal & Tumor
- RNA extraction
- Linear amplification
- Affymetrix Genechip (54,000 probes)
- qRT-PCR validation

Stroma



Epithelium



■ Normal
■ Tumor

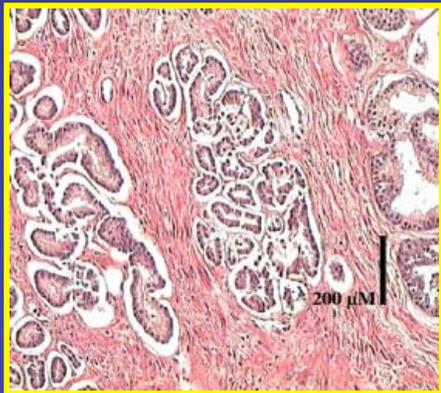
- Tumor-associated stroma shows 44 differentially expressed genes compared with normal stroma
- Some genes are currently under evaluation as potential molecular target.

Gene Promoter Methylation in Prostate Tumor-Associated Stromal Cells

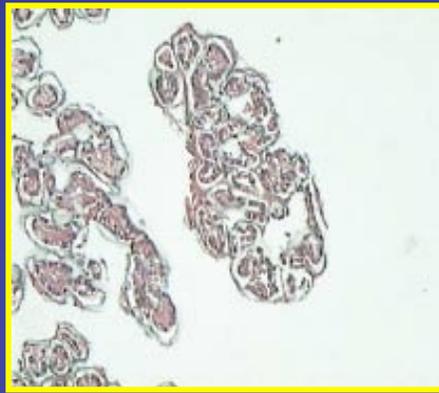
Jeffrey A. Hanson, John W. Gillespie, Amelia Grover, Michael A. Tangrea, Rodrigo F. Chuaqui, Michael R. Emmert-Buck, Joseph A. Tangrea, Stephen K. Libutti, W. Marston Linehan, Karen G. Woodson

J Natl Cancer Inst 2006;98:255 – 61

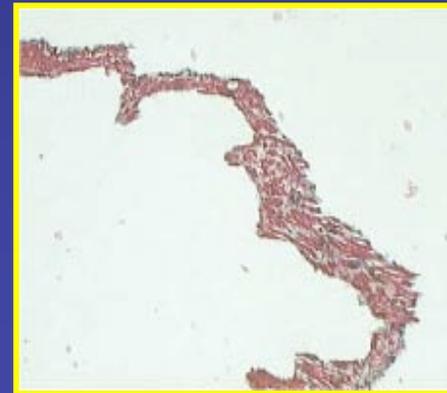
- LCM and xMD applied for microdissection of epithelial cells (keratins) and stroma cells (vimentin)
- Several genes (*GSTP1*, *RARB2*) show promoter hypermethylation not only in the carcinoma cells but also the stroma cells



Tissue Section:
Tumor Area



Tumor Epithelium
LCM sample



Tumor-associated Stroma
LCM sample

The genetics of cancer—a 3D model

Kristina A. Cole^{1,2}, David B. Krizman³ & Michael R. Emmert-Buck^{1,3}

¹Pathogenetics Unit, Laboratory of Pathology, National Cancer Institute, Bethesda, Maryland 20892, USA. ²Department of Pathology, University of Maryland School of Medicine, Baltimore, Maryland 21201, USA. ³Cancer Genome Anatomy Project, Office of the Director, National Cancer Institute, Bethesda, Maryland 20892, USA. e-mail: mbuck@helix.nih.gov

