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## Immunohistochemistry Staining for Tumor-associated Macrophage Polarization in Murine Subcutaneous Colon Tumor Allografts

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**[Abstract]** Tumor-associated macrophages are the most abundant immune cells in the tumor microenvironment and may express both anti-tumor and pro-tumor functions depending on their polarization towards an M1 (classical) or M2 (alternative) phenotype, respectively. This dual role has yet to be understood in many cancers, such as colon cancer, although it is known that pro-tumor functions include tumor growth, angiogenesis, immunosuppression, and matrix remodeling. Thus, tumor-associated macrophages have gained clinical momentum as cancer targets. To better understand the role of tumor-associated macrophages in the tumor microenvironment in response to various anti-cancer therapies, investigators typically implement an immunohistochemistry panel. However, immunohistochemistry protocols differ widely in their use of target surface markers, and many use intracellular antigens or only stain for single surface markers to quantify macrophage polarization, making reproducibility and study-to-study comparisons difficult. In response, we have developed a reliable and reproducible double immunostaining approach to quantify polarization of tumor-associated macrophages in murine tissue. Specifically, this immunohistochemistry staining protocol was developed for murine subcutaneous colon tumor allografts; however, this protocol can be readily modified to stain any murine tissue in allograft, xenograft, or orthotopic tumor models. This immunohistochemistry staining protocol is meant to be used and modified by investigators studying the causal effects between anti-cancer therapies and tumor-associated macrophages in murine tumors and to aid in reproducibility, consistency, and better study-to-study comparisons across the field.

**Keywords:** Murine, Tumor, Allograft, Immunofluorescence, Tumor-associated Macrophage, M1-M2 Polarization

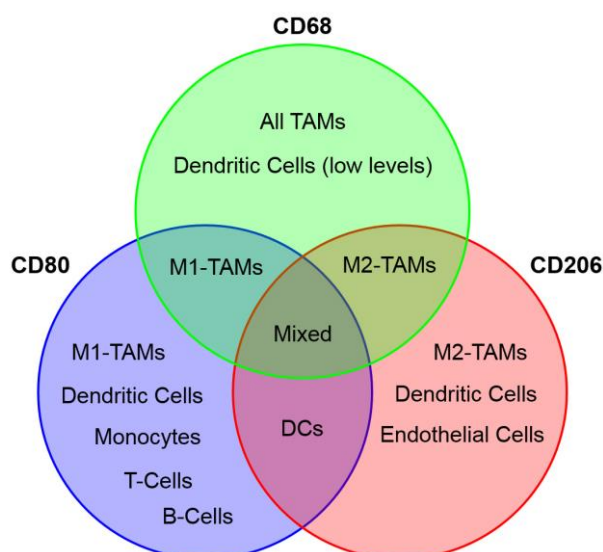
**[Background]** Tumor-associated macrophages (TAMs) are the most abundant immune cells in the tumor microenvironment (TME) in most cancers (Noy and Pollard, 2014), and also have the most substantial and pervasive effect of any immune cell in the TME (Chen *et al.*, 2005; Allavena *et al.*, 2008; Erreni *et al.*, 2011; Marech *et al.*, 2016). TAMs have been shown to have both anti-tumor and pro-tumor functions, depending on whether they are polarized more towards an M1 (classical) or M2 (alternative) phenotype (Chen *et al.*, 2005), respectively, and their physical location within a tumor (Marech *et al.*, 2016). In reality, M1 and M2 TAMs are the extremes of a continuum of intermediate cells which may have both anti-tumor and pro-tumor functions. Broad anti-tumor functions of classically activated M1-polarized TAMs include inflammation and immune response (Sugita *et al.*, 2002; Funada *et al.*, 2003; Zhou *et al.*, 2010). On the other hand, pro-tumor functions of alternatively activated M2-polarized TAMs

include tumor growth, angiogenesis, immunosuppression, and matrix remodeling (Liu and Cao, 2015). In most cancers, TAMs are skewed more towards the pro-tumor M2-phenotype (Yang and Zhang, 2017); therefore, TAMs have gained clinical momentum as immunotherapy targets for cancer. Currently, there are over 30 clinical trials targeting TAMs; strategies include reducing monocyte recruitment to the tumor and reprogramming M2-TAMs to M1-TAMs. Yang and Zhang provide a comprehensive review of TAM immunotherapy strategies (Yang and Zhang, 2017). Still many other groups are actively investigating TAMs in the basic science and pre-clinical realms (Cassetta *et al.*, 2016; He *et al.*, 2017; Jarosz-Biej *et al.*, 2018; Suarez-Lopez *et al.*, 2018). However, TAMs in some cancers, like colorectal cancer (CRC), simultaneously have both detrimental and beneficial effects on the patient (Zhong *et al.*, 2018).

