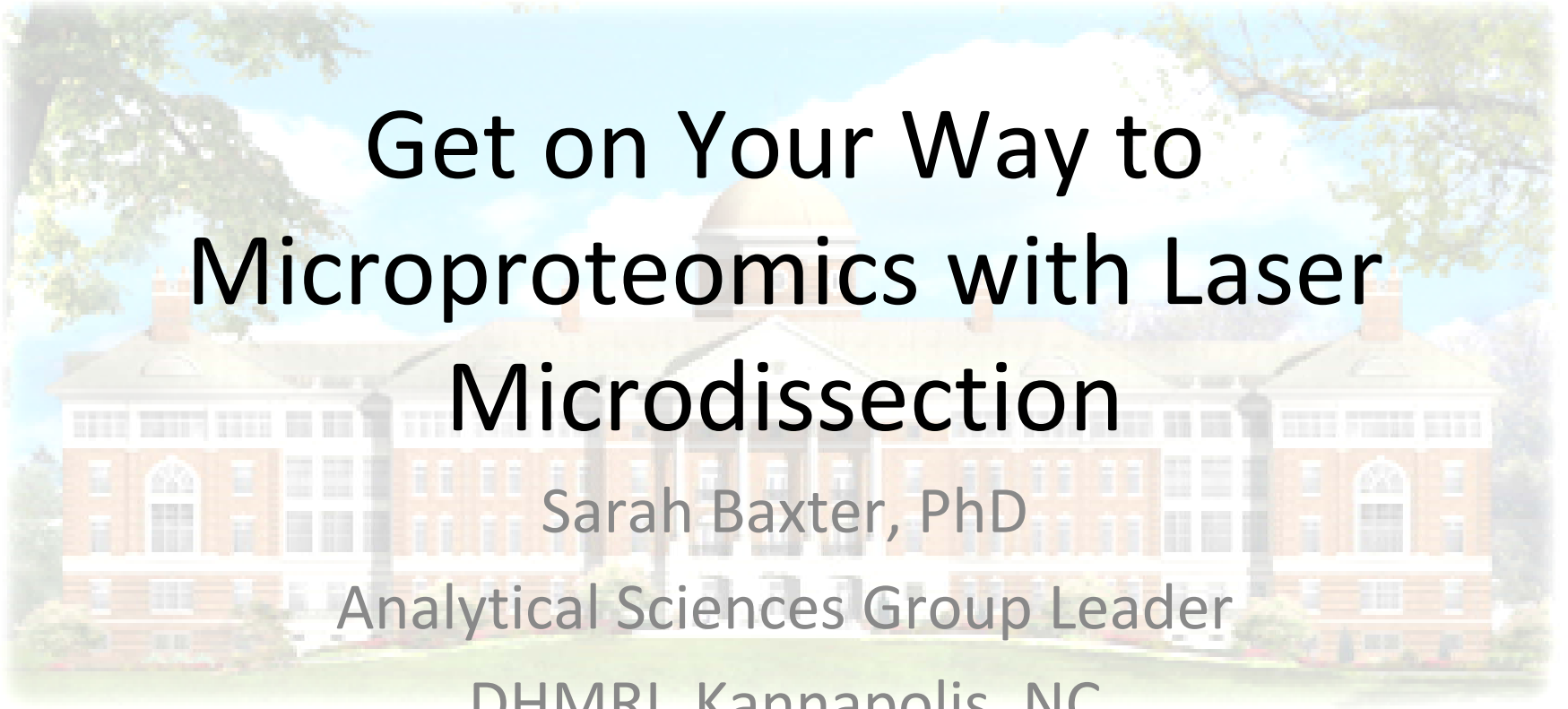
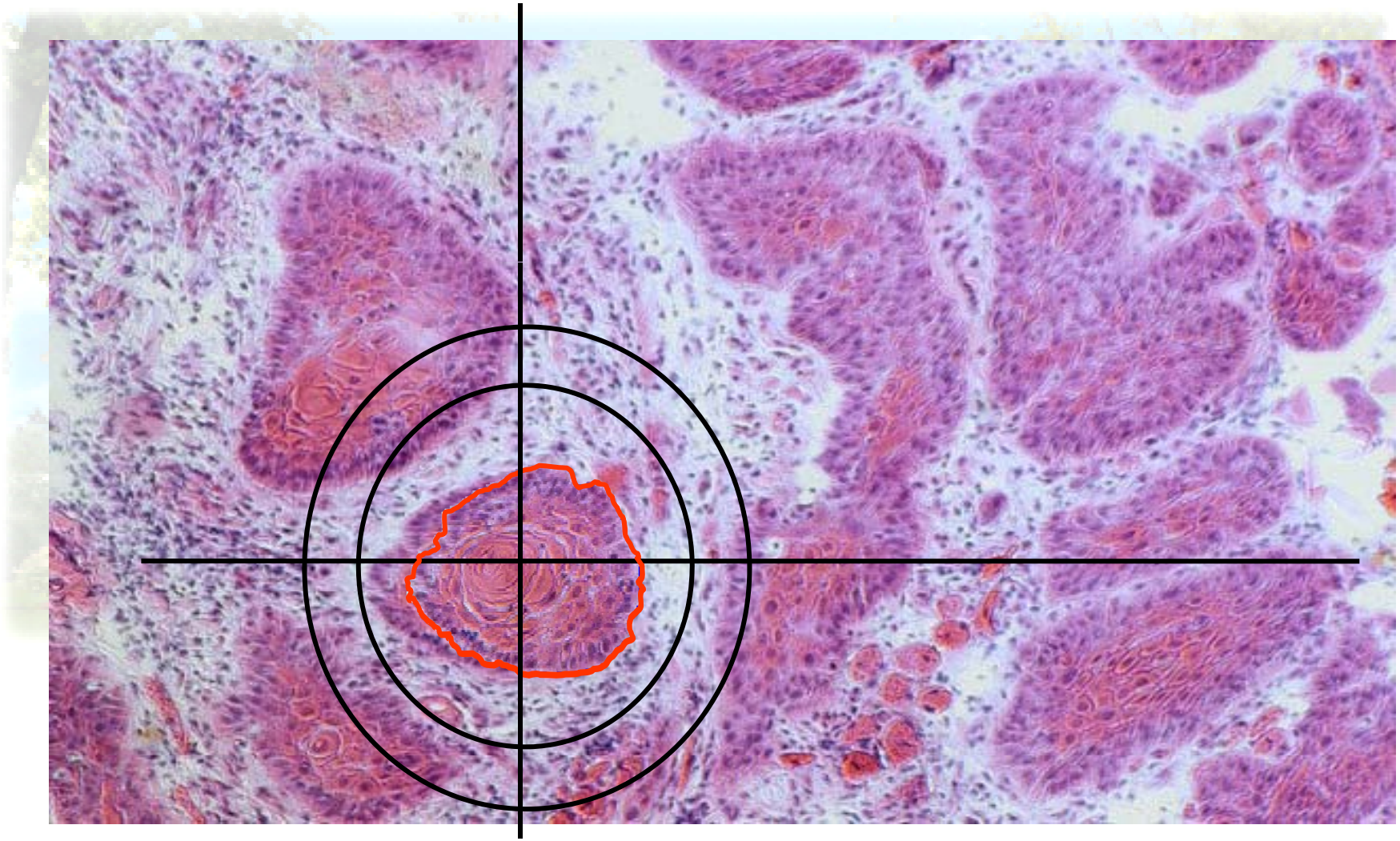


