SITC, 2024, Poster # 72

Enhanced signal removal for fast and highly multiplexed immunofluorescence staining with broad reagent and assay compatibility using CellScape Precise Spatial Proteomics

Thore Böttke*[1]; Arne Christians[1]; Marcus Böttcher[1]; Jannik Boog[1]; Julia Feldhaus[1]; Anke Brix[1]; Daniel Jimenez-Sanchez[1]; Oliver Braubach[1]

1 Bruker Spatial Biology, St. Louis, MO, USA

*Presenting Author

Introduction

- The demand for high-plex biomarker detection *in situ* has surged in recent years.
- Various cyclic immunofluorescence (IF) staining techniques have been developed to increase biomarker multiplex capabilities.
- The removal of fluorescence signals after each detection cycle is achieved by antibody stripping, antibody barcoding, or photobleaching.
- Each method poses challenges to the design & conduct of multiplex immunofluorescent (mIF) assays.
- We developed EpicIF™ technology (Enhanced photobleaching in cyclic Immunofluorescence), a workflow for the CellScape™ Precise Spatial Proteomics platform that enables quick, gentle, and specific photobleaching of common photostable fluorophores *in situ*.

Methods

- *In vitro* photobleaching with tunable LEDs demonstrates the effectivity & specificity of EpicIF-based photobleaching of organic fluorophores.
- *In situ* experiments on FFPE tissues were performed to test the effect of the EpicIF workflow on sample integrity and epitope stability after several rounds of photobleaching.
- Pixel intensity calculations were used to assess the speed and effectivity of EpicIF technology in IF experiments.
- A custom 48-plex mIF assay was developed on the CellScape platform to demonstrate streamlined mIF assay design and performance while retaining robust signal removal and epitope preservation.

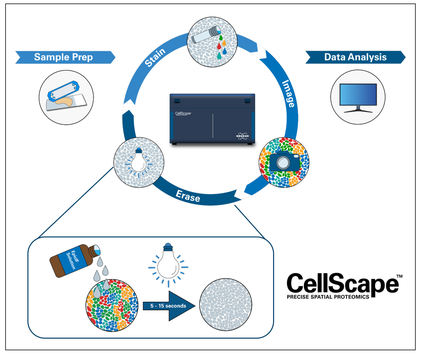

Figure 1. The EpicIF workflow on the CellScape platform. The instrument uses cycles of staining, imaging, and signal removal to detect biomarkers with spatial context at single-cell resolution. EpicIF™ Solution facilitates signal removal via filtered photobleaching.

Fast and selective photobleaching *in vitro*

Fluorescence dyes of the rhodamine, fluoresceine, cyanine and BODIPY classes can be photobleached using EpicIF Solution *in vitro* (**Fig. 2A**), with bleaching rates >100 times faster than using PBS (**Fig. 2B**). EpicIF photobleaching is dependent on excitation wavelength, where optimal fluorophore decolorization is achieved with the excitation wavelength of the fluorophore (**Fig. 2A**). This eliminates the need for energy-rich UV light for photobleaching, thus establishing the gentle nature of EpicIF. We also demonstrate selective bleaching of AlexaFluor™ (AF) 594 (Thermo) in a mixture with AF488 in **Fig. 2C** using a selectively tuned LED. White light, on the other hand, is able to bleach both fluorophores if specificity is not required (**Fig. 2D**).

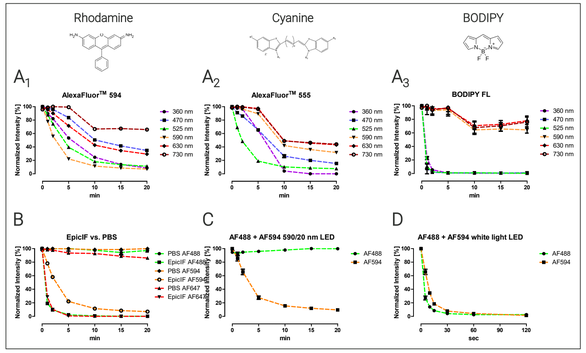

Figure 2: *In vitro* photobleaching of common fluorophores using EpicIF. Dyes were dissolved in EpicIF Solution. Irradiation was performed with selectively tuned LEDs set to an output power of 2.52 mW/cm². White light LED with an output power of 1,600 mW/cm². The fluorescence intensity at t=0 min (before irradiation) was set as 100%. Data shown represent the mean + SD of n=3.

