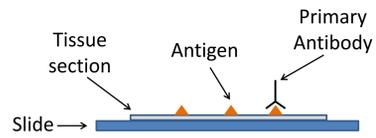


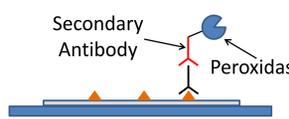
Background

The goal of microdissection is to capture pure populations of cells for downstream molecular analysis. Manual microdissection is time consuming, labor-intensive, and limited by manual dexterity, making it impractical for accumulation of sufficient tissue for meaningful analysis¹. Laser Capture Microdissection (LCM) eliminates the manual component, but remains too time-consuming and costly for routine use.

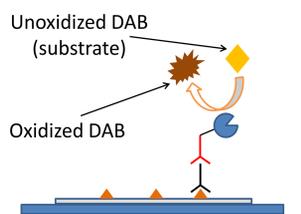
Target Activated Microdissection (TAM) offers fast, precise, operator-independent capture of desired cell types. The procedure begins with immunohistochemistry to stain target cells.



A primary antibody binds a specific target biomarker molecule (antigen).

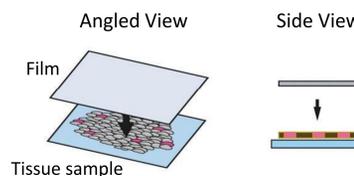


A secondary antibody with peroxidase activity is added and binds the primary antibody.

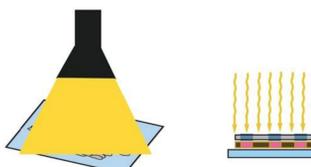


Diaminobenzidine (DAB) is added and oxidized by the peroxidase, forming a brown precipitate stain within the desired targets.

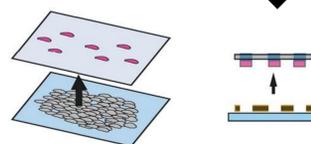
Once the tissue slide has been IHC stained, it is ready for microdissection with TAM. This involves three main steps:



A transfer tape with an ethylene vinyl acetate (EVA) polymer layer is then placed on top of the specifically labelled tissue slide.



When uniformly illuminated by a brief pulse from a Xenon flashlamp, the immunostain heats and focally melts the overlying EVA.



After cooling (ms), the EVA forms a bond with the tissue targets, which are then removed when the transfer tape is peeled from the slide.

Objectives

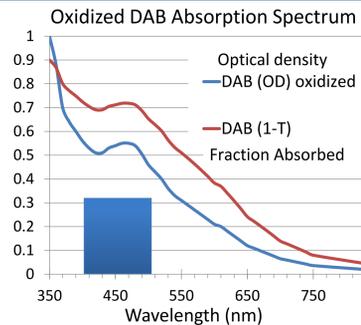
A method to quantitatively assess the efficiency and specificity of transfer is needed to evaluate the advantages TAM offers in speed, ease, and specificity. Such a method would also aid in selection of appropriate transfer films and dosimetry, preferably based on image analysis of the stain distribution and prior knowledge of the stained target localization, in order to achieve optimal dissection of specific tissue targets.

The specific objectives are:

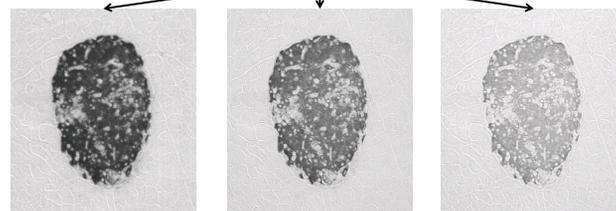
1. Develop a quantitative method to accurately assess efficiency and specificity of transfer.
2. Demonstrate specific capture of nuclei from IHC stained cells when IHC biomarkers are not in the cell nucleus.

Evaluating Transfer Efficiency

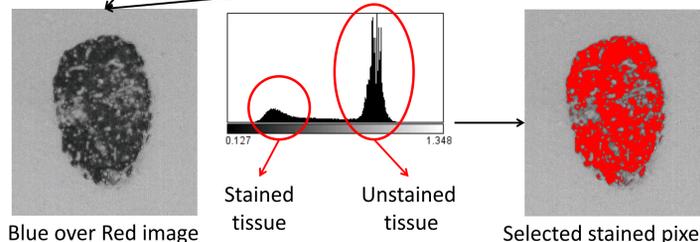
The absorption spectrum of oxidized DAB is shown to the right. The blue box indicates the region excited by the flashlamp. DAB's spectral properties are such that it absorbs more strongly in the blue wavelength range than in the red. We can take advantage of this to differentiate stained tissue from unstained tissue by splitting the image into its RGB channels and computing the blue over red ratio as shown below.