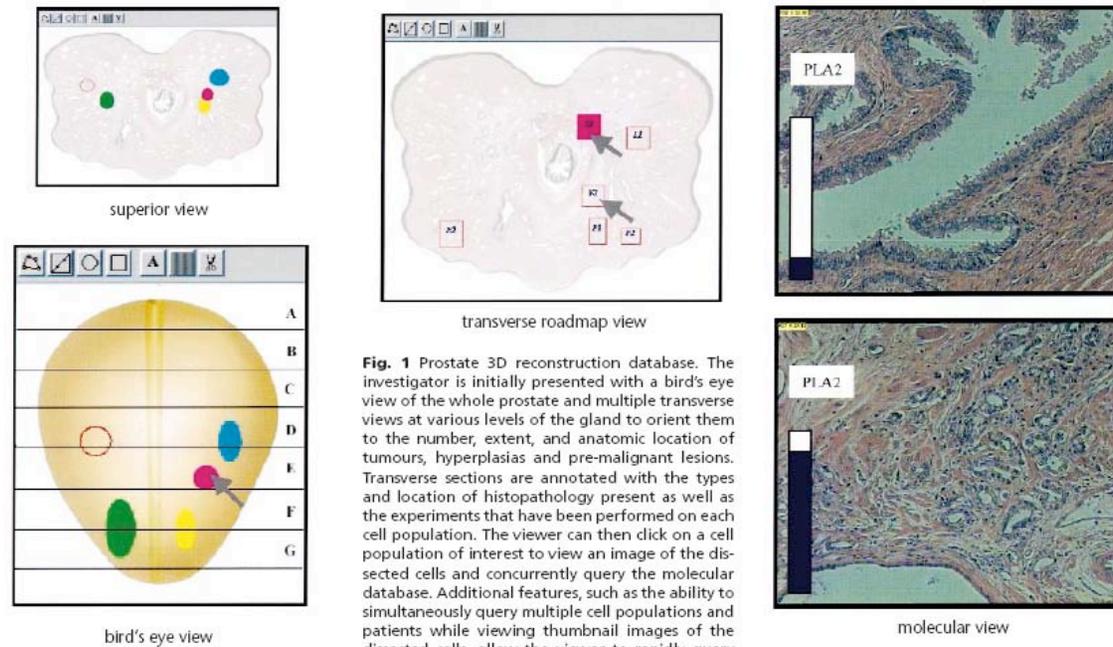


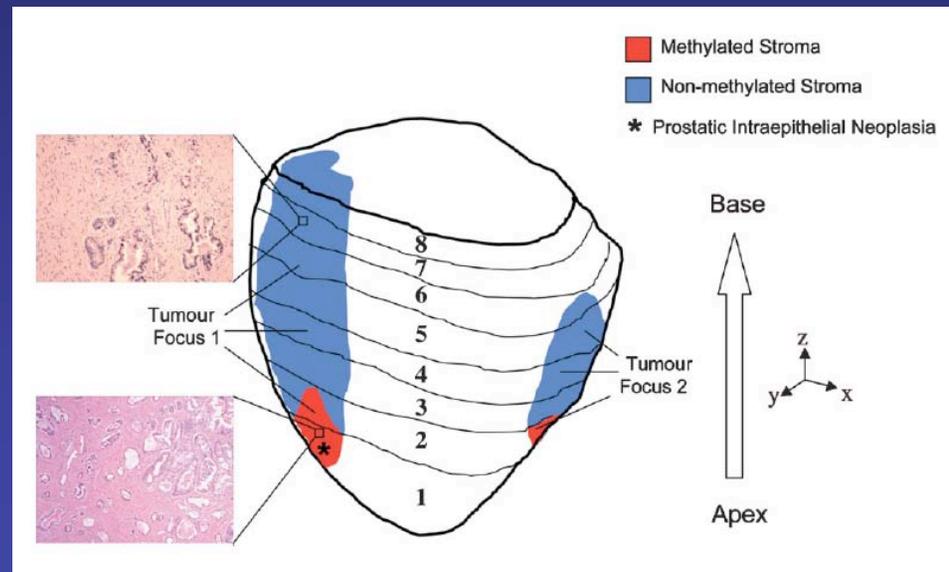
Fig. 1 Prostate 3D reconstruction database. The investigator is initially presented with a bird's eye view of the whole prostate and multiple transverse views at various levels of the gland to orient them to the number, extent, and anatomic location of tumours, hyperplasias and pre-malignant lesions. Transverse sections are annotated with the types and location of histopathology present as well as the experiments that have been performed on each cell population. The viewer can then click on a cell population of interest to view an image of the dissected cells and concurrently query the molecular database. Additional features, such as the ability to simultaneously query multiple cell populations and patients while viewing thumbnail images of the dissected cells, allow the viewer to rapidly query gene expression profiles across a spectrum of samples and/or patients.

Original Paper

Identification of a unique epigenetic sub-microenvironment in prostate cancer[†]

J Rodriguez-Canales,¹ JC Hanson,² MA Tangrea,¹ HS Erickson,¹ PS Albert,³ BS Wallis,¹ AM Richardson,¹ PA Pinto,⁴ WM Linehan,⁴ JW Gillespie,^{4,5} MJ Merino,⁶ SK Libutti,⁷ KG Woodson,² MR Emmert-Buck^{1*} and RF Chuaqui¹

- A prostate specimen with cancer
- Whole mount sections from apex to base
- LCM mapping: epithelium and stroma (normal and tumor) from apex to base
- DNA extraction, methylation analysis of *GSTP1* promoter (pyrosequencing)
- 3D reconstruction and molecular-histopathology integration



- Tumor epithelium is hypermethylated from apex to base.
- Tumor-associated stroma is hypermethylated at the apical pole of prostate cancer.