Fast and gentle photobleaching *in situ*

Sample integrity

We next tested the effects of repeated cycles of the EpicIF workflow on tissue integrity and epitope stability.

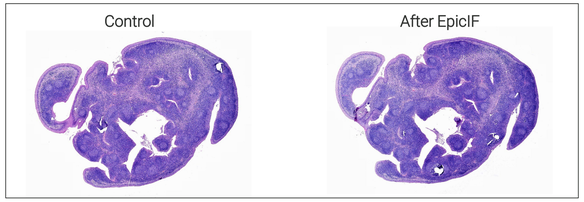

Figure 3: H&E Stained FFPE tonsil sections. 5 µm FFPE tonsil sections were H&E stained after a standard photobleaching assay (left, control) or after 10 cycles of EpicIF photobleaching (right). The pathologist report noted that both sections are "*very acceptable in terms of staining intensity and preservation of cellular architecture*" and "*I do not see differences between experimental conditions*".

Bleaching Speed

The *in situ* effectivity of EpicIF is demonstrated on an FFPE tonsil section stained with CD45RO-AF594 (**Fig. 4**). The fluorescence signal is completely removed after as little as 5 seconds.

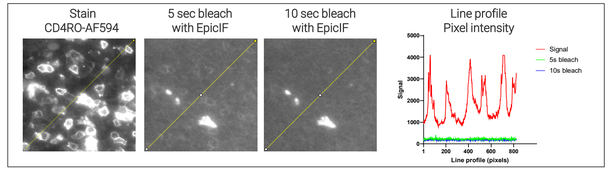

Figure 4: Measuring the *in situ* bleaching speed of the EpicIF workflow on the CellScape platform. The pixel intensity of the line (yellow) was measured before and after 5 or 10 sec of photobleaching with EpicIF technology. The pixel intensities are shown in the graph (right).

Epitope Stability

Serial FFPE tonsil sections were photobleached for 10 cycles with or without using EpicIF Solution, stained with standard immune markers, and analyzed for cell densities and other metrics (**Fig. 5**).

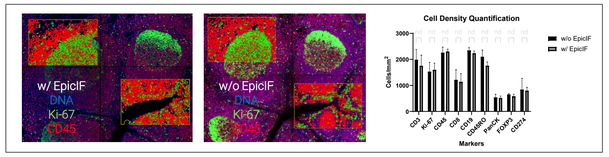

Figure 5: Epitope stability after 10 rounds of EpicIF photobleaching. Two ROI of serial sections were analyzed for the cell densities of 9 different markers. Results show comparable values for section photobleached with or without EpicIF technology.

FFPE tonsil sections were stained with a 48-plex immunofluorescence assay. Comprehensive and robust staining of immune cells was observed throughout (**Fig. 6**).

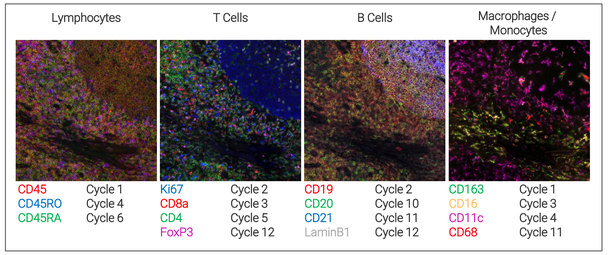

Figure 6: Immunofluorescence stain of a FFPE tonsil section. A FFPE tonsil section was stained with 48 antibodies in 12 cycles (4 markers per cycle) using EpicIF. Different subsets of immune cells were stained and detected over multiple cycles, regardless of cycle position in the mIF assay. Weakly expressing antigens, like FoxP3, were detectable in late cycles, indicating preservation of epitopes through the assay.