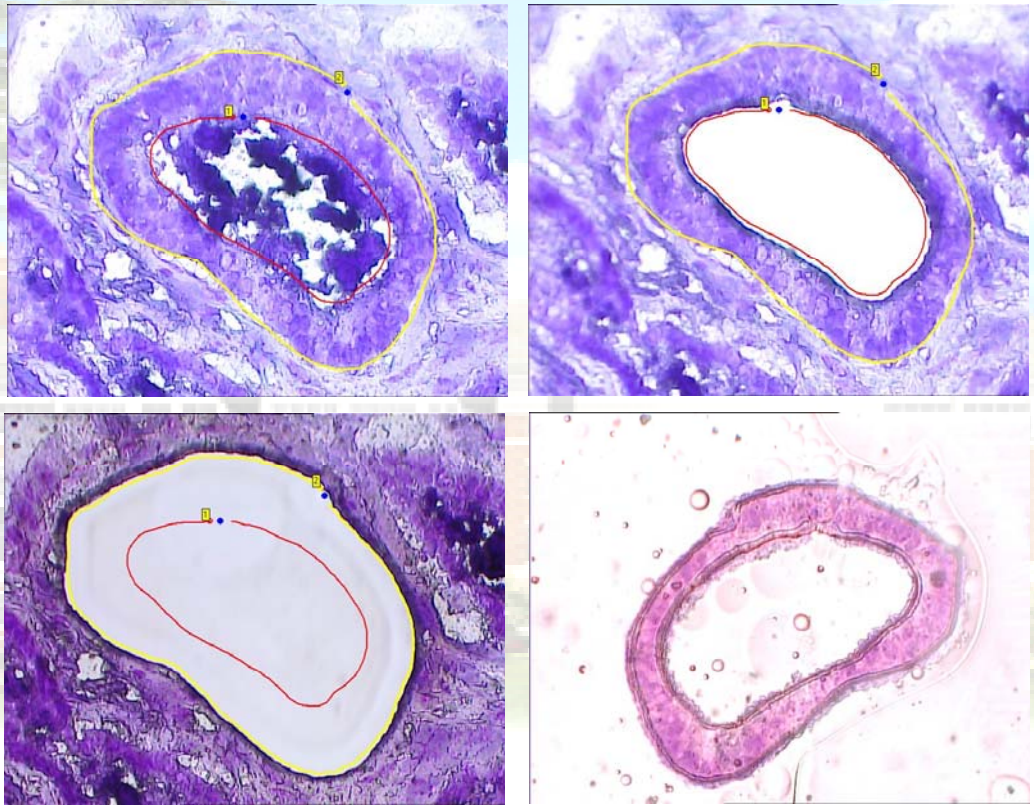
Get on Your Way to Microproteomics with Laser Microdissection

Sarah Baxter, PhD
Analytical Sciences Group Leader
DHMRI, Kannapolis, NC

Why Microdissection?



Selective non-contact LCM



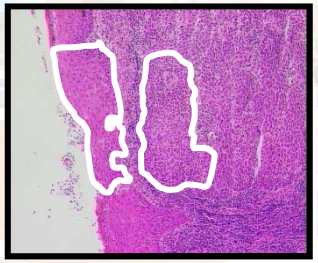
- selective elimination of unwanted material
- clear cut between selected and unwanted specimen

frozen section, human prostate duct, Cresyl Violet stain, 40x obj.

Images by courtesy of Dr. T. Schlomm, UKE Hamburg

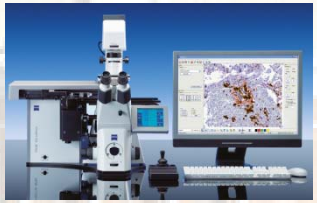
Laser Microdissection for a variety of protein analysis methods

Frozen tissue
e.g., mouse liver



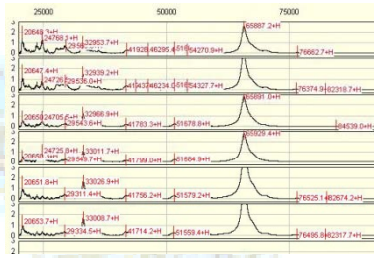
Pharyngeal
Epithelium:
tumor – non-tumor