LCM-based Tumor microenvironment studies

- Application of laser microdissection technology and 3D approach allowed us to find of a distinctive pattern of molecular alterations in the stroma associated to the cancer cells in tissue samples
- Biological meaning still unclear: paracrine cross-talking, epithelial-mesenchymal transition?
- Cancer is a disease of the tissue as a whole (microenvironment) and not a disease of just the “tumor” cell population
- Currently we are doing a follow up study of 3D prostate mapping with 4-5 new cases

Laser Capture Microdissection Core Facility

- Location: Bldg 10, Room B1B37
- Freely open to NIH research community
- Availability of LCM instruments, cryostat and laboratory set up for molecular extraction
- Assistance:
 - Laser Microdissection techniques
 - Tissue handling (cryostat, staining, -80 freezer space for temporary storage of samples)
 - Molecular analysis from LCM samples
 - Pathology consultation for LCM and research

LCM Core Facility: Currently Available Instruments



**PixCell II (4)
LCM system (IR Laser)**



**Veritas, MDS (1)
(IR & UV Laser, Automatic)**



**Arcturus XT, MDS (1)
(IR & UV Laser, Automatic)
(On Order)**



Leica Cryostat CM1850UV



**Pathology consultation and slide review
(1 upright microscope; Future: double head
microscope with imaging system)**

Selected Collaborations

- Pathogenetics Unit: follow up of Prostate Cancer 3D mapping project (epigenetic changes in tumor stroma)
- Lab of Medical Biophysics & CIT: xMD applications & development
- Genetics Branch and Proteomics core (Lab of Pathology): Epigenetics Profiling of Prostate Cancer using xMD sampling, DNA methylation array (Keith Killian) and histone reverse phase array (Chris Devor and Dr Levens)
- Genetics Branch (Dr Konrad Huppi), siRNA expression in prostate cancer and tumor microenvironment study
- LCM, pathology and molecular assistance for 50 users in the last year from diverse NIH institutes

Future Directions LCM core

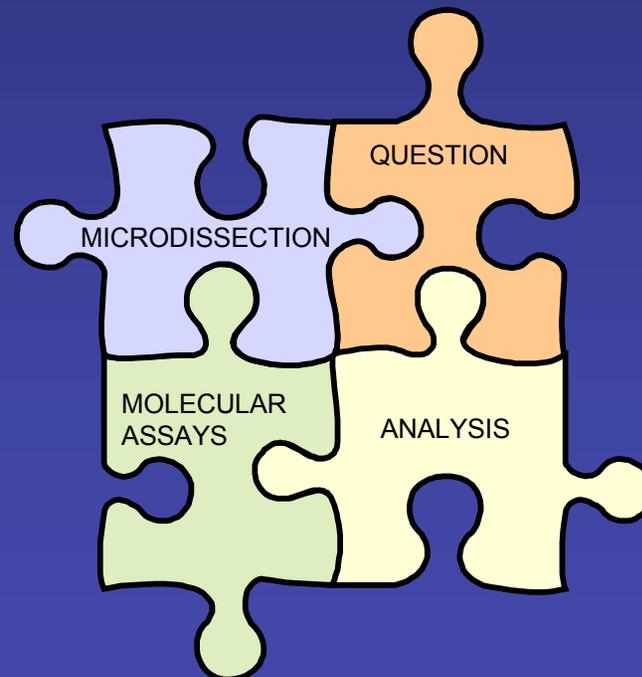
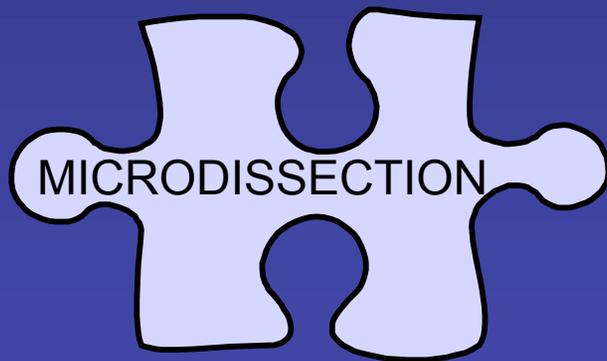
- Expand collaborations:
 - Laboratory of Pathology: research, resident rotations
 - Molecular Profiling Core: LCM, research on downstream analysis from LCM samples
 - OBBR: research on tissue preservation and molecule extraction from LCM samples
- Setting up a more functional website (microdissection.nih.gov) including online schedule and registration, protocols, follow-up & feed-back
- We would like to offer complete evaluation of biomolecules recovery from dissected samples

LCM Core Future Directions: New Challenges

- Improve methods for working with dissected tissues.
- Push the limits of the technology.
- Incorporate other technologies in conjunction with microdissection.
- Laser microdissection and molecular analysis technology development

Challenges and Goals

- Well Defined and Realistic
- Project design and downstream analysis will depend on the goals
- Remember that LCM is a tool to accomplish a goal



Challenges:

- Downstream Analysis
- Target Cells
- Biomolecules
 - Quantity
 - Quality

Downstream Analysis

- What do we need for our assay?
- The honest answer is: It Depends

TABLE 1 | Recommended cellular yields from microdissected tissue for downstream analyses.