Multiplex immunofluorescence staining using EpicIF on the CellScape platform

A FFPE breast cancer section was stained over 12 cycles with commercially available antibodies (**Fig. 7A**). The staining plan can be seen in **Table 1**. To assess the effectivity of the EpicIF workflow, marker over marker crosstalk analysis was performed (**Fig. 7B**). The average Pearsons´s r=0.239 shows that no correlation between different markers with the same fluorophore cycle over cycle. Different markers with high correlations are due only to biological colocalization (**Fig. 7C**). This demonstrates the effectivity of EpicIF technology for removing fluorescence signals, which would not be possible with normal photobleaching and common photostable fluorophores.

Cycle 1	CD3	CD45	CD163	HLA-DR
Cycle 2	CD19	Ki-67	CD14	CD279
Cycle 3	CD16	SMA	CD8a	ATP1A1
Cycle 4	CD11c	CD31	CD45RO	CD138
Cycle 5	CD56	CD34	CD274	CD66b
Cycle 6	CD208	CD45RA	Beta-Actin	CD4

Cycle 7	Col4	Vimentin	Beta-Cat	Caveolin-1
Cycle 8	GranzymeB	PCNA	EGFR	CD38
Cycle 9	LaminB1	CK14	CK19	Galectin-3
Cycle 10	CD23	CK18	CD21	CD20
Cycle 11	Pan-Cadherin	CD68	HistoneH3	MUC1
Cycle 12	E-cadherin	panCK	FoxP3	CD103

Table 1: Staining plan 48-plex. 48 markers (+DNA) were stained following this plan over a total of 12 cycles.

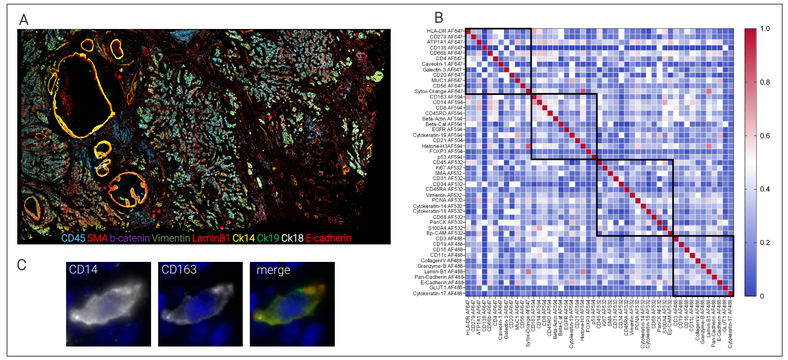

Figure 7: 48-plex immunofluorescence stain of a FFPE breast cancer section. A FFPE breast cancer section (Invasive Ductal Carcinoma) was stained with 48 antibodies in 12 cycles using EpicIF technology. **(A)** Overview with selected markers as indicated at the bottom of the picture. **(B)** Channel-based marker cross correlation. Markers with the same fluorophore are marked with boxes. **(C)** Higher correlations between certain markers (r=0.71 for CD14/CD163) were confirmed to be due to biological spatial colocalization rather than incomplete signal removal by EpicIF. Note that co-localizing markers have distinct subcellular biological expression patterns (e.g. CD14 and CD163 in **C**), which confirms complete signal removal with EpicIF, as well as preservation of subcellular marker expression patterns.

Conclusions

- EpicIF technology enables the quick and selective photobleaching of commonly used organic fluorophores.
- Assays that rely on a fluorescence readout are compatible with EpicIF, including immunofluorescence and HCR RNA FISH (see Poster 78).
- H&E stains and cell density calculations showed gentle photobleaching with EpicIF did not affect tissue integrity and epitope stability.
- Cycle over cycle cross correlation as well as pixel intensity measurements showed complete fluorescence signal elimination in as little as 5 seconds.

Contact

Presenter: Thore Böttke

thore.boettke@bruker.com

For more information, visit www.BrukerSpatialBiology.com

Use QR code to request more information or a quote.