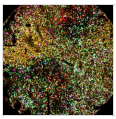

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Automated Multi-plex Immunofluorescence with TSA for CD4, CD8, FOXP3, CD21, PD1 and CD68 in Follicular Lymphoma

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ABSTRACT

Observing immune cell infiltration and spatial organisaion in the tumour microenvironment of follicular lymphoma is important for the development of novel biomarkers for prognosis and treatment selection. However the identification of multiple immune cell sub-populations is limited in routine single-plex immunohistochemical staining.

Our goal was to develop and validate an automated protocol for multiplex immunofluoresence using tyramide signal amplification, the Opal™ 7 color Kit (Akoya Biosciences) and the Ultra Discovery (Roche) autostainer, which would enable concurrent visualisation on the same tissue sections of:

- T follicular helper cells ($CD4^+$)
- cytotoxic T cells ($CD8^+$)
- Tregs ($CD4^+FOXP3^+$)
- Macrophages ($CD68^+$)
- $PD1^+$ lymphocytes
- DAPI nuclear counterstain

We aimed in addition to observe the spatial arrangement of these cells in reference to the follicle areas, therefore the CD21 marker, which is expressed in mature B cells and follicular dendtritic cells located in the follicles was added in the panel.

We validated quantitatively the agreement between single-plex and multiplex assays by comparing stained area in sequential sections of a follicular lymphoma FFPE tissue micro-array (TMA). The TMA was constructed from a follicular lymphoma cohort of 44 patients collected retrospectively from the archives of the Christie NHS Foundation Trust (Manchester), with ethical permission granted by the Central Manchester Multi-centre Research Ethical Committee (03/08/016). The protocol was automated with the DISCOVERY ULTRA IHC/ISH Roche autostainer and stained areas were quantified using the Indica Labs' Area Quantification Fluorescence module (HALO software).

Statistically significant linear correlations were seen between single-plex and multiplex assays (R^2 >0.72, $p<7.1\times10^{-14}$, see attached) for all markers.Therefore, this multiplex immunofluorescence (mIF) protocol can be used to conserve tissue and observe immune cell spatial organisation in follicular lymphoma.

Protocol validation.pdf

GUIDELINES

This protocol has been validated for use with follicular lymphoma FFPE tissue sections from lymph node tumour biospies (4µm thichness).

A new spectral library and separate validation should be carried out before use with other types of tissue where the levels of expression may vary. Instructions for detailed optimisation steps required to set up a new protocol are provided by Akoya Biosciences.

Acknowledgements:
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Morgan who shared an optimised chromogenic protocol for the CD4 antibody; these were used as base for development of the current setup.

MATERIALS

NAME	CATALOG #	VENDOR
Prolong Gold	P36930	Thermo Fisher Scientific
CONFIRM anti-CD4 (SP35) Rabbit Monoclonal Primary Antibody	790-4423	Roche
Anti-CD68 antibody mouse monoclonal [KP1] to CD68	(ab955)	Abcam
Anti-FOXP3 antibody mouse monoclonal [236A/E7]	(ab20034)	Abcam
Anti-PD1 antibody [NAT105] (ab52587) Mouse monoclonal	(ab52587)	Abcam
CD8 (Concentrate) Monoclonal Mouse Anti-Human CD8 Clone C8/144B	M710301-2	Agilent Technologies
CD21 (2G9) Mouse Monoclonal Antibody	121M-14	Cell Marque
EZ Prep (10x)	950-102	Roche
Opal 7-Color Automation IHC Kit 50 Slides	NEL821001KT	Akoya Biosciences
DISCOVERY UltraMap anti-Ms HRP (RUO)	760-4313	Roche
DISCOVERY UltraMap anti-Rb HRP (RUO)	760-4315	Roche
DISCOVERY Inhibitor	760-4840	Roche
DAPI	62248	Thermo Fisher Scientific
Dulbecco's Phosphate Buffered Saline (PBS)	D8537	

MATERIALS TEXT

Vectra 3.0 Automated Quantitative Pathology Imaging System, 200 Slide Microscope

Vectra CLS142338

DISCOVERY ULTRA IHC/ISH Autostainer

Ventana 05 987 750 001 / N750-DISU-FS

Light Sensitive Dispenser
User-fillable/Prep Kits
Roche 760-205

Building a spectra library

1 A spectral library is initially built using positive controls of follicular lymphoma tissue to enable unmixing the 6 fluorophore spectra and DAPI in the multi-plex experiment. To build the library, the spectrum of each individual fluorophore is acquired from single-plex experiments.