Renal Cell
Carcinoma:
tumor – non-tumor



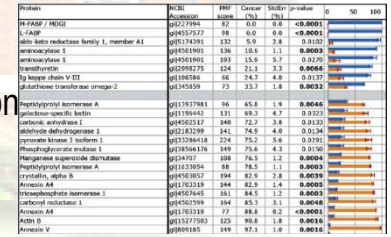
Laser Microdissection

SELDI:
Test on
influence of
different stains

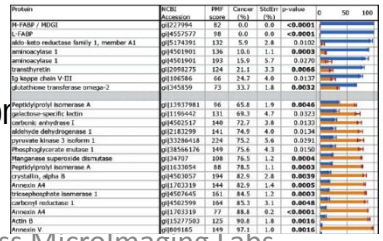


2D SDS-PAGE:
Comparison of
protein expression
in tumor versus
non-tumor

MALDI:
Comparison of
protein expression
in tumor versus
non-tumor



LC/MS:
Comparison of
protein expression
in tumor versus
non-tumor

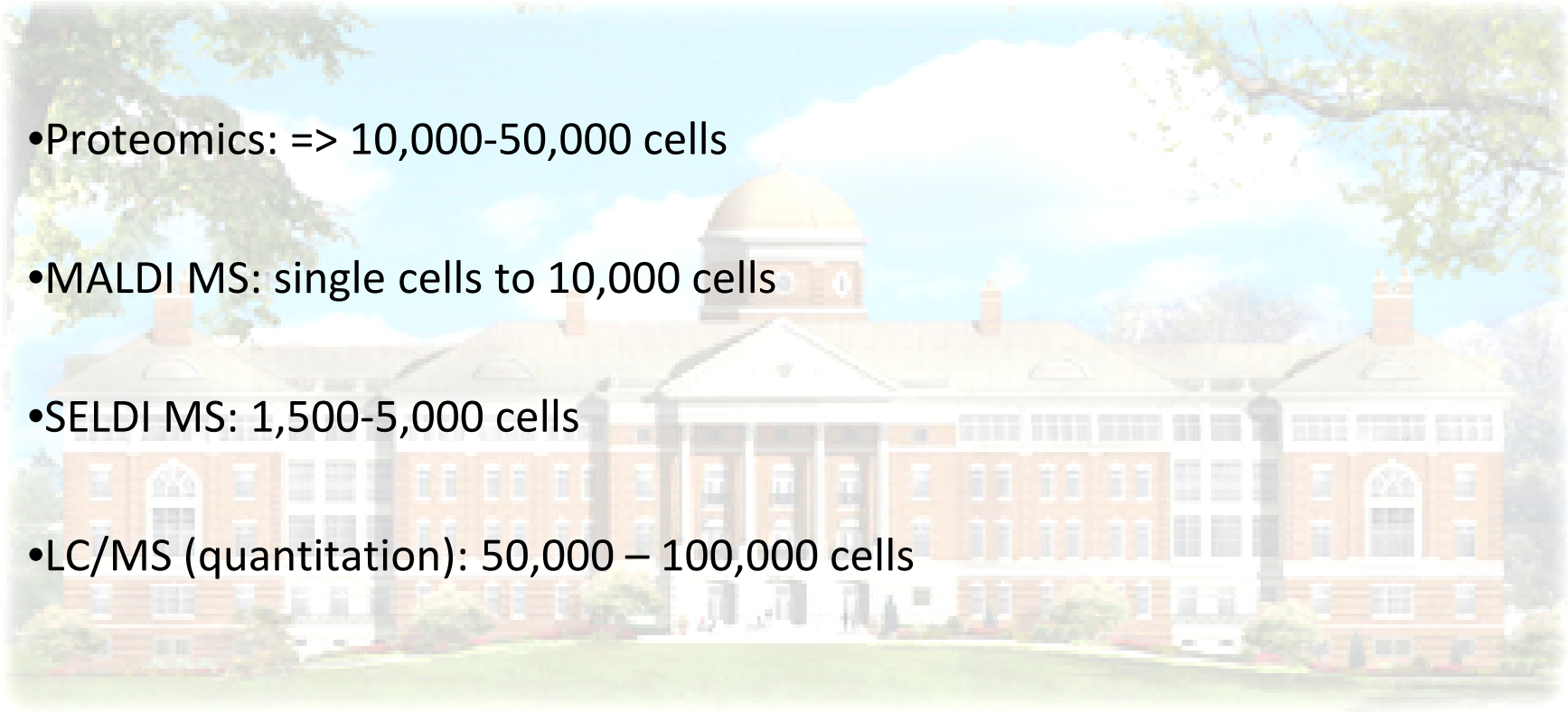


Proteomics can be utilized for molecular profiling of captured cells

- Proteomic analysis can be leveraged to determine the proteome, protein expression changes and/or PTMs for collected cells

- Proteome analysis of LCM cells has been applied to evaluate:
  - Proteome of individual cell sub-populations
  - Molecular progression of cancer (normal, preneoplastic and cancer)
  - Tumor vs invasive cells
  - Monitor cells for efficacy of treatment

Amount of source material required



- Proteomics: => 10,000-50,000 cells
- MALDI MS: single cells to 10,000 cells
- SELDI MS: 1,500-5,000 cells
- LC/MS (quantitation): 50,000 – 100,000 cells

- LCM cells can be pooled from different slides/ sections into one cap to reach the necessary number of cells

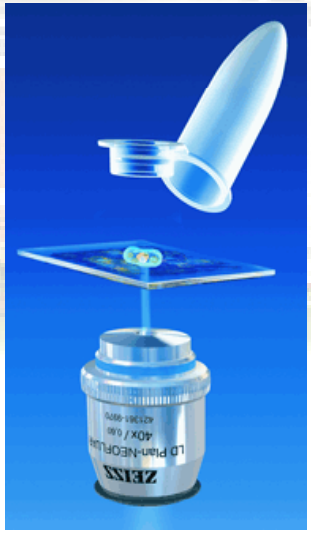
Espina V. et al, Nature Protocols 1(2): 2006