Molecule	Methodology/assay	Cellular yield/area of microdissection
DNA	Loss of heterozygosity	100–1,000 cells
DNA	Imprinting/DNA methylation	200 cells
gDNA	Genetic mosaic analysis	2,000 cells
RNA	cDNA library construction	25,000 cells (93 ng total RNA)
		5,000 cells (14.7–18.6 ng total RNA)
RNA	Gene-expression arrays	100 cells from FFPE
RNA	Real-time RT-PCR	1,400 cells
		0.8–1.0 × 10 ⁶ μm ²
		200 and 1,000 cells
		22,000 cells/37.5 ng RNA
		10,000 cells/40 ng RNA from maize
RNA	QRT-PCR	single cell
		100 cells/1 reaction or 2,000 cells/200 μl
Protein	Western blot	4,000–5,000 cells
		500 cells (optimized blotting procedure)
		2,500 cells
Protein	2D gel electrophoresis	8,000–10,000 cells
		50,000–100,000 cells
		3.7 mm ² area
		10,000 cells (100–200 μg in 350 μl)
		20,000–25,000 cells
		50,000 cells
Protein	2D-DIGE	30,000 cells/40 μl
Protein	Molecular profiling: reverse-phase protein microarray	5,000–30,000 cells
Protein	Mass spectrometry: MALDI or LC/MS-MS	50,000–100,000 cells (ICAT and LC/MS)
		10,000–15,000 cells
		25,000 μm ²
		300 microvessels
Protein	Mass spectrometry: SELDI	1,500 cells
		3,000–5,000 cells

