Validation of triplex CD3, CD8 and FOXP3 IHC of tumor-infiltrating lymphocytes in follicular lymphoma

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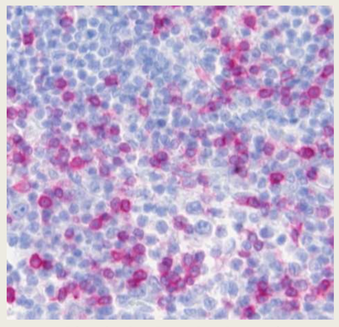
Abstract

It is clear that immune cells play many sometimes conflicting roles in the tumor microenvironment and it would be extremely useful to be able to visualize the distributions of multiple phenotyped immune cells in-situ in solid tumors. However, obtaining phenotypic information about the various cells that play these roles in and around the tumor has been a challenge. Existing methods can either deliver phenotypic information on homogenous samples (e.g., flow cytometry) or morphologic information on single immunomarkers (standard IHC). These limitations can be largely overcome through a multiplexed staining, imaging and analysis methodology using standard clinical FFPE tissue sections. Although multiplex methods have been shown to be particularly useful not much has been presented on the validation of such methods. We present here a validation of the method for CD3, CD8 and FOXP3 in tissue microarray containing triplex follicular lymphoma cores from 40 subjects [24 male, 16 female, age 35 to 75 years at diagnosis, median 55 years , 2- 171 months follow-up]. This involves a sequential multi-marker labeling for 3 antigens and a counterstain; automated multispectral imaging to separate chromogens; and an automated analysis that can quantitate the per-cell marker expression, determine the cellular phenotype, count these cells separately in the tumor compartment and in the stroma and provide high-resolution images of their distributions. The IHC for each marker was optimized in as single-stain IHC and then those specifications used as a part of the triplex stain. The samples were scored using an automated scoring methodology and the results from the triplexed method are compared to analyses done on singly stained sections with excellent correlation (R greater than 0.9 in all cases), showing that multiplexed staining methods can replicate standard IHC methods while maintaining the inter-distribution and visualization of the markers in a single section.

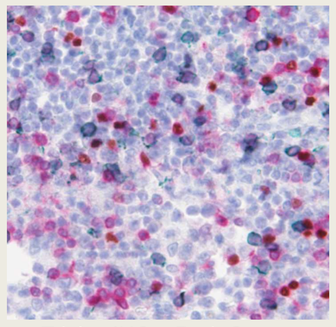
Methodology

Gold Standard

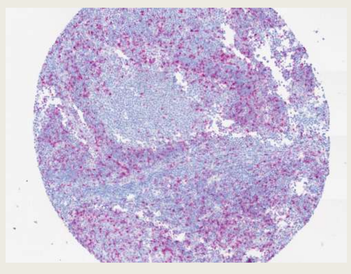


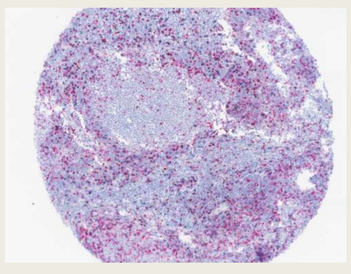
Single IHC – CD3 Red

New Method



Triplex IHC – Red, Green, Brown





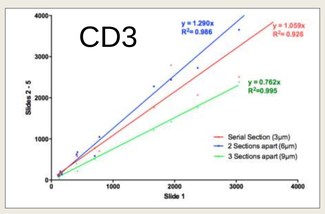
Can we derive the same quantitative information from multiplex IHC as from single IHC?