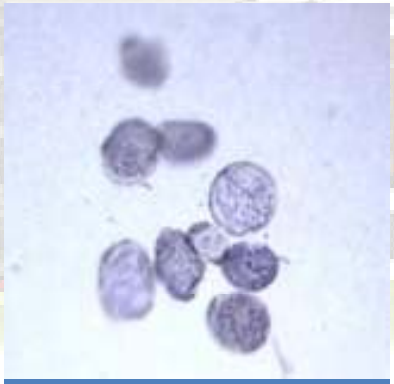
Non-Contact LCM / Pick-Up LCM

Laser Microdissection using
Non-Contact LCM

Collection of:
- single cells, nuclei, chromosomes
- specific cell areas, tumor cells

here: glomeruli of a kidney sample

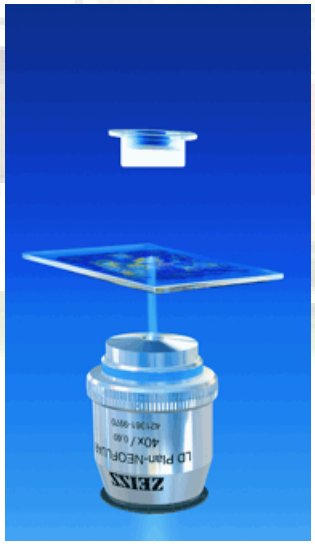




First step:
non-contact LCM of glomeruli

Laser Microdissection using
Pick-Up LCM

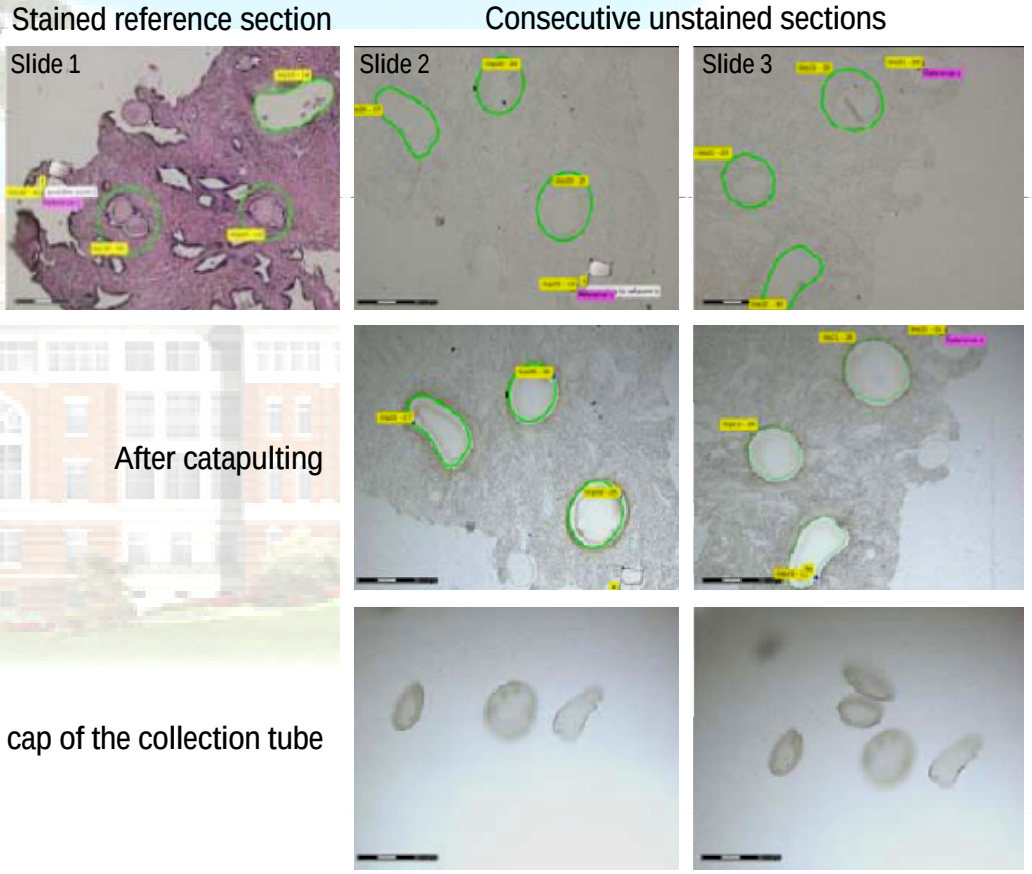
Collection of:
- large areas of homogeneous tissue
- captured in one piece
- for proteomic or metabolic profiling



Second step:
pick-up LCM of kidney tissue

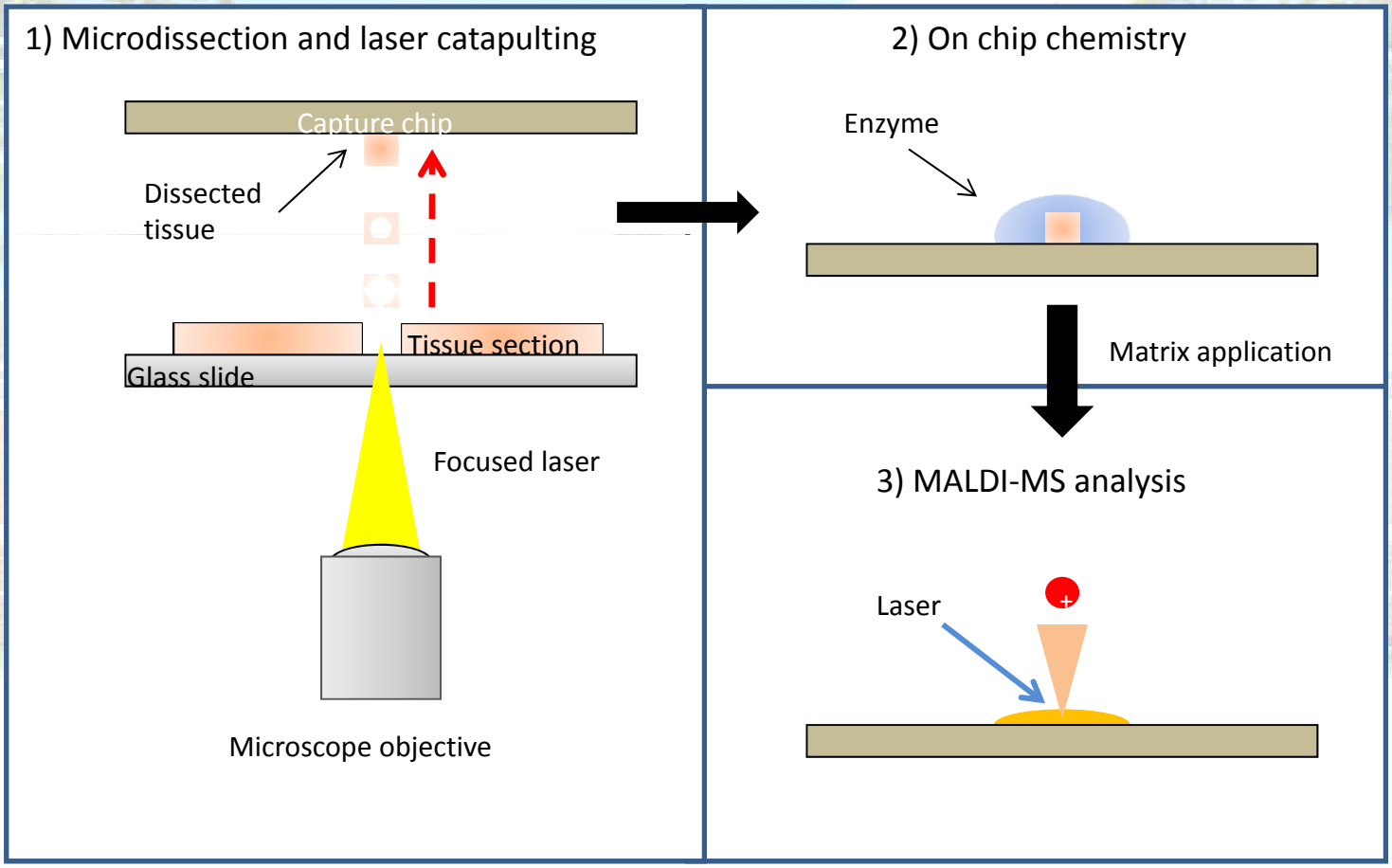
Software-assisted Positioning for Serial Sections