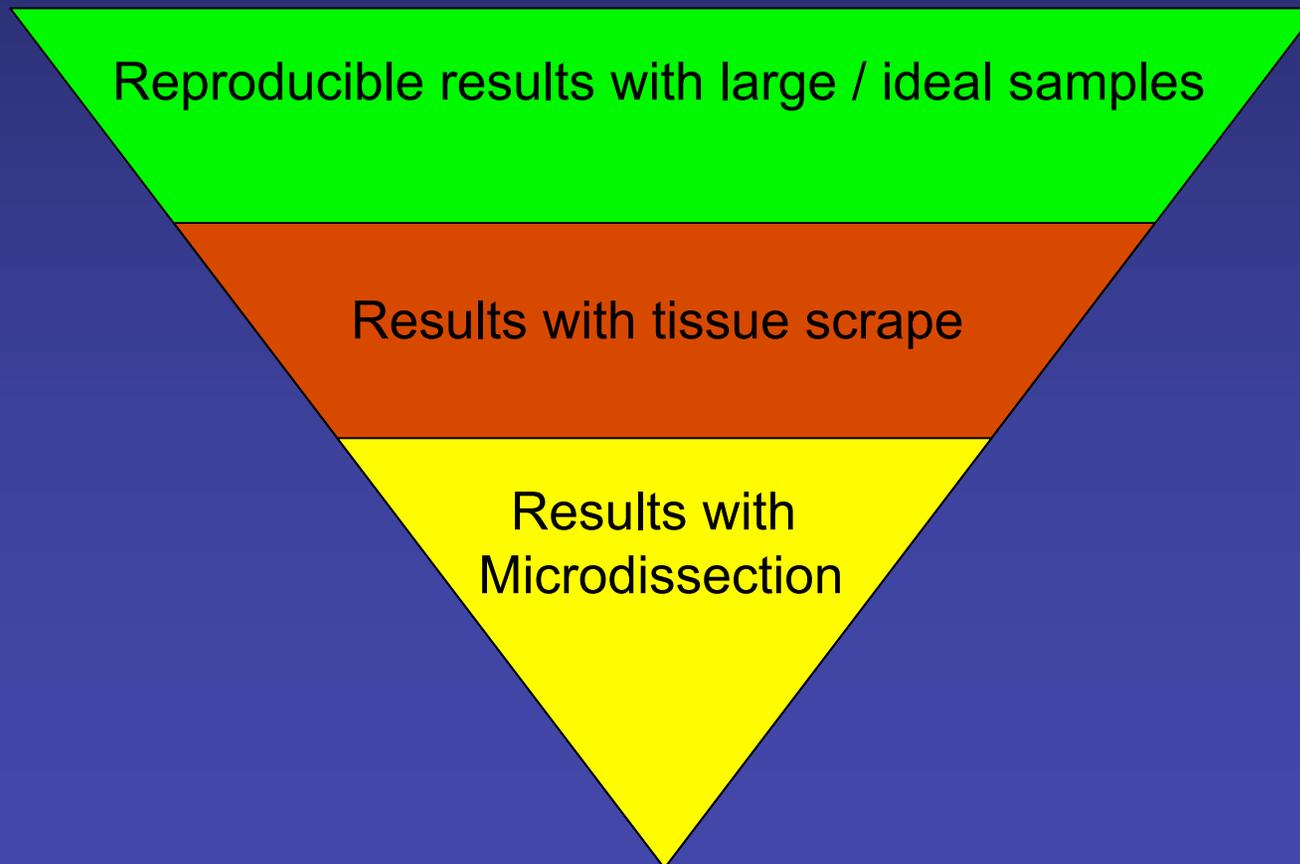
Espina et. al. 2006

Downstream Analysis

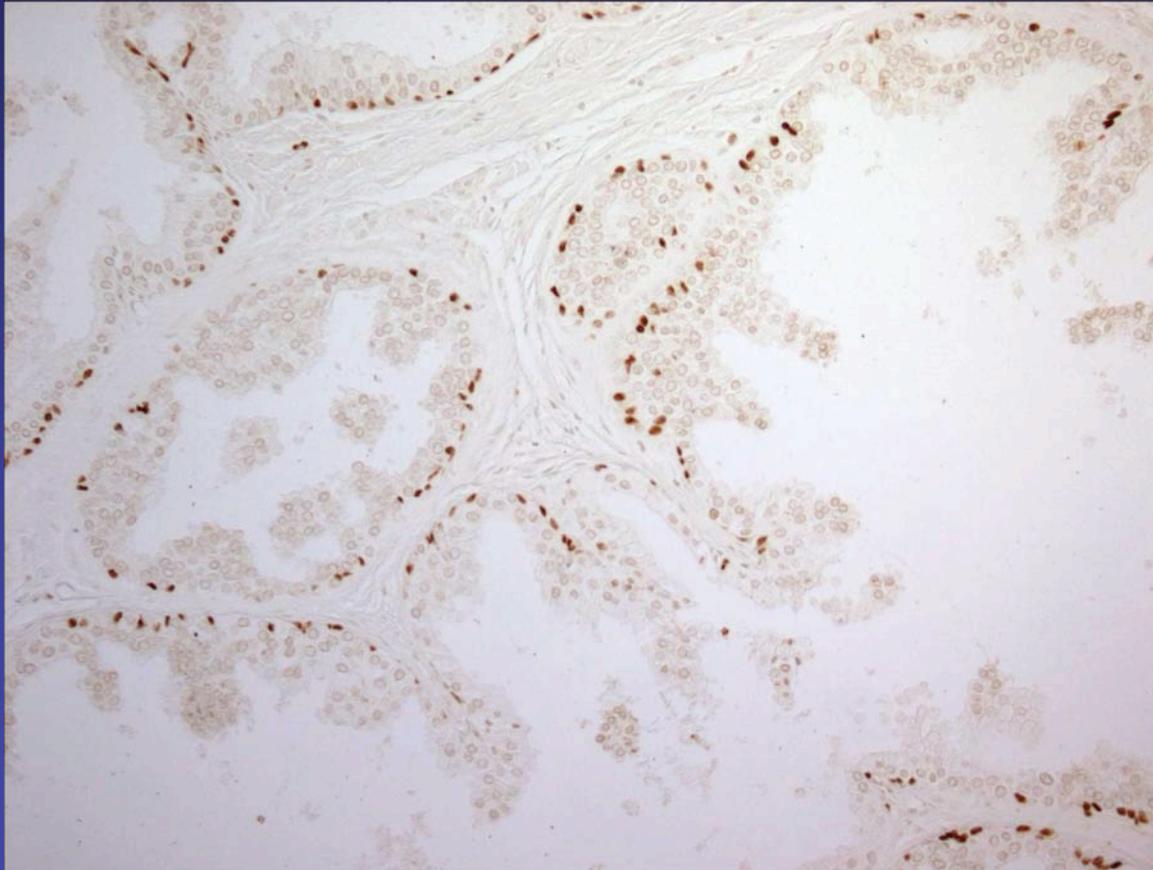
- Replicates
- Effects of processing and sample amount
- Effects of amplification
- Alternatives
 - RNA - Q-PCR vs. Microarray
 - DNA methylation Q-PCR vs. Pyrosequencing assay