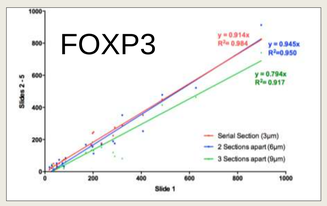
Level of agreement:

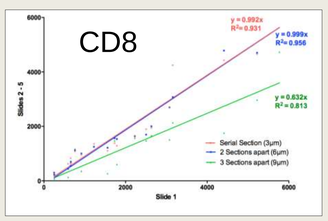
- Validation of triple stain method for TILs enumeration

Assumption: Serial adjacent sections should be near-identical in biomarker characteristics

Determination of acceptable correlation coefficients and bias between single stained serial sections

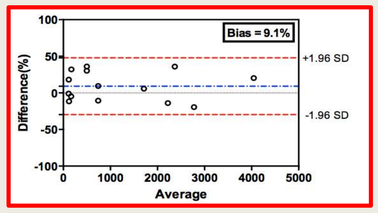


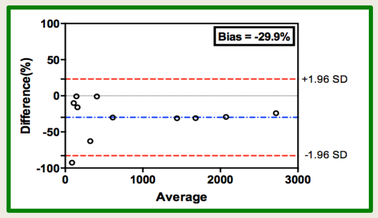


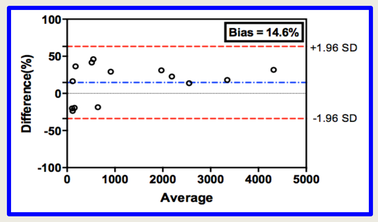


1st Index section vs.

- 2nd section (3µm)
- 3rd section (6µm)
- 4th section (9µm)

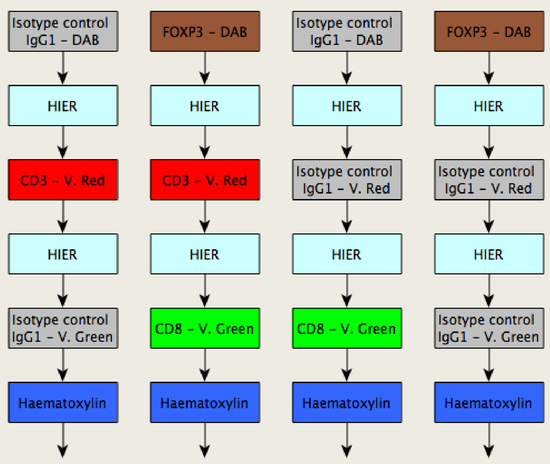






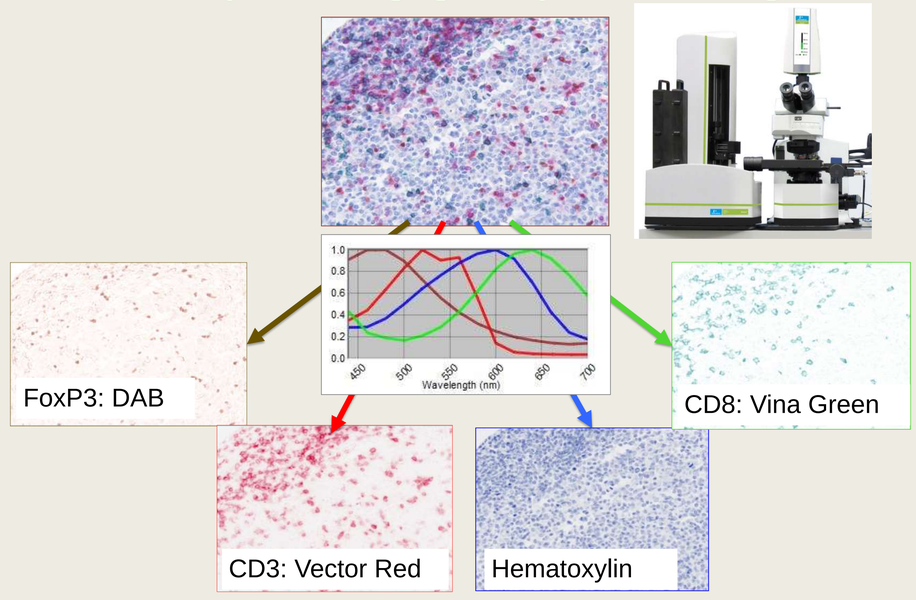
Conclusion: Serial sections (up to 6µm) are suitable for assay comparison