PALM RoboSoftware-assisted Laser Microdissection from unstained tissue sections



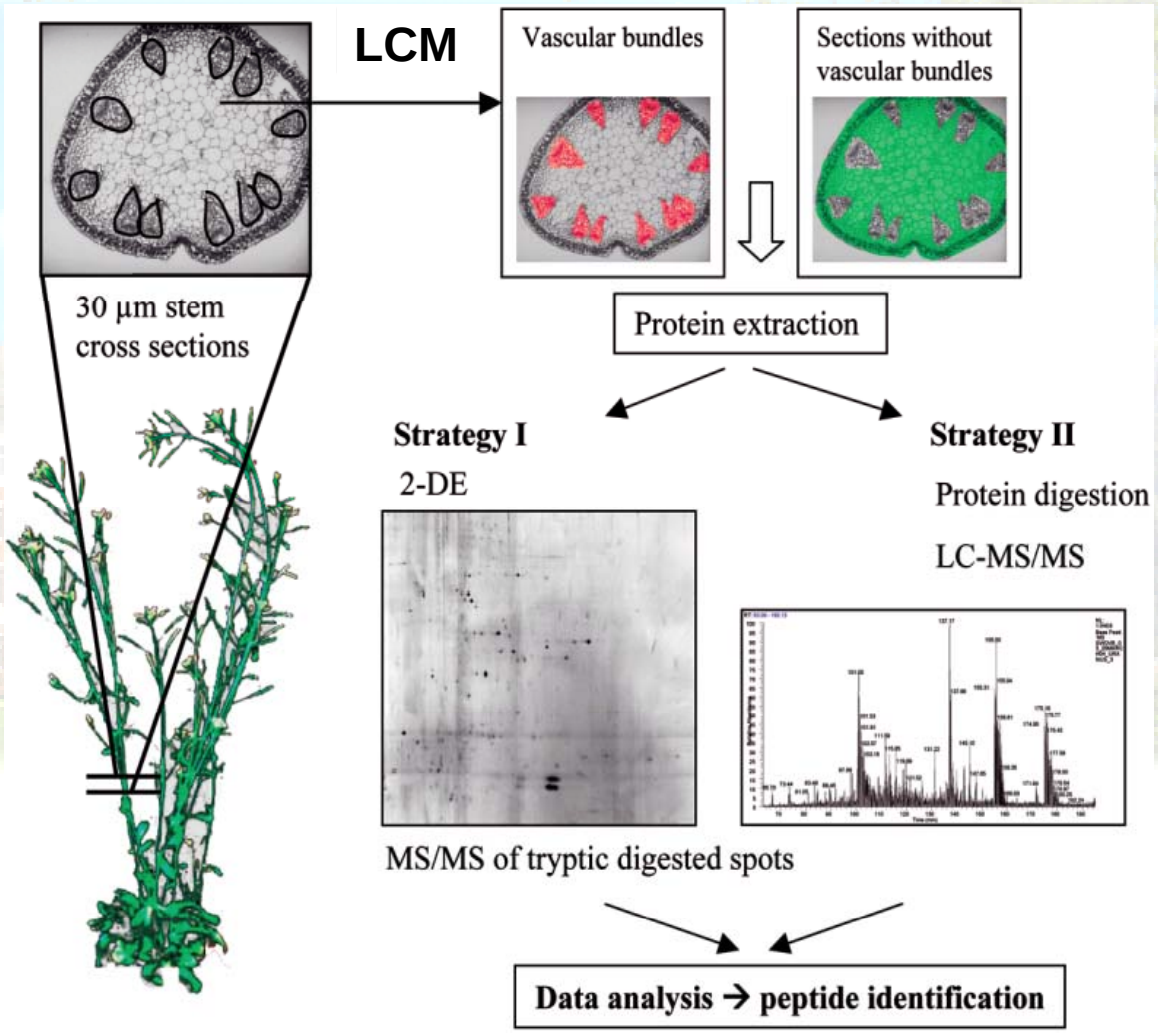
Downstream protein analysis from unstained samples