Downstream Analysis

Pilot study



Target Cells



Rodriguez-Canales (unpublished)

- How abundant?
- Prostate:
 - Basal cells vs. total epithelium
 - p63 labeled IHC

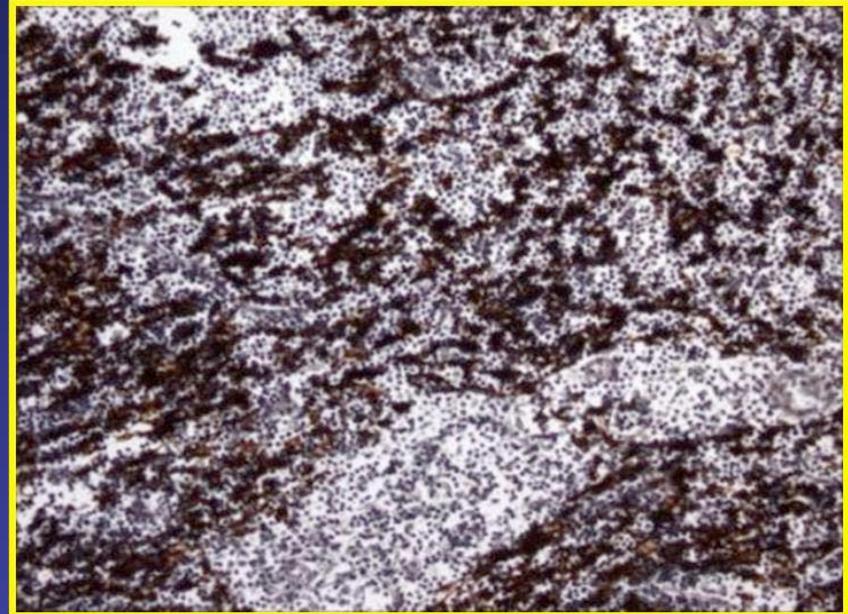
Target Cells



- How abundant?
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Rodriguez-Canales (unpublished)

Target Cells

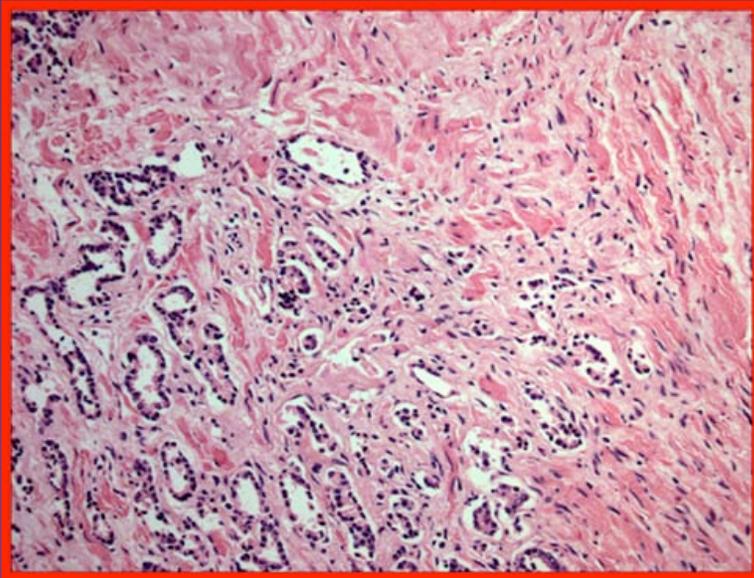


How easy to dissect?

- Complex milieu or is there a distinct area of the tissue
- Dissecting the Epithelial cells from the esophagus is relatively easy (Left, H&E)
- Dissection of thymic epithelial tumor cells from lymphocytes is almost impossible (Right, AE1/AE3 IHC stain)