The dual-role of TAMs in CRC has yet to be fully understood. It has recently been shown that macrophages induce resistance to fluorouracil-based chemotherapy, and that this TAM-induced resistance may contribute to the poor chemotherapy response in some CRC patients (Zhang *et al.*, 2008; Zhang *et al.*, 2016). Currently, a gap in colon cancer research is how therapy affects TAMs in the colorectal TME, and, in turn, how altering TAM population and polarization affects tumor therapeutic response. Therefore, this immunohistochemistry staining protocol was developed to stain for TAMs in murine subcutaneous colon tumor allografts, although this protocol can be used for other murine tissue. In this article, Balb/c mice were subcutaneously injected in the left flank with CT26 murine colon carcinoma cells. Tumors grew until they reached 75 mm<sup>3</sup>. Following three days of additional tumor growth, untreated tumors were dissected, flash-frozen in isopentane and liquid nitrogen, and stored at -80 °C until cryosectioning, and then sectioned at a thickness of 5 µm. The scope of this article is on macrophage immunohistochemistry of murine tumors, not on the tumor model itself. Therefore, this protocol can be readily modified to stain any murine tissue in allograft, xenograft, or orthotopic tumor models.

This direct immunohistochemistry staining method uses three primary-conjugated macrophage antibodies: anti-CD68, anti-CD80, and anti-CD206. CD68 is a pan-macrophage surface marker (Gordon *et al.*, 2014), CD80 is a cell surface marker for M1-type macrophages (Zhou *et al.*, 2017), and CD206 is a cell surface marker for M2-type macrophages (Kigerl *et al.*, 2009). CD68, CD80, and CD206 are markers for other cell types as well but were chosen such that (CD68<sup>+</sup>/CD80<sup>+</sup>)-cells were considered M1-TAMs, (CD68<sup>+</sup>/CD206<sup>+</sup>)-cells were considered M2-TAMs, and (CD80<sup>+</sup>/CD206<sup>+</sup>)-cells were considered dendritic cells (DCs). Cells expressing all macrophage surface markers were considered to have a mixed M1-M2 phenotype (Figure 1).

With this simple and reproducible method, we are able to accurately stain for M1 and M2 macrophages. To ensure long-term relevancy and usability of this protocol, this work also provides an easy-to-follow mathematical analysis of antibody concentrations so that readers can easily modify and optimize this protocol for their specific test system. This work is relevant for investigators developing and/or testing TAM-targeting cancer immunotherapies in mice.



**Figure 1. A double stain technique was used to distinguish M1- and M2- polarized macrophages.** CD68<sup>+</sup>/CD80<sup>+</sup> cells were considered M1 TAMs and CD68<sup>+</sup>/CD206<sup>+</sup> cells were considered M2 TAMs. Additionally, CD68<sup>+</sup>/CD80<sup>+</sup>/CD206<sup>+</sup> TAMs were considered to have a mixed phenotype.

## Materials and Reagents

### A. Consumables

1. Paper towel
2. Aluminum foil
3. Coverslip
4. 10 µl sterile universal Fit S<sup>3</sup> polymer pipette tips (VWR, catalog number: 89140-160)
5. 100 µl sterile universal Fit S<sup>3</sup> polymer pipette tips (VWR, catalog number: 89140-162)
6. 1,000 µl sterile universal Fit S<sup>3</sup> polymer pipette tips (VWR, catalog number: 89217-468)
7. SuperSpin<sup>TM</sup> microcentrifuge tubes (VWR, catalog number: 20170-038)
8. EasyDip<sup>TM</sup> slide staining rack (VWR, catalog number: CA87000-132)
9. Superfrost<sup>®</sup> plus micro slide (VWR, catalog number: 48311-703)
10. VistaVision<sup>TM</sup> cover glasses, No. 1, 22 x 50 mm (VWR, catalog number: 16004-314)

### B. Animal

1. Balb/c mice (aged 10 weeks)

### C. Antibodies

1. Brilliant Violet 421<sup>TM</sup> anti-mouse CD80 [stock solution concentration: 100 µg/ml, staining concentration: 5 µg/ml (1:20), storage temperature: 2-8 °C undiluted, shelf-life: ~3 years (lot-specific)] (BioLegend, catalog number: 104725)
2. Alexa Fluor<sup>®</sup> 488 anti-mouse CD68 [stock solution concentration: 500 µg/ml, staining

concentration: 5 µg/ml (1:100), used at 5 µg/ml, storage temperature: 2-8 °C undiluted, shelf-life: ~3 years (lot-specific)] (BioLegend, catalog number: 137012)