Coupling of LCM with MALDI MS for profiling of intact peptides and proteins



Combination of LCM and LC-MS/MS

Arabidopsis thaliana
stem tissue



Schad et al.,
Electrophoresis, 2005, 26

Sample preparation is key for proteomics analysis

- Preparation goal is to extract proteins and minimize protein loss
- Key procedure steps include:
  - Tissue fixation
  - Tissue staining
  - Protein extraction
  - Protein separation
  - Sample analysis

Tissue Fixation

- Formalin fixation may negatively impact protein analysis
  - Decreases protein yield 10-100 fold
  - Slow fixative process, inconsistent fixative time frames within a tissue
  - Buffers for extracting proteins from fixed tissue are commercially available

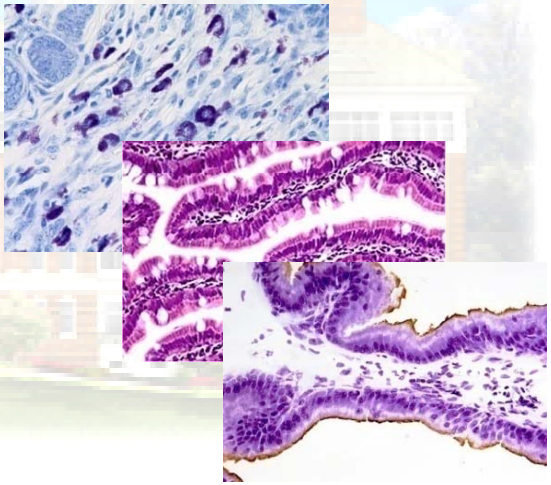
- Minimal fixation is preferred
  - Fresh/frozen tissue
  - Precipitation fixatives
    - Ethanol/xylene
    - Can be performed after sectioning (brief fixation)
  - Paraffin embedding
    - Results in some protein loss
    - Tissue processing can aid in proteomic analysis

Tissue Sectioning: Frozen Tissue

- Do not allow tissue to dry on slide at RT
- Store frozen sections at -80C prior to LCM
- Can use a stained or unstained frozen tissue section
- Can add proteinase inhibitors to staining solutions (protein)
- Minimize LCM session
- Protein quality will degrade rapidly after staining
- Do not refreeze a stained tissue section
- Once a tissue has been stained, LCM must be completed

Tissue Staining

- Staining can result in loss of cellular proteins or inhibit protein extraction
- Light staining improves visualization
- Reminder: Use the same staining procedure for all samples in a study set
- Preferred options include:
  - **Staining an adjacent section for tissue navigation**
  - **Hematoxylin** (in the absense of eosin)
  - Cresyl violet, Toluidine blue, Methylene blue
  - Fluorescent dyes
  - Immunostaining



- Can add protease inhibitors to staining solutions

Protein Extraction

- Physical disruption
  - Homogenization
  - Ultrasonication
  - Freeze-thaw
  - Pressure cycling
  - Bead mills
- Chemical disruption
  - Buffer kits (T-Per, Pierce)
  - Denaturation
  - Urea, thiourea
  - MS friendly detergents (Rapigest, PPS Silent Surfactant)
  - Enzymatic lysis
  - Buffers for FFPE samples
- For chemical disruption, 1 µL extraction buffer per 1,000 cells (100 ng protein)

Protein Extraction Solutions Evaluated

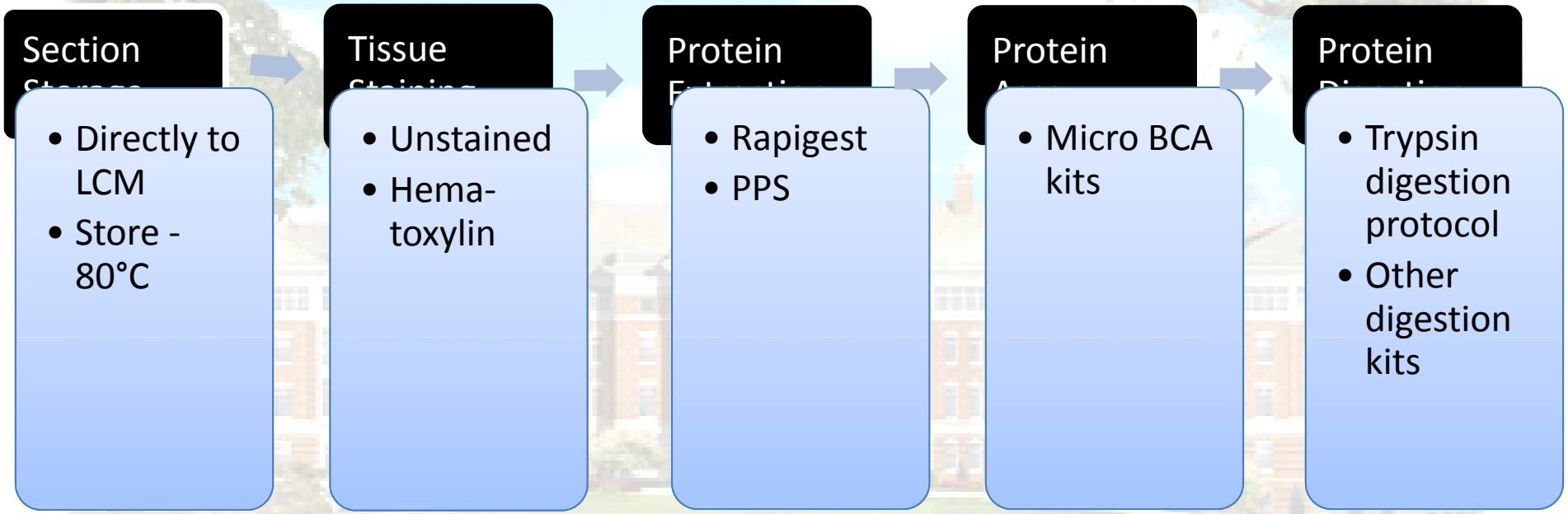
- 100mM Ammonium Bicarbonate (ABC)
- 100mM Tris-HCl pH 8 (TRIS)
- PPS Silent Surfactant (PPS)- 2% solution
- T-PER Tissue Protein Extraction Reagent (TPER)
- Rapigest SF surfactant (Rapigest)

Protein Extraction Results (BCA Assay)