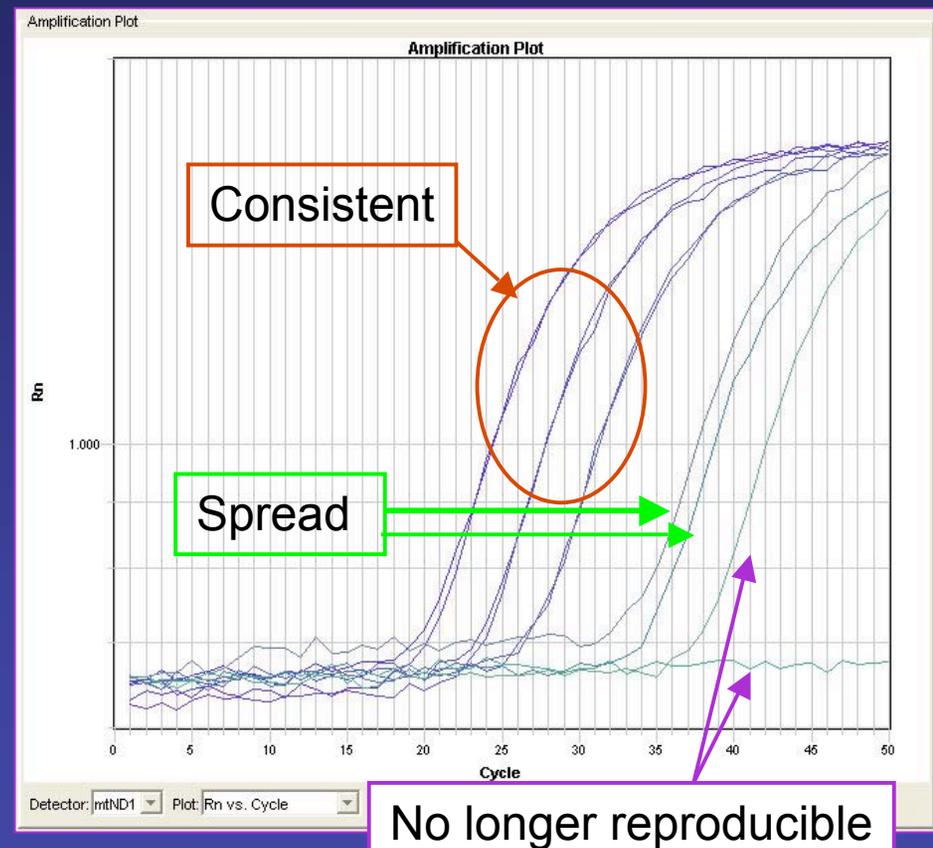
Target Cells

- How to visualize
 - How similar are they to the surrounding contaminating cells
 - Prostate Carcinoma and Tumor associated Stroma
 - Stain choice H&E (left) vs. Cytokeratin IHC (Right) allows the identification of epithelial cells in the stroma and allows for a more pure dissection



Biomolecules: Quantity and Quality

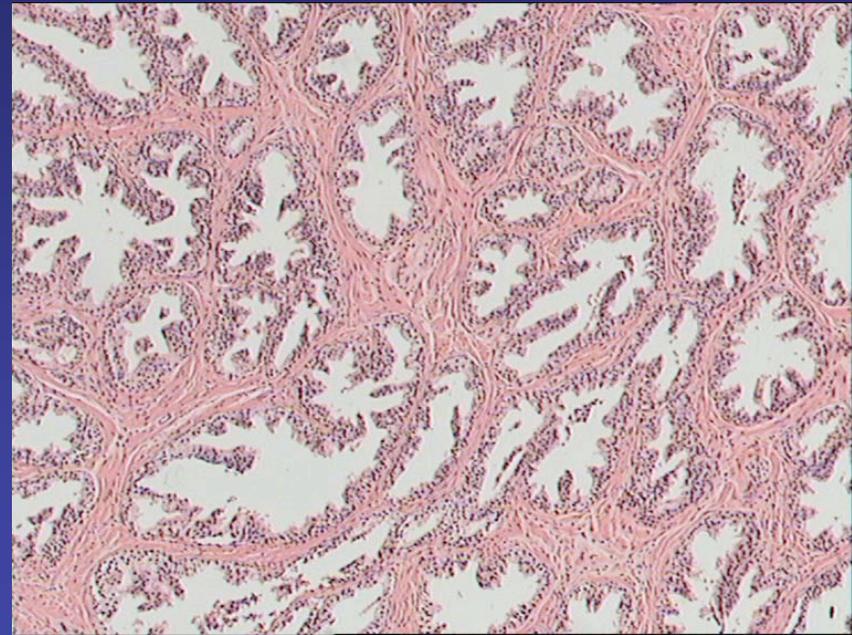
- Minimal amount of biomolecules needed
 - Limit of detection
 - Limit of reproducibility
 - Quantification vs. Detection
 - Image mtD1 qPCR



Biomolecules: Quantity and Quality

Amount of tissue to
obtain using
microdissection

- Area needed
 - Amount of target molecule per cell
 - Number of reactions to be performed
- Time Factors
 - Degradation during dissection (RNA)
 - Microdissection collection time