The single-plex experiments are run with the same parameters and positions as in the multiplex experiment; all the pre-treatment steps are kept but only one antibody and its fluorphore are applied in the same order as for the multi-plex.

The order of antibodies and corresponding fluorophores:

Table 1. Order of antibodies and corresponding fluorophores.

Position	Antibody	Fluorophore
1	CD4	Opal 620
2	CD68	Opal 650
3	CD8	Opal 540
4	CD21	Opal 570
5	FOXP3	Opal 520
6	PD1	Opal 690
7	DAPI	

The single-plex experiments are described below. The antibodies and fluorophores can be either pipetted manually on the slide by interupting the automated protocol or prepared in a dispenser and applied automatically. For the multi-plex experiment which lasts 17 hours, we suggest the use of dispensers, whereas the single-plexes can be done manually with more flexibility.

The dilutions of antibodies and fluorophores can be found in Tables 2 and 3, respectively and are the same for multi-plex and single-plex experiments.

6h

1.1

CD4/620 Single-plex

Deparafinisation

In the autostainer:

Warm up slide to 69°C and incubate for 3 cycles of 8 minutes each

Pre-treatment and blocking

Warm up slide to 95°C and incubate for 40 minutes (CC1)

Apply one drop of DISCOVERY Inhibitor and incubate for 4 minutes

1st Cycle

- Warm up slide to 36°C
- Automatically apply 'one drop' of primary **CD4 antibody** (pre-diluted dispenser) and incubate for **16 minutes**
- Apply one drop of **Ultramap anti-Rb HRP** secondary and incubate for **16 minutes**
- Hand-pipette 100µl of the **620** diluted with TSA and incubate for **16 minutes**

2nd Cycle

- Pre-treatment: Warm up slide to 95°C and incubate for **16 minutes** (CC1)

3rd Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

4th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

5th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

6th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

Washing

Remove the slides from the autostainer and was with EZ prep diluted 1:10 with distilled water (3 x 5 minutes).

Coverslip

Two drops of prolong gold antifade reagent (without DAPI) is used per slide

After coverslipping, let slides dry in a dark place for at least 2 hours before scanning

The automated protocol that should be programmed in the Ventana Discovery Ultra autostainer can be found here:

987 CD4.PDF

8h

1.2 **CD68/650 Single-plex**

Deparafinisation

In the autostainer:

Warm up slide to 69°C and incubate for 3 cycles of 8 minutes each

Pre-treatment and blocking

Warm up slide to 95°C and incubate for 40 minutes (CC1)

Apply one drop of DISCOVERY Inhibitor and incubate for 4 minutes

2nd Cycle

- Pre-treatment: Warm up slide to 95°C and incubate for **16 minutes** (CC1)
- Warm up slide to 36°C
- Hand-pipette 100 µm of diluted **CD68** and incubate for **60 minutes**
- Apply one drop of **Ultramap anti-Ms HRP** secondary and incubate for **16 minutes**
- Hand-pipette 100µm **650** diluted with TSA and incubate for **16 minutes**

3rd Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

4th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

5th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

6th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

Washing

Remove the slides from the autostainer and was with EZ prep diluted 1:10 with distilled water (3 x 5 minutes).

Coverslip

Two drops of prolong gold antifade reagent (without DAPI) is used per slide

After coverslipping, let slides dry in a dark place for at least 2 hours before scanning

992 CD68.PDF

8h

1.3 **CD8/540 Single-plex**

Deparafinisation

In the autostainer:

Warm up slide to 69 °C and incubate for 3 cycles of 8 minutes each

Pre-treatment and blocking

Warm up slide to 95°C and incubate for 40 minutes (CC1)

Apply one drop of DISCOVERY Inhibitor and incubate for 4 minutes

2nd Cycle

- Pre-treatment: Warm up slide to 95°C and incubate for **16 minutes** (CC1)

3rd Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)
- Warm up slide to 36°C
- Hand-pipette 100 µm of diluted **CD8** and incubate for **32 minutes**
- Apply one drop of **Ultramap anti-Ms HRP** secondary and incubate for **16 minutes**
- Hand-pipette 100 µm of diluted **540** and incubate for **16 minutes**

4th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

5th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

6th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

Washing

Remove the slides from the autostainer and was with EZ prep diluted 1:10 with distilled water (3 x 5 minutes).