					Average	Std Dev		[protein] ug/mL in sample
ABC-U1	0.076	0.086	0.098		0.087	0.011		1.6
ABC-U2	0.076	0.086	0.083		0.082	0.005		0.5
ABC-CV1	0.063	0.066	0.066		0.065	0.002		7.8
ABC-CV2	0.067	0.073	0.069		0.070	0.003		5.8
Tris-CV1	0.047	0.049	0.043		0.046	0.003		15.9
Tris-CV2	0.090	0.102	0.051		0.081	0.027		0.8
TPER_CV1	0.083	0.096	0.084		0.088	0.007		2.1
TPER-CV2	0.074	0.069	0.071		0.071	0.003		5.0
Rapigest-CV1	0.311	0.308	0.317		0.312	0.005		**99.6**
Rapigest-CV2	0.351	0.368	0.240		0.320	0.070		**102.9**
PPS-CV1	0.121	0.117	0.126		0.121	0.005		**16.7**
PPS-CV2	0.115	0.129	0.117		0.120	0.008		**16.3**

*TPER and Tris extraction solvents did result in some absorbance suppression for a BSA control

Recommended Procedure for LC/MS Analysis of LCM Cells



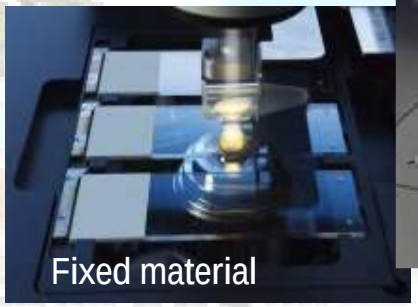
Common Problems for Proteomics Experiments

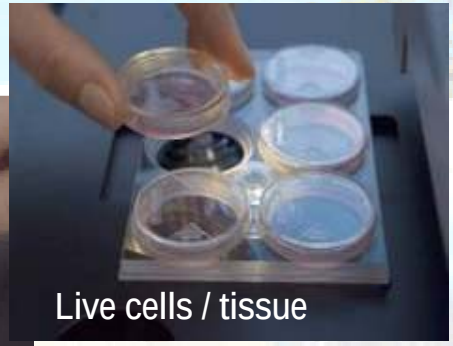
Problem	Possible reason	Solution
Decreased protein yield	Protein degradation before freezing, processing, and fization	Evaluate tissue quality, ensure tissue was procured and processed in a timely manner; snap freeze, embed in OCT or place in ethanol fixative within 5-10 min or procurement; add protease inhibitors
	Degradation of protein during staining and microdissection	Add protease inhibitors Limit time of microdissection for frozen sections Microdisessect cells immediately after staining
	Formalin-, parapformaldeydyde or glutaraldehyde-fixed tissue	Use frozen ethanol-fixed tissue
	Inadequate number of cells	Solubilize several caps in 1 volume of extraction buffer Use the minimal volume of extraction buffer per cap Use one cap to microdissect cells from multiple tissue sections

Espina V. et al, Nature Protocols 1(2): 2006

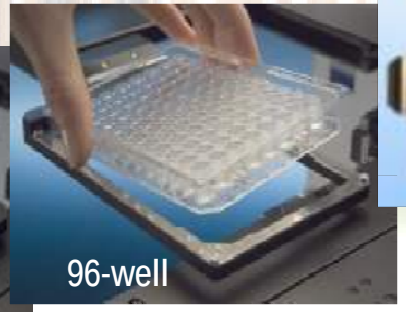
Technical Developments: From Single Experiments to Higher Throughput

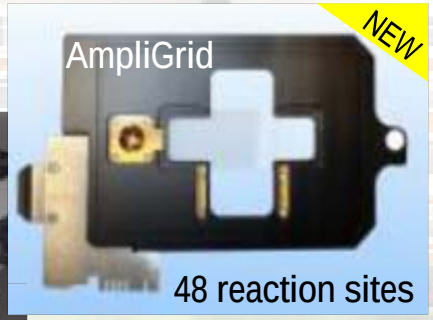
Stage inserts:



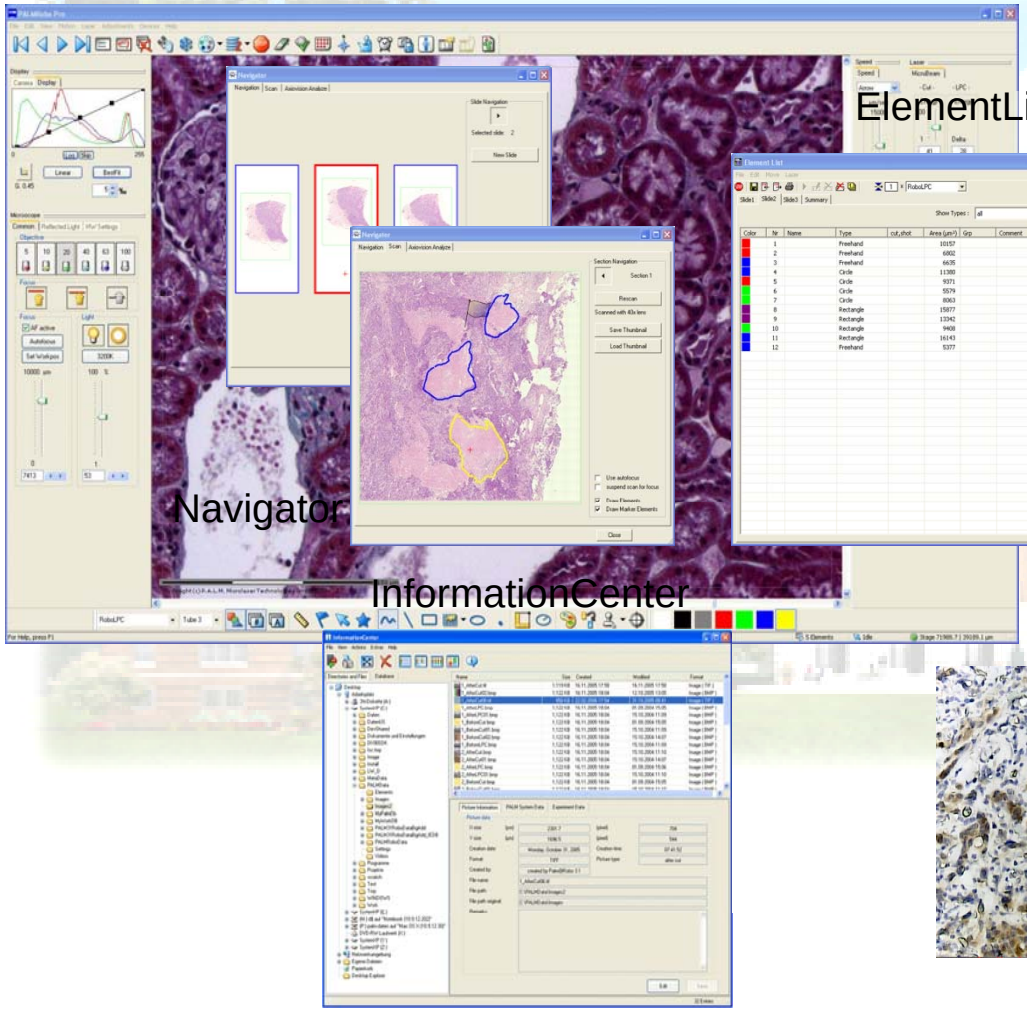


Collectors:





PALM RoboSoftware
User-friendly from Routine to Advanced Applications

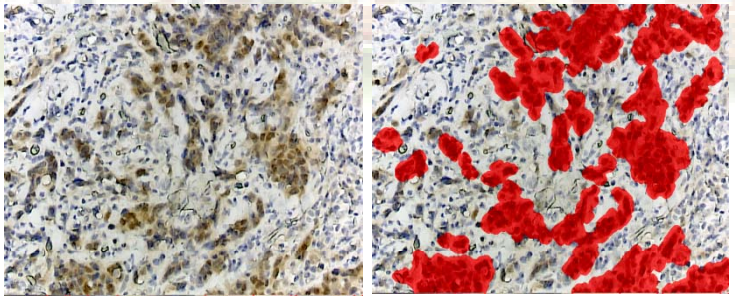


Basic version for routine applications:

- Navigator (overview image)
- Element List
- Information Center

Pro version with optional modules for advanced applications:

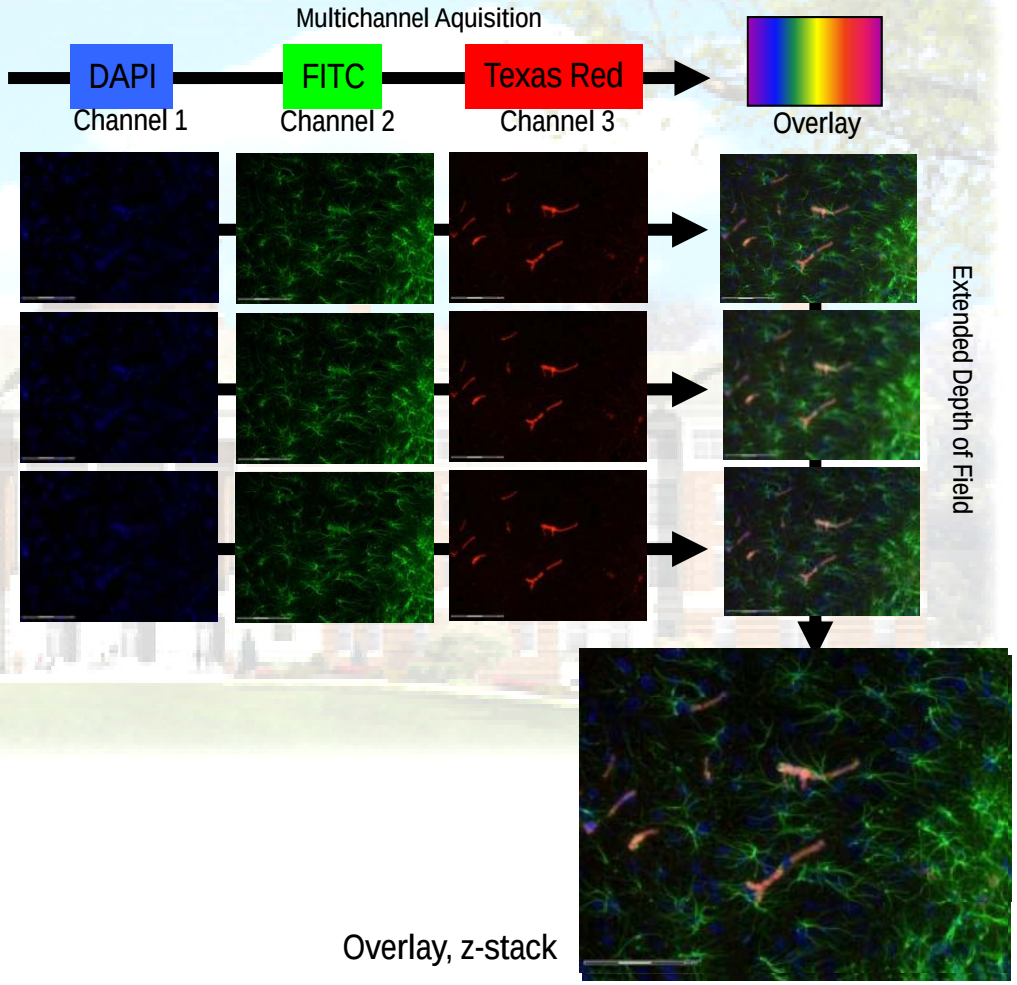
- Database mode for clinical use
- Automated pattern recognition
- Multi-channel fluorescence
- Z-Stacking



ImageAnalysis

PALM MicroBeam
Single experiment to full automation

- Multi channel Fluorescence/ Extended Focus
  Combine both features for perfect fluorescence imaging



Special Thanks

- Zeiss Microimaging Labs

www.zeiss.de/microdissection

- DHMRI
  - Jim Carlson
  - Nidhi Sharma