3. Alexa Fluor® 594 anti-mouse CD206 [stock solution concentration: 500 µg/ml, staining concentration: 4 µg/ml (1:125), storage temperature: 2-8 °C undiluted, shelf-life: ~3 years (lot-specific)] (BioLegend, catalog number: 141726)

#### D. Reagents

1. Acetone (storage temperature: -20 °C, shelf-life: 4 years) (VWR, catalog number: BDH1101-4LP)
2. Phosphate-buffered saline (PBS), 1x without Calcium and Magnesium (storage temperature: 2-8 °C, shelf-life: 3 years) (VWR, catalog number: 45000-446)
3. Triton™ X-100 (storage temperature: room temperature, shelf-life: 2 years) (Sigma Aldrich, catalog number: X100-100ML)
4. Tween® 20 (storage temperature: room temperature, shelf-life: 3 years) (Sigma-Aldrich, catalog number: P9416-100mL)
5. Goat serum (storage temperature: -20 °C, shelf-life: 2 years) (Sigma-Aldrich, catalog number: G9023-10ML)
6. Sodium azide (NaN<sub>3</sub>) (storage temperature: room temperature) (Sigma-Aldrich, catalog number: S2002-25G)

*Note: This compound is toxic (GHS06), a health hazard (GHS08), and an environmental hazard (GHS09). It should be handled with protective clothing in a certified fume hood and disposed of at an approved waste disposal site per institutional regulations.*

7. Bovine serum albumin (BSA) (storage temperature: 2-8 °C, shelf-life: 1 year) (Sigma-Aldrich, catalog number: A8806-5G)
8. Universal antibody dilution buffer (storage temperature: room temperature, shelf-life: 2 years) (Sigma-Aldrich, catalog number: U3510-100ML)
9. Isopentane (storage temperature: room temperature, shelf-life: 2 years) (VWR, catalog number: AA19387-AP)
10. Liquid nitrogen (Airgas, catalog number: NI NF180LT22)
11. Quick-dry nail polish
12. Fluoromount-G® slide mounting medium (storage temperature: room temperature, shelf-life: 15 months) (VWR, catalog number: 100241-874)
13. Macrophage antibody cocktail (see Recipes)
14. Blocking solution (see Recipes)
15. PBS-T washing solution (see Recipes)

#### Equipment

1. Tweezer



2. 0.5-10 µl ergonomic high performance single-channel mechanical pipettor (VWR, catalog number: 89079-962)
3. 10-100 µl ergonomic high performance single-channel mechanical pipettor (VWR, catalog number: 89079-968)
4. 100-1,000 µl ergonomic high performance single-channel mechanical pipettor (VWR, catalog number 89079-974)
5. Linear pipettor stand (VWR, catalog number: 40000-272)
6. 80-Place storage system (VWR, catalog number: 30128-282)
7. EasyDip™ slide staining jars, white (VWR, catalog number: CA87000-126)
8. Low temperature organic liquid filled thermometer (VWR, catalog number: 89062-908)
9. SlideTray™ 20 slide humidity chamber with black lid (VWR, catalog number: 102097-504)
10. Laboratory bench and table protector with leakproof and moisture barrier (VWR, catalog number: 89126-790)
11. -20 °C and -80 °C freezers
12. Water bath (VWR, catalog number: 89501-464)
13. Upright microscope (Nikon, model: Eclipse Ni-U)
14. Monochrome digital camera (Nikon, model: DS-Qi1Mc)
15. PC-based camera control unit (Nikon, model: DS-U3)
16. SOLA Light Engine® fluorescent lamp (Lumencor, catalog number: SOLA SM 6-LCR-SB)
17. DAPI filter set (Chroma Technology, catalog number: 49000)
18. FITC filter set (Chroma Technology, catalog number: SP101)
19. Texas red filter set (Chroma Technology, catalog number: 41004)
20. Objective lens, 20x/0.50NA (Nikon, catalog number: CFI Plan Fluor 20X)

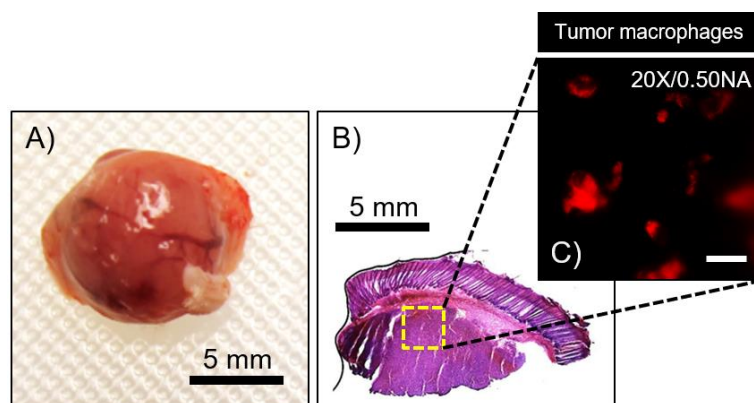
## **Software**

1. NIS-Elements F Ver4.60.00 for 64bit edition (Nikon, <https://www.nikoninstruments.com/Products/Software>)
2. ImageJ bundled with 64-bit Java 1.8.0\_112 (National Institutes of Health and the Laboratory for Optical Computational Instrumentation, <https://imagej.nih.gov/ij/download.html>)

