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Immunofluorescence for detection of ALFA-tag in S. rosetta V.1

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Fredrick leon¹, David Booth²

¹UCSF; ²University of California, San Francisco



David Booth

University of California, San