Mouse pancreas islet IHC-stained for insulin



Blue Channel Green Channel Red Channel

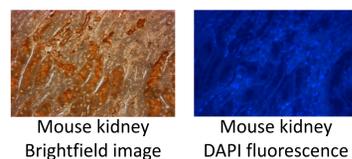


The intensity of the selected stained pixels is analyzed to determine the stain density of the islet. Specifically, for each pixel the optical density (OD) of DAB is given by $OD = -\log_{10}(I_i/N)$, where I_i is the intensity of the pixel i and N is the average unstained tissue pixel intensity. The sum of all the pixel ODs gives us an estimate of the stain density of the islet.

This analysis is performed on index-matched (cover-slipped) images taken of each islet on the tissue section before and after microdissection, as well as on the transfer tape with the captured targets, using a 20x objective.

Evaluating Transfer Specificity

Nuclear fluorescent staining allows visualization of nuclei to determine specificity. DAPI is a common fluorescent stain that binds DNA, excites in the UV wavelength range with a maximum at 358 nm, and emits in the blue wavelength range with a maximum of 461 nm.

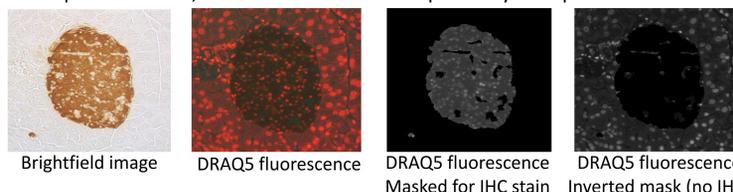


Mouse kidney Brightfield image

Mouse kidney DAPI fluorescence

Though bright and easy to detect, DAPI's fluorescence has high background and is attenuated within targets by DAB's high UV/blue OD.

As a replacement for DAPI, we tested and chose DRAQ5, a fluorescent stain that excites in the red with a 647 nm maximum and emits in the far-red >665 nm. By selecting stained cells from the brightfield image using the same method described above and masking the fluorescence image as shown below for a mouse pancreas islet, we can determine the specificity of capture.



Brightfield image

DRAQ5 fluorescence

DRAQ5 fluorescence Masked for IHC stain

DRAQ5 fluorescence Inverted mask (no IHC)

Results

The images below depict transfer of IHC-stained insulin-producing β cells in mouse pancreas. With increasing light dose, percent capture increases up to threshold then levels off at ~70%.

	2 pulses 0.13 J/cm ²	2 pulses 0.29 J/cm ²	8 pulses 0.29 J/cm ²
Before microdissection			
After microdissection			
Film pre-DRAQ5 staining			
Film post-DRAQ5 staining			
Percent capture	25.1%	69.8%	71.7%
Percent error	1.7%	8.9%	6.7%

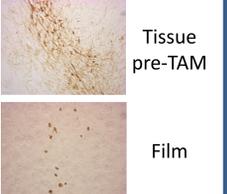
DRAQ5 imaging followed by masking to view IHC-stained and -unstained areas separately show capture of nuclei from stained beta cells and little to no capture of nuclei from adjacent unstained alpha and gamma cells in islets of Langerhans.

	2 pulses 0.1 J/cm ²	2 pulses 0.2 J/cm ²	8 pulses 0.2 J/cm ²
IHC-Stained Area			
Unstained Area			

Conclusions and Future Work

We report here the use of a fluorescent DRAQ5 counterstain of cell nuclei and transmission and fluorescence microscope image analysis to evaluate specificity (only stained cells and their nuclei) and efficiency (fraction of stain captured) of transfer as TAM parameters are varied. From these microscope images, we can determine those pixels with DAB stain above some threshold and all cell nuclei in the tissue sections before and after transfer and in the tissue captured by fTAM on the transfer tape. We also demonstrate specific transfer of nuclei from IHC-stained cells without need for nuclear immunostain, with ~70% capture efficiency above a threshold of 2 pulses at 0.2 J/cm² and little to no nuclei captured from unstained cells.

Ongoing work includes optimizing and analyzing transfer on different types of tissue such as dopaminergic neurons from the substantia nigra of rat brain as shown to the right. In addition, dose-response analyses are needed to correlate DAB stain density with light dose required for complete transfer.



Tissue pre-TAM

Film

Acknowledgements

I was fortunate to receive guidance and support throughout the summer from a long list of mentors and collaborators that includes: Robert Bonner, Brandon Harvey, Tom Pohida, Nicole Morgan, Stephen Hewitt, Randall Pursley, Jeffrey Hanson, Pyry Koivula, and Oshea Johnson. I'd also like to thank NIH, the BESIP program, and Bob Lutz for the opportunity to complete this research.

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- ¹Emmert-Buck et al. (8 Nov 1996). Laser capture microdissection. *Science*, 274(5289):998-1001.
- ²Tangrea et al. (Dec 2004). Expression microdissection: operator-independent retrieval of cells for molecular profiling. *Diagnostic Molecular Pathology*, 13(4):207-12.