## **Procedure**

The scope of this article is on macrophage immunohistochemistry of murine tumors, not on the tumor model itself; however, the tumor model is briefly described here. Five Balb/c mice (aged 10 weeks) were subcutaneously injected with  $1 \times 10^5$  CT26 cells (passage 3) in sterile saline into the left flank until the tumors reached a volume of 75 mm<sup>3</sup> (Greening *et al.*, 2018a and 2018b). Three days after reaching this volume, the five untreated mice were euthanized, and the tumor was dissected for TAM analysis (Figure 2). This staining and imaging procedure works best with flash frozen tumors. Therefore, upon dissection,

tumors were placed in a cryomold and covered completely in optimal cutting temperature (OCT). The cryomold with OCT and tumor was flash frozen in isopentane chilled in liquid nitrogen at  $-75^{\circ}\text{C}$  to  $-77^{\circ}\text{C}$  for at least 15 s and stored permanently in  $-80^{\circ}\text{C}$  until sectioning. The tumor was sectioned at  $5\text{ }\mu\text{m}$ , although sections up to  $10\text{ }\mu\text{m}$  are acceptable. Once sectioned, slides were stored for up to one month at  $-80^{\circ}\text{C}$  before staining. All antibody solutions and stained slides were handled in darkness.



**Figure 2. Murine colon tumor allografts were (a) dissected and flash frozen.** Tumors were (b) sectioned at  $5\text{ }\mu\text{m}$  thickness (H&E shown for clarity, yellow box not to scale). Tumor sections were (c) stained for TAMs and imaged with a 20x objective at 10 regions of interest (ROI) along the section. Scale bar =  $30\text{ }\mu\text{m}$ . Images enhanced for publication.

A. Preparing slides for staining (20 min)

*Note: Keep at least 90 ml of acetone stored at  $-20^{\circ}\text{C}$  for at least 1 h prior to start of procedure.*

1. Remove  $5\text{ }\mu\text{m}$ -thick slides from  $-80^{\circ}\text{C}$ .
2. Place all slides in  $-20^{\circ}\text{C}$ .
3. Keep the acetone for 20 min at  $-20^{\circ}\text{C}$ .
4. Prepare macrophage antibody cocktail (see Recipes).
5. Set out four EasyDip™ slide staining jars.
6. Fill 3 staining jars with 90 ml of room temperature sterile PBS-T (see Recipes).
7. Remove blocking solution aliquots (see Recipes) from  $4^{\circ}\text{C}$  and set in 80-Place Storage System at room temperature.
8. 20 min after Step A3, remove acetone from  $-20^{\circ}\text{C}$ .
9. Fill final staining jar with 90 ml of  $-20^{\circ}\text{C}$  acetone.
10. Place low-temperature thermometer in 1<sup>st</sup> staining jar with acetone.

B. Fixation with acetone (10 min)

1. When the acetone reaches  $-3^{\circ}\text{C}$ , remove slides from  $-20^{\circ}\text{C}$ .
2. Place slides (up to 12) in the EasyDip™ slide staining rack.
3. Place the staining rack (with slides) in cold acetone for 10 min.
4. Remove from acetone and air dry for 5 s.

C. Wash with PBS-T (#1) (5 min)

1. Place the staining rack in PBS-T (2<sup>nd</sup> staining jar) for 2 min.
2. Dip the staining rack in PBS-T for 1 min such that each “dip” is 3 s (20 total dips).
3. Swirl staining rack inside slide staining jar for 1 min such that each “swirl” is 1 s (60 total swirls).
4. Keep staining rack in PBS-T unmoving for an additional 1 min.

D. Adding blocking solution (2 h)

1. Remove staining rack from PBS-T (2<sup>nd</sup> staining jar).
2. Remove 3 slides from the staining rack.
3. Keep the other slides (if there are more than 3) in the staining rack and place back in PBS-T.
4. Using a paper towel, dry the area around the tissue sections without touching the tissue sections.  
*Note: A hydrophobic barrier PAP pen may be used to keep reagents localized on the tissue specimen but is not required.*
5. Add 50-100 µl (depending on size of tissue section) blocking solution to each tissue section.
6. Place slides (with blocking solution) in a dark humidified chamber at room temperature.
7. Repeat Steps D1-D6 for additional slides.
8. Incubate in the dark humidified chamber at room temperature for 60 min.
9. Reapply 50-100 µl blocking solution to each tissue section.
10. Incubate in the dark humidified chamber at room temperature for 60 min.

