Five Biomarker Multiplex Immunofluorescence Staining without Spectral Unmixing using a MegaStoke Dye

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ABSTRACT: Due to the broad spectral emission of fluorescent dyes one is limited to imaging five fluors, *e.g.* DAPI, 490, 550, 650 and 750 nm, without requiring spectral unmixing using standard fluorescent microscopes and filter sets. The requirement for nuclear staining with DAPI, leaves four fluors for detection of biomarkers. Here we report the ability to increase imaging to six colors and five biomarkers on a standard imager by including a megastokes dye, *i.e.* a dye with a Stokes shift > 30 nm. We demonstrate the rapid, simultaneous whole slide staining and imaging of six colors and five biomarkers, CD3, CD4, CD8, CD20 and panCK on a single tissue section. Using Cell IDx's UltraPlex Tag-based technology this only requires a single antigen retrieval step and two step staining procedure using cocktails of antibodies without the need for spectral unmixing.

UltraPlex Fluorescent Multiplex Technology

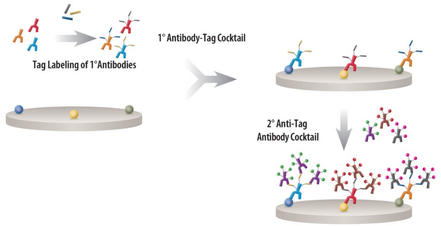

Figure 1: Schematic showing staining of tissue sections using modified Tag-labeled primary antibodies and detection using fluor-labeled anti-Tag antibodies.

METHODS: Primary antibodies, CD3, CD4, CD8, CD20 and panCK were conjugated to 5 UltraPlex Tags, UT012, UT014, UT015, UT019, and UT021. anti-Tag antibodies were conjugated to fluorophors, CL490, CL550, CL650, CL750 and megastoke dye CL500MS. Tissues were initially incubated with a cocktail of Tag-modified primary antibodies for 1 hour followed by washing and incubation with a cocktail of fluor-labeled anti-Tag antibodies. Following washing and coverslipping the slides were imaged on the Leica Versa imager. It is to be noted that the four CD antibodies are rabbit monoclonal antibodies and PanCK is a mouse monoclonal antibody.

Fluorescent Dyes Spectral Properties

Fluor	Absorbance (nm)	Emission (nm)	Extinction Coefficient ($M^{-1}cm^{-1}$)	Chroma Filter Set Catalog #
CL-490	491	515	73,000	49308
CL-550	550	575	150,000	49303
CL-650	654	672	250,000	49006
CL-750	759	780	240,000	49007
CL500MS	500	630	50,000	custom

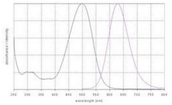

Figure 2: Table including spectral properties of fluorescent dyes and Chroma filter sets used (www.chroma.com) (upper) and absorbance and emission spectra of MegaStoke Dye CL500MS.