Single and multiplex IHC staining protocol

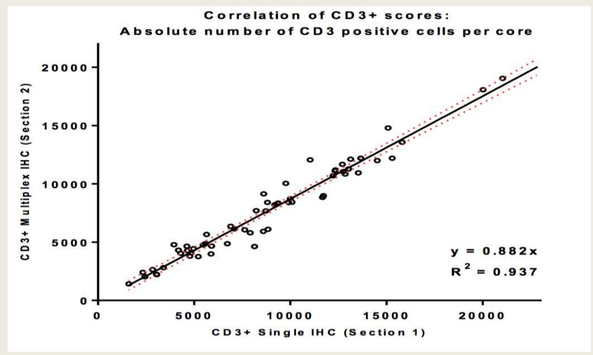


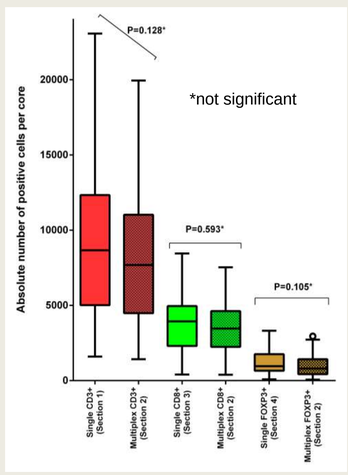
All tissue sections submitted to same reagent exposures. Primary antibodies were replaced by isotype controls in single IHC slides

Multispectral imaging and spectral unmixing



Results





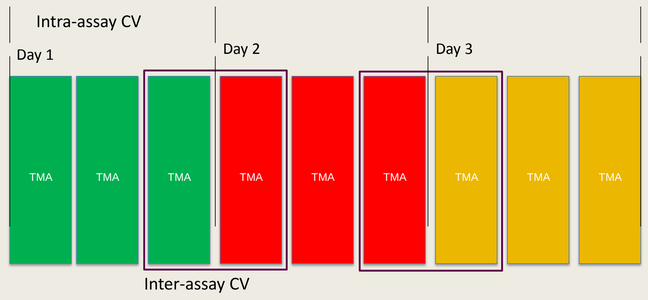
Mann-Whitney U Test: Multiplex vs. Single IHC

Biomarker	Goodness of fit (R^2)	Gradient of LOBF	*P Value*
CD3	0.937	0.882	*P<0.0001*
CD8	0.926	0.950	*P<0.0001*
FOXP3	0.953	0.895	*P<0.0001*

No statistically significant difference between absolute quantitation of positive cells in cores of multiplex vs. single IHC in adjacent tissue sections for each biomarker

MSI analysis software with multiplex IHC can be used to quantify cells based upon the co-expression of up to three biomarkers

Repeatability and Reproducibility



	CD3			CD8			FoxP3		
Assay	Single	Multiplex		Single	Multiplex		Single	Multiplex	
Intra- assay CV (%)	20	23	P=0.97	22.2	15	P=0.41	21	20	P=0.83
Inter- assay CV (%)	24	25		22.4	22		21	23	

Conclusions

- Multiplex chromogenic IHC used in conjunction with multispectral imaging techniques provides:
  - Objective, reproducible, high-throughput alternative to current gold standard techniques in TIL studies
  - Quantitative solution to immunophenotyping and cellular enumeration in FFPE tissue sections
- Sections more than 6 um apart do not give well correlated results!
- Technique transferrable to any biomarker study aiming to detect spatial co-localization and distribution of multiple protein targets in archival tissue sections
- Can be expanded to up to 8 biomarkers using Opal™ immunofluorescence labelling

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