Coverslip

Two drops of prolong gold antifade reagent (without DAPI) is used per slide

After coverslipping, let slides dry in a dark place for at least 2 hours before scanning

989 CD8.PDF

8h

1.4 **CD21/570 Single-plex**

Deparafinisation

In the autostainer:

Warm up slide to 69°C and incubate for 3 cycles of 8 minutes each

Pre-treatment and blocking

Warm up slide to 95°C and incubate for 40 minutes (CC1)

Apply one drop of DISCOVERY Inhibitor and incubate for 4 minutes

2nd Cycle

- Pre-treatment: Warm up slide to 95°C and incubate for **16 minutes** (CC1)

3rd Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

4th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)
- Apply one drop of DISCOVERY Inhibitor and incubate for 4 minutes
- Warm up slide to 36°C
- Hand-pipette 100 µm of diluted **CD21** and incubate for **60 minutes**
- Apply one drop of **Ultramap anti-Ms HRP** secondary and incubate for **24 minutes**
- Hand-pipette 100 µm of diluted **570** and incubate for **16 minutes**

5th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

6th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

Washing

Remove the slides from the autostainer and was with EZ prep diluted 1:10 with distilled water (3 x 5 minutes).

Coverslip

Two drops of prolong gold antifade reagent (without DAPI) is used per slide

After coverslipping, let slides dry in a dark place for at least 2 hours before scanning

988 CD21.PDF

8h

1.5 **FOXP3/520 Single-plex**

Deparafinisation

In the autostainer:

Warm up slide to 69°C and incubate for 3 cycles of 8 minutes each

Pre-treatment and blocking

Warm up slide to 95°C and incubate for 40 minutes (CC1)

Apply one drop of DISCOVERY Inhibitor and incubate for 4 minutes

2nd Cycle

- Pre-treatment: Warm up slide to 95°C and incubate for **16 minutes** (CC1)

3rd Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

4th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

5th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)
- Warm up slide to 36°C
- Hand-pipette 100 µm of diluted **FOXP3** and incubate for **60 minutes**
- Apply one drop of **Ultramap anti-Ms HRP** secondary and incubate for **16 minutes**
- Hand-pipette 100 µm of diluted **520** and incubate for **16 minutes**

6th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

Washing

Remove the slides from the autostainer and was with EZ prep diluted 1:10 with distilled water (3 x 5 minutes).

Coverslip

Two drops of prolong gold antifade reagent (without DAPI) is used per slide

After coverslipping, let slides dry in a dark place for at least 2 hours before scanning

990 FOXP3.PDF

8h

1.6 **PD1/690 Single-plex**

Deparafinisation

In the autostainer:

Warm up slide to 69°C and incubate for 3 cycles of 8 minutes each

Pre-treatment and blocking

Warm up slide to 95°C and incubate for 40 minutes (CC1)

Apply one drop of DISCOVERY Inhibitor and incubate for 4 minutes

2nd Cycle

- Pre-treatment: Warm up slide to 95°C and incubate for **16 minutes** (CC1)

3rd Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

4th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

5th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

6th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)
- Warm up slide to 36°C
- Hand - apply (pipette) 110µl of diluted **PD1** and incubate for **32 minutes**
- Apply one drop of **Ultramap anti-Ms HRP** secondary and incubate for **16 minutes**
- Hand - apply 110µl of diluted **690** and incubate for **16 minutes**

Washing

Remove the slides from the autostainer and was with EZ prep diluted 1:10 with distilled water (3 x 5 minutes).

Coverslip

Two drops of prolong gold antifade reagent (without DAPI) is used per slide

After coverslipping, let slides dry in a dark place for at least 2 hours before scanning

996 PD1.PDF

5h

1.7 **DAPI**

In this step the slide is run through the autostainer, without application of antibodies or fluorophores. Then it is removed, washed and DAPI is applied.