H & E stain of Prostate

Biomolecules: Quantity and Quality

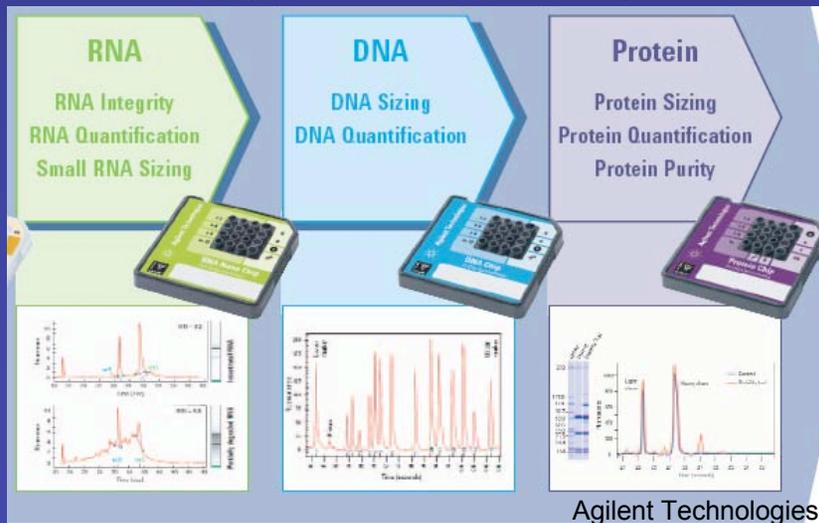
- Storage / Fixation
 - Fresh > Frozen > EFPE > FFPE
- Staining
 - Identification vs. recovery
 - Suboptimal microscope visualization
 - Loss of biomolecules
 - Interference with extraction
 - Interference with molecular assays
- Isolation
 - Purification vs. loss in processing
 - Harshness vs. recovery

Biomolecules: Quantity and Quality

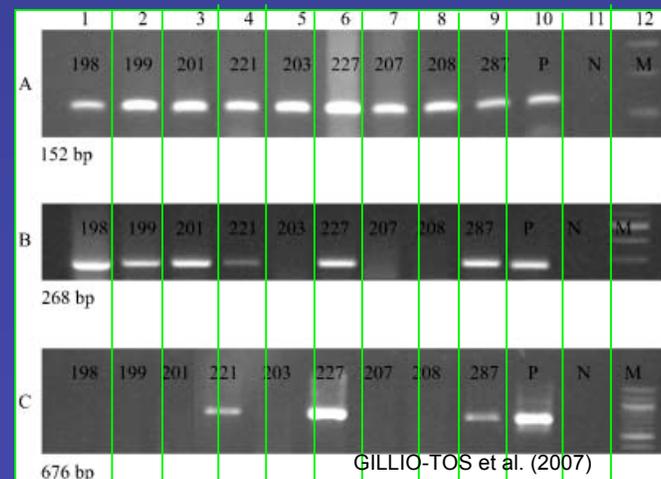
Tests

- Total amount vs. Usable amount
- Suitability of microdissected samples to test
 - Isolation methodologies
 - How much sample is used
 - In study vs. in development

Bioanalyzer: Lab on a chip



β -globin primers for assessment of DNA Length



Project Design Considerations

- Goals
- Pathology evaluation
 - Target cells
 - Tissue Samples
 - Staining
- Downstream analysis
 - Microdissection technique
 - Biomolecules (Quantity and Quality)

Setting up a Project Involving Microdissection

1. Initial meeting
2. Pathology review of the slides/tissues
3. Decision on Microdissection Technique
4. Test of Feasibility
5. Perform Study

The earlier in the study planning that microdissection is evaluated the greater the chance for it's successful incorporation

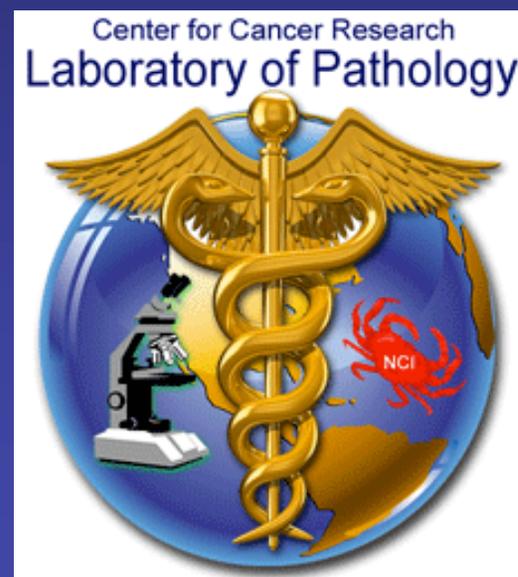
Laser Microdissection Core Facility

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