E. Adding macrophage antibody cocktail (2 h)

*Note: The following steps must be performed in a dark room.*

1. Remove macrophage antibody cocktail from 4 °C and remove aluminum foil.
2. Remove 1 slide from the dark humidified chamber.
3. Using a paper towel, dry the area around the tissue sections without touching the tissue sections.
4. Add 100 µl of macrophage antibody cocktail to each tissue section.
5. Place slide with macrophage antibody cocktail in a dark humidified chamber.
6. Repeat Steps E2-E5 for additional slides.
7. Incubate in the dark humidified chamber at room temperature for 2 h.

F. Wash with PBS-T (#2) (5 min)

*Note: The following steps must be performed in a dark room.*

1. Remove the dark humidified chamber from 4 °C.
2. Place all slides back in staining rack.
3. Place the staining rack in PBS-T (3<sup>rd</sup> staining jar) for 2 min.
4. Dip the staining rack in PBS-T for 1 min such that each “dip” is 3 s (20 total dips).
5. Swirl staining rack inside slide staining jar for 1 min such that each “swirl” is 1 s (60 total swirls).
6. Keep staining rack in PBS-T unmoving for an additional 1 min.



#### G. Wash with PBS-T (#3) (5 min)

*Note: The following steps must be performed in a dark room.*

1. Place the staining rack in PBS-T (4<sup>th</sup> staining jar) for 2 min.
2. Dip the staining rack in PBS-T for 1 min such that each “dip” is 3 s (20 total dips).
3. Swirl staining rack inside slide staining jar for 1 min such that each “swirl” is 1 s (60 total swirls).
4. Keep staining rack in PBS-T unmoving for an additional 1 min.

#### H. Mounting slides

*Note: The following steps must be performed in a dark room.*

1. Remove staining rack from PBS-T (4<sup>th</sup> staining jar).
2. Set all slides face-up on paper towel.
3. Using a paper towel, dry the area around the tissue sections without touching the tissue sections.
4. Add 1-2 drops of Fluoromount-G® Slide Mounting Medium to each tissue section.
5. Gently place 22 x 50 mm coverslip on each slide.
6. Using tweezers, gently press out air bubbles.
7. Wipe off any excess mounting medium.
8. Let slides sit for 5 min.
9. Apply quick-dry nail polish along the edges of the coverslip. Ensure edges are completely covered to prevent drying out of slides.
10. Leave slides to dry for 1 h.
11. Store slides permanently in the dark at 4 °C.

### **Data analysis**

#### A. Imaging

1. Remove slides from 4 °C, but keep in the dark.
2. Place slide under a microscope.
3. Turn on fluorescent lamp, microscope, and camera.
4. Open imaging software.
5. Change the fast focus to 1,280 x 1,024 no binning.
6. Change the quality capture to 1,280 x 1,024 no binning.
7. Set the exposure to 100 ms.
8. Set the gain to 1x.
9. Switch to the 20x objective.
10. Use the DAPI filter and bring the CD80<sup>+</sup> cells into focus.
11. Image the CD80-stained cells.

*Note: In the 5 µm thick slide, some cells may be in-focus while others are out-of-focus. Starting 1 µm beneath the tissue surface, adjust the z-axis travel 1 µm between each step and take at least 4 images of the same location in the x-y plane (Figure 3).*

12. Without moving tissue location, switch to the FITC filter and bring the CD68<sup>+</sup> cells into focus.
13. Image the CD68-stained cells.

*Note: See the note under step A11 (Image the CD80-stained cells).*

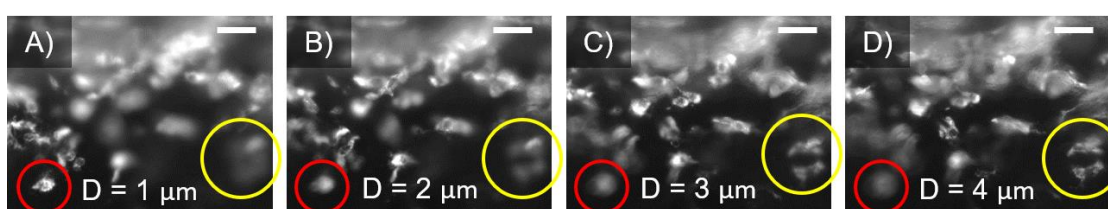
14. Without moving tissue location, use the Texas Red filter and bring the CD206<sup>+</sup> cells into focus.
15. Image the CD206-stained cells.