Deparafinisation
In the autostainer:
Warm up slide to 69°C and incubate for 3 cycles of 8 minutes each
Pre-treatment and blocking
Warm up slide to 95°C and incubate for 40 minutes (CC1)
Apply one drop of DISCOVERY Inhibitor and incubate for 4 minutes
2nd Cycle
- Pre-treatment: Warm up slide to 95°C and incubate for **16 minutes** (CC1)

3rd Cycle
- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

4th Cycle
- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

5th Cycle
- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

6th Cycle
- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

Washing
Remove the slides from the autostainer and was with EZ prep diluted 1:10 with distilled water (3 x 5 minutes).
DAPI
Dilute DAPI 1:2000 with PBS, add one drop on the slides
Let rest in a dark slide box for 10 minutes
Rinse with TBST then water
Coverslip
Two drops of prolong gold antifade reagent (without DAPI) is used per slide
After coverslipping, let slides dry in a dark place for at least 2 hours before scanning

993 AF.PDF

5h

1.8 **Autofluorescence**

The spectrum of autofluorescence can also be captured by running a slide through the autostainer deparafinisation and pretreatments without application of any antibodies and fluorophores, nor DAPI.

Deparafinisation
In the autostainer:
Warm up slide to 69°C and incubate for 3 cycles of 8 minutes each
Pre-treatment and blocking
Warm up slide to 95°C and incubate for 40 minutes (CC1)
Apply one drop of DISCOVERY Inhibitor and incubate for 4 minutes
2nd Cycle
- Pre-treatment: Warm up slide to 95°C and incubate for **16 minutes** (CC1)

3rd Cycle
- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

4th Cycle
- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

5th Cycle
- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

6th Cycle
- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

Washing
Remove the slides from the autostainer and was with EZ prep diluted 1:10 with distilled water (3 x 5 minutes).
Coverslip
Two drops of prolong gold antifade reagent (without DAPI) is used per slide
After coverslipping, let slides dry in a dark place for at least 2 hours before scanning

993 AF.PDF

2 Extracting the fluorophore spectra

Vectra 3.5 microscope is used to take snapshots of stained areas in the single-plex slides (withouth DAPI).

Snapshots are taken as multispectral images (MSI). Exposure times are set automatically for the filters relevant to each fluorophore. The filters where we do not expect to see signal from a fluorophore are set to the default value (150 ms for the MSI).

When auto-exposing, the exposure times at 20x magnification should each be between 20-100ms for all filters. Higher exposure times would mean that the signanl is in fact mostly autofluorescence. Lower exposure times could result in bleed through between the different markers in the multi-plex experiment. If exposure times need to be increased, the fluorophores can be diluted further to lower the signal's brightness.

Cells we see as positive for each fluorophore should appear only on the filters where we expect the fluorophore to emit. If these cells are seen in unexpected filters then they may be autofluorescing red blood cells instead of true positives, or the fluorophore might be too bright and should be diluted more.

InForm 2.4 software (PerkinELmer) is used to build the spectral library from these snapshots.

When using the new spectral library to unmix the single-plex snapshots used to build it (and any other images) the signal to noise ratio, i.e. the intensity units on stained cells versus the intensity of the background, should be ideally more than 10:1, and at least 5:1.

Multiplex experiment

17h

3 **Multi-plex staining**

1048 automated.PDF

The protocol is fully automated, except for the final steps, and lasts approximately 17 hours. For this reason we suggest running overnight and carrying out the final cycle (which is manual), washing, DAPI and coverslipping in the morning.

It is important that the single-plex experiments are satisfactory before the multi-plex experiments are started.

Preparation before starting

All antibodies and fluorophores will be pre-diluted and added in user fillable dispensers to be applied automatically by the autostainer. The dispensers can be used for at least a week following preparation (potentially longer but we have not tested this).

The following antibody dispensers are prepared by diluting the primary concentrates with the Antibody Diluent (included in the Opal kit). At least 1ml is added in each dispenser.

Table 2. Primary antibody dilutions. Antibodies are diluted with the antibody diluent included in the Opal kit.