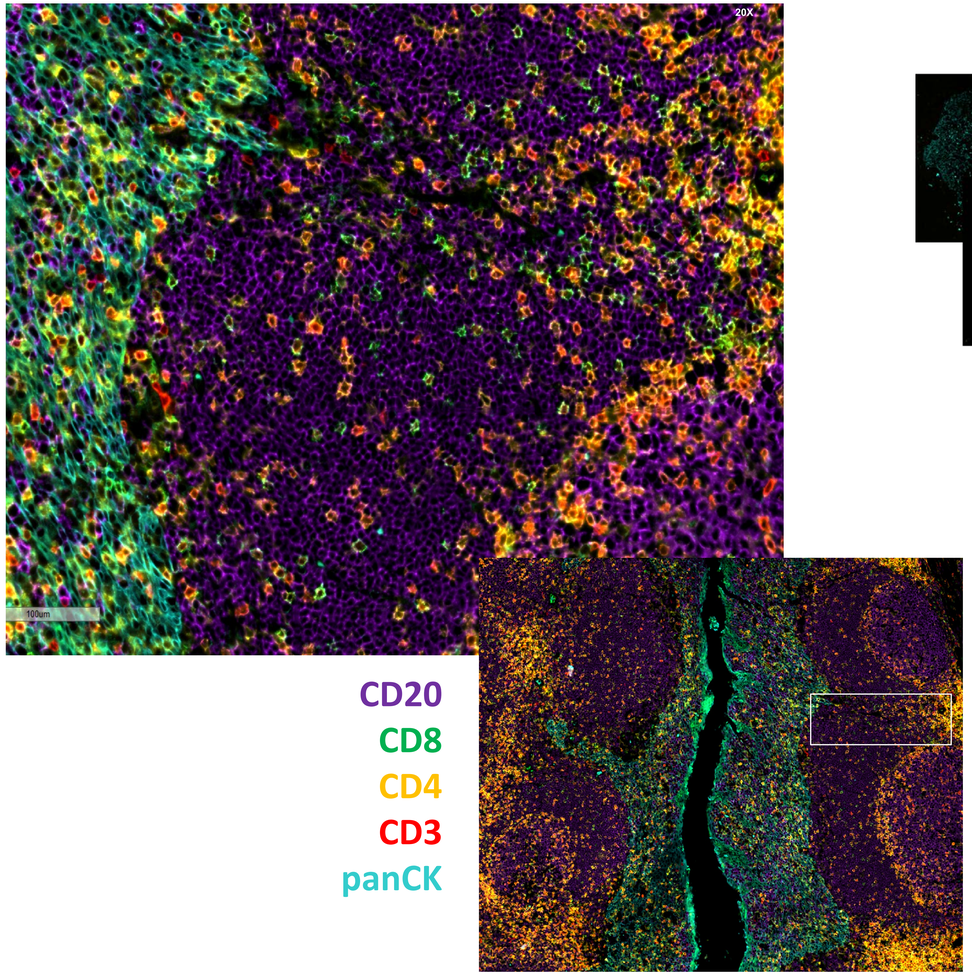

Figure 2: Five-plex image on tonsil tissue of CD3 (red), CD4 (yellow), CD8 (green), CD20 (purple) and PanCK (teal) using four standard fluorescent dyes and megastoke dye CL500MS on panCK.

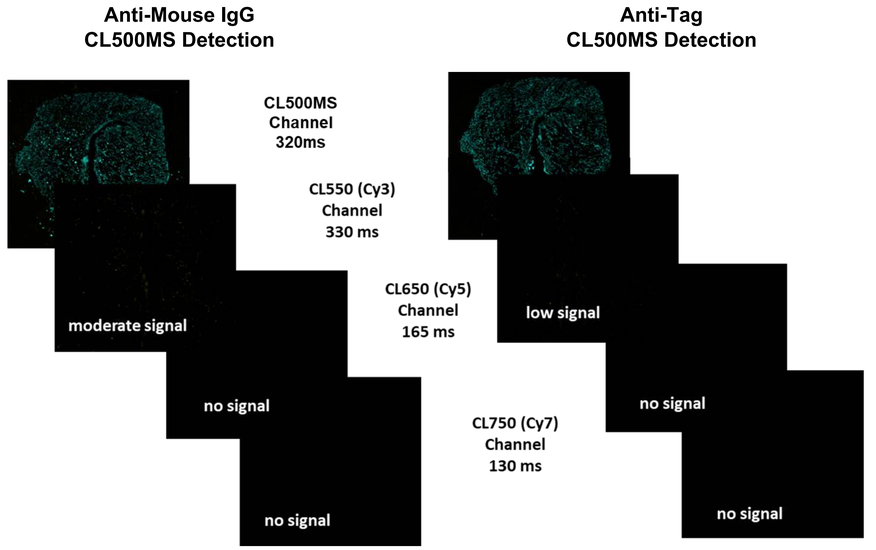

Figure 3: Scans of tonsil tissue stained with PanCK/α-mouse-CL500MS (left) and PanCK-UT012/α-UT012-CL500MS (right) in the CL500MS, 550, 650 and 750 channels. Both PanCK/α-mouse-CL500MS (left, top) and PanCK-UT012/α-UT012-CL500MS (right, top) produced strong staining however the PanCK/α-mouse-CL500MS signal did bleed into the 550 channel more significantly than PanCK-UT012/α-UT012-CL500MS.

UltraPlex Fluorescent Multiplex IHC: A Paradigm Shifting and Disruptive Technology

- Primary antibody species independent
- Standard two-hour, two-step staining protocol employed
- Signals equivalent to fluorescent secondary antibodies
- Significant time savings
- High throughput compatible
- Ability to detect multiple co-localized markers
- Data readily transferrable to image analysis software
- View as fluorescent or "virtual brightfield" overlaid on H&E sections

CONCLUSION: This study demonstrated the ability to fluorescently stain and image 5 biomarkers using a standard fluorescent imager without the requirement for spectral unmixing. This successful imaging of 5 biomarkers without spectral unmixing, employing a single antigen retrieval step and using antibodies from any species further expands the utility of **Cell IDx's *UltraPlex*** technology.

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