*Note: See the note under step A11 (Image the CD80-stained cells).*

16. Switch to a new tissue location and repeat Steps A10-A15.

17. Acquire images from the desired amount of tissue locations.

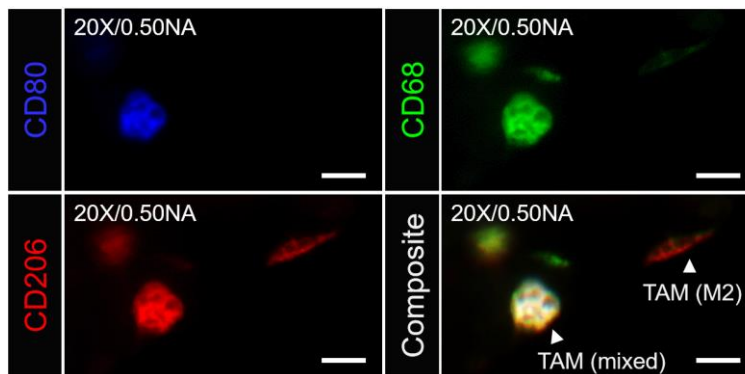
*Note: In this study, 10 regions-of-interests (ROI) per tumor were imaged (image area = 31,100  $\mu\text{m}^2$ ).*



**Figure 3. Multiple images in varying z-axis depths of the same x-y location may be needed for accurate TAM counting.** Notice the red-circled cell starts in-focus at (A) and is out-of-focus at (D). Alternatively, notice the yellow-circled cells start off out-of-focus at (A) and are in-focus at (D). Each image (A-D) represents a 1  $\mu\text{m}$  step along the z axis. Scale bars = 40  $\mu\text{m}$ . 20x/0.50NA objective used for each image. Images enhanced for publication. This part of the protocol may be modified depending on tissue section thickness and desired accuracy when quantifying image-based cell counts.

#### B. TAM counting and polarization

1. Count CD68<sup>+</sup> cells in each ROI.
2. Count CD80<sup>+</sup> cells in each ROI.
3. Count CD206<sup>+</sup> cells in each ROI.
4. Determine the number of CD68<sup>+</sup>/CD80<sup>+</sup> cells in each ROI. These are M1-polarized TAMs.
5. Determine the number of CD68<sup>+</sup>/CD206<sup>+</sup> cells in each ROI. These are M2-polarized TAMs.
6. Determine the number of CD68<sup>+</sup>/CD80<sup>+</sup>/CD206<sup>+</sup> cells in each ROI. These are TAMs with a mixed M1/M2 phenotype (Figure 4).



**Figure 4.** Image of several TAMs imaged with DAPI (for CD80<sup>+</sup>), FITC (for CD68<sup>+</sup>), and Texas Red (for CD206<sup>+</sup>) filter sets. The composite image (ImageJ) shows a TAM with an M2 phenotype and a TAM with a mixed M1-M2 phenotype. Scale bars = 30  $\mu$ m. 20x/0.50NA objective used for each image.

7. If desired, determine the number of CD80<sup>+</sup>/CD206<sup>+</sup> cells in each ROI. There are dendritic cells.
8. Calculate the average number of M1 TAMs (included TAMs with mixed phenotype) per image area.

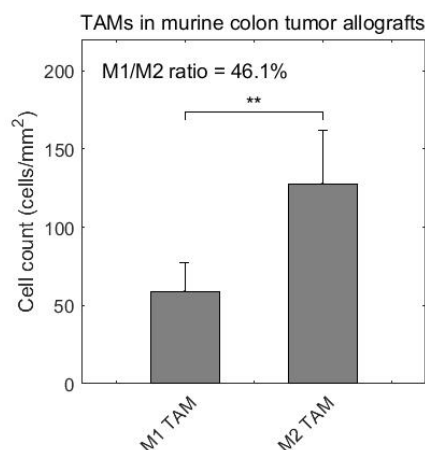
$$TAM_{M1} = (CD68^+/CD80^+ \text{ cells}) + (CD68^+/CD80^+/CD206^+ \text{ cells})$$

9. Calculate the average number of M2 TAMs (including TAMs with mixed phenotype) per image area.

$$TAM_{M2} = (CD68^+/CD206^+ \text{ cells}) + (CD68^+/CD80^+/CD206^+ \text{ cells})$$

10. Calculate average M1/M2 ratio per mouse (Figure 5).

$$TAM_{ratio} = \left( \frac{TAM_{M1}}{TAM_{M2}} \right)$$

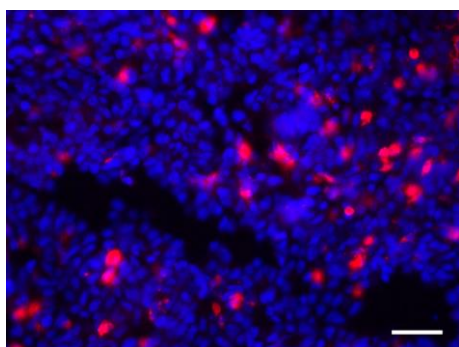


**Figure 5.** TAMs in subcutaneous murine colon tumor allografts. In untreated Balb/c-CT26

tumors, 3 days after the tumor reaches a volume of 75 mm<sup>3</sup>, there is a significant difference ( $P < 0.01$ ) between the number of M1 ( $58.8 \pm 18.7$  cells/mm<sup>2</sup>) and M2 TAMs ( $127.5 \pm 34.2$  cells/mm<sup>2</sup>) in the tumor, resulting in an M1/M2 ratio of 46.1%.

## Notes

This protocol can be modified to include nuclear staining. Figure 6 shows a Balb/c-CT26 subcutaneous colon tumor allograft tissue section stained with DAPI and CD206 only.



**Figure 6. An untreated Balb/c-CT26 subcutaneous colon tumor allograft tissue section co-stained with DAPI and Alexa Fluor<sup>®</sup> 594 anti-mouse CD206.** Scale bars = 100  $\mu$ m. 20x/0.50NA objective used for each image.

A limitation of this staining protocol is the absence of a reliable stain for DCs. In addition to being a commonly used pan-macrophage marker (Gordon *et al.*, 2014), CD68 can also stain for DCs at low levels in some tissues such as the lung (Yu *et al.*, 2016, Figure 1). To control for the possibility of DCs, this protocol may be modified to include a panel of DC-specific markers for murine non-lymphoid tissues on adjacent tissue sections (Yu *et al.*, 2016).

The following calculations aid in reproducibility of results and modification of the protocol for specific test systems. Stock antibody concentrations and amount of antibodies per vial are accurate up to the publication of this article and are subject to change based on manufacturer lot specifications. The desired dilution from stock were experimentally-determined in lab for the Balb/c-CT26 murine subcutaneous allograft model and may be different per specific test system. The volume of macrophage antibody cocktail represents the total volume of diluted antibody mix placed on the tissue section via micropipette before incubation at room temperature for 2 h. The average area of our tissue sections was  $\sim 50$  mm<sup>2</sup>. Thus, we recommend a macrophage antibody cocktail volume per tissue section area of  $\sim 2$   $\mu$ l/mm<sup>2</sup> (100  $\mu$ l total per section).

The proceeding calculations determine the volume of concentrated stock antibody to add to the macrophage antibody cocktail, and how many trials can be performed with these settings.

1. Brilliant Violet 421<sup>™</sup> anti-mouse CD80

## Constants

Stock Antibody Concentration*	100 µg/ml
Desired Dilution from Stock*	1:20
Amount of Antibodies per Vial*	100 µg
Volume of Macrophage Antibody Cocktail per Tissue Section**	100 µl

\* Values subject to change based on manufacturer specifications and recommendations

\*\* Value depends on size of tissue section

## Calculations

Desired Volume of Stock per Trial	$\left[\frac{100 \mu\text{l}}{1}\right] \cdot \left[\frac{1}{20}\right] = 5 \mu\text{l}$
Desired Antibody Amount per Trial	$\left[\frac{100 \mu\text{l}}{1}\right] \cdot \left[\frac{1}{20}\right] \cdot \left[\frac{100 \mu\text{g}}{\text{ml}}\right] \cdot \left[\frac{1 \text{ ml}}{1000 \mu\text{l}}\right] = 0.5 \mu\text{g}$
Desired Antibody Concentration in Macrophage Antibody Cocktail	$\left[\frac{1}{20}\right] \cdot \left[\frac{100 \mu\text{g}}{1 \text{ ml}}\right] = 5 \mu\text{g/ml}$
Trials per Vial of Antibodies	$\left[\frac{100 \mu\text{g}}{1}\right] \cdot \left[\frac{1}{0.5 \mu\text{g}}\right] = 200$

## 2. Alexa Fluor® 488 anti-mouse CD68

### Constants

Stock Antibody Concentration*	500 µg/ml
Desired Dilution from Stock*	1:100
Amount of Antibodies per Vial*	100 µg
Volume of Macrophage Antibody Cocktail per Tissue Section**	100 µl

\* Values subject to change based on manufacturer specifications and recommendations

\*\* Value depends on size of tissue section

### Calculations

Desired Volume of Stock per Trial	$\left[\frac{100 \mu\text{l}}{1}\right] \cdot \left[\frac{1}{100}\right] = 1 \mu\text{l}$
Desired Antibody Amount per Trial	$\left[\frac{100 \mu\text{l}}{1}\right] \cdot \left[\frac{1}{100}\right] \cdot \left[\frac{500 \mu\text{g}}{\text{ml}}\right] \cdot \left[\frac{1 \text{ ml}}{1000 \mu\text{l}}\right] = 0.5 \mu\text{g}$
Desired Antibody Concentration in Macrophage Antibody Cocktail	$\left[\frac{1}{100}\right] \cdot \left[\frac{500 \mu\text{g}}{1 \text{ ml}}\right] = 5 \mu\text{g/ml}$
Trials per Vial of Antibodies	$\left[\frac{100 \mu\text{g}}{1}\right] \cdot \left[\frac{1}{0.5 \mu\text{g}}\right] = 200$

## 3. Alexa Fluor® 594 anti-mouse CD206

### Constants

Stock Antibody Concentration*	500 µg/ml
Desired Dilution from Stock*	1:125
Amount of Antibodies per Vial*	100 µg
Volume of Macrophage Antibody Cocktail per Tissue Section**	100 µl

\* Values subject to change based on manufacturer specifications and recommendations

\*\* Value depends on size of tissue section

## Calculations

Desired Volume of Stock per Trial	$\left[\frac{100 \mu l}{1}\right] \cdot \left[\frac{1}{125}\right] = 0.8 \mu l$
Desired Antibody Amount per Trial	$\left[\frac{100 \mu l}{1}\right] \cdot \left[\frac{1}{125}\right] \cdot \left[\frac{500 \mu g}{ml}\right] \cdot \left[\frac{1 ml}{1000 \mu l}\right] = 0.4 \mu g$
Desired Antibody Concentration in Macrophage Antibody Cocktail	$\left[\frac{1}{125}\right] \cdot \left[\frac{500 \mu g}{1 ml}\right] = 4 \mu g/ml$
Trials per Vial of Antibodies	$\left[\frac{100 \mu g}{1}\right] \cdot \left[\frac{1}{0.4 \mu g}\right] = 250$

## Recipes

### 1. Macrophage antibody cocktail

*Note: The following steps must be prepared in the dark.*

- Determine the number of tissue sections to be stained
- Add 100  $\mu l$  of universal antibody dilution buffer, per number of tissue sections, to microcentrifuge tube

*Note: This volume of 100  $\mu l$ /section can be changed based on the size of the specific tissue section. More than one microcentrifuge tube may be used if staining multiple tissue sections requiring > 1 ml.*

- Add Brilliant Violet 421™ anti-mouse CD80 to universal antibody dilution buffer at 5  $\mu g/ml$  (i.e., 1:20 dilution from 100  $\mu g/ml$  stock)
- Add Alexa Fluor® 488 anti-mouse CD68 to universal antibody dilution buffer at 5  $\mu g/ml$  (i.e., 1:100 dilution from 500  $\mu g/ml$  stock)
- Add Alexa Fluor® 594 anti-mouse CD206 to universal antibody dilution buffer at 4  $\mu g/ml$  (i.e., 1:125 dilution from 500  $\mu g/ml$  stock)
- Gently and sufficiently pipette up and down to mix the macrophage antibody cocktail
- Wrap microcentrifuge tubes with macrophage antibody cocktail with aluminum foil and store at 4 °C until ready for use

### 2. Blocking solution

- Bring sterile PBS to 37 °C in a water bath
- Bring goat serum to room temperature

*Note: The following steps must be prepared in a certified biological safety cabinet.*

- Add 4% (v/v) of goat serum to PBS
- Add 0.5% (w/v) BSA to PBS
- Add 0.1% (w/v) NaN<sub>3</sub> to PBS
- Perform sterile vacuum filtration of the blocking solution
- Add 0.1% (v/v) Triton™ X-100 to PBS



- h. Add 0.05% (v/v) Tween® 20 to PBS
- i. Shake solution vigorously until Triton™ X-100 and Tween® 20 have dispersed  
*Note: If needed, solution can be placed in a 37 °C water bath for 5-10 min to aid dispersion.*
- j. Aliquot blocking solution into sterile microcentrifuge tubes
- k. Store blocking solution at 4 °C (can be stored for up to 2 months)
3. PBS-T washing solution
  - a. Bring sterile PBS to 37 °C in a water bath  
*Note: The following steps must be prepared in a certified biological safety cabinet.*
  - b. Add 0.2% (v/v) of Tween® 20 to PBS (1 ml)
  - c. Shake solution vigorously until Triton™ X-100 and Tween® 20 have dispersed  
*Note: If needed, solution can be placed in a 37 °C water bath to aid dispersion.*
  - d. Store PBS-T washing solution at room temperature

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### **Competing interests**

The authors declare that there are no conflicts of interests or competing interests related to this article.

### **Ethics**

The study was approved by the University of Arkansas Institutional Animal Care and Use Committee (IACUC #18060).

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