Antibody	Volume Ratio
CD4	Pre-diluted

CD8	1:450
CD68	1:40
FOXP3	1:60
CD21	1:25
PD1	1:150

The PD1 antibody should only be used withing 2 days of diluting and preparing the dispenser. Thus the steps of the final cycle (6th) when PD1 is applied are preferably done by manually pipetting freshly prepared reagent onto the slide. Therefore no dispensers are prepared for PD1 and the corresponding Opal 690 fluorophore.

Light sensitive dispensers are preferably used for the fluorophores. The fluorophores are diluted with the Tyramide Signal Amplification liquid that is included in the Opal kit.

Table 2. Fluorophore dilutions. Fluorophores are diluted with the Tyramide Signal Amplification liquid included in the Opal kit.

Fluorophore	Fluorophore Dilution (Volume ratio)
Opal 520	1:75
Opal 540	1:150
Opal 570	1:60
Opal 620	1:150
Opal 650	1:75
Opal 690	1:75

Caution: User fillable dispensers if prepared incorectly could dispense less or more than the nominal 100µl per 'drop'. Please refer to manufacturer's instructions.

24m

3.1 **Deparafinisation**
In the autostainer:
Warm up slide to 69°C and incubate for 3 cycles of 8 minutes each

44m

3.2 **Pre-treatment and blocking**
Warm up slide to 95°C and incubate for 40 minutes (CC1)
Apply one drop of DISCOVERY Inhibitor and incubate for 4 minutes

48h

3.3 **1st Cycle**
- Warm up slide to 36°C
- Automatically apply 'one drop' of primary **CD4 antibody** (pre-diluted) and incubate for **16 minutes**
- Apply one drop of **Ultramap anti-Rb HRP** secondary and incubate for **16 minutes**
- Apply one drop of the **620** dispenser and incubate for **16 minutes**

1h 48m

3.4 **2nd Cycle**
- Pre-treatment: Warm up slide to 95°C and incubate for **16 minutes** (CC1)
- Warm up slide to 36°C
- Apply one drop of **CD68** dispenser and incubate for **60 minutes**
- Apply one drop of **Ultramap anti-Ms HRP** secondary and incubate for **16 minutes**
- Apply one drop of the **650** dispenser and incubate for **16 minutes**

1h 12m

3.5 **3rd Cycle**

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)
- Warm up slide to 36°C
- Apply one drop of **CD8** dispenser and incubate for **32 minutes**
- Apply one drop of **Ultramap anti-Ms HRP** secondary and incubate for **16 minutes**
- Apply one drop of the **540** dispenser and incubate for **16 minutes**

1h 48m

3.6 **4th Cycle**

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)
- Apply one drop of DISCOVERY Inhibitor and incubate for 4 minutes
- Warm up slide to 36°C
- Apply one drop of **CD21** dispenser and incubate for **60 minutes**
- Apply one drop of **Ultramap anti-Ms HRP** secondary and incubate for **24 minutes**
- Apply one drop of the **570** dispenser and incubate for **16 minutes**

1h 40m

3.7 **5th Cycle**

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)
- Warm up slide to 36°C
- Apply one drop of **FOXP3** dispenser and incubate for **60 minutes**
- Apply one drop of **Ultramap anti-Ms HRP** secondary and incubate for **16 minutes**
- Apply one drop of the **520** dispenser and incubate for **16 minutes**

1h 12m

3.8 **6th Cycle**

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)
- Warm up slide to 36°C
- Hand - apply (pipette) 110µl of diluted **PD1** and incubate for **32 minutes**
- Apply one drop of **Ultramap anti-Ms HRP** secondary and incubate for **16 minutes**
- Hand - apply 110µl of diluted **690** and incubate for **16 minutes**

15m

3.9 **Washing**

Remove the slides from the autostainer and wash with EZ prep diluted 1:10 with distilled water (3 x 5 minutes).

10m

3.10 **DAPI**

Dilute DAPI 1:2000 with PBS, add one drop on the slides
Rest in a dark slide box for 10 minutes
Rinse with TBST then water

3.11 **Coverslip**

Two drops of prolong gold antifade reagent (without DAPI) is used per slide
After coverslipping, let slides dry in a dark place for at least 2 hours before scanning

4 Scanning and unmixing

While scanning the multi-plex images all filters are auto-exposed in the Vectra microscope.

After scanning multispectrally at the same resolution as the single-plexes, the markers can be unmixed in inForm 2.4 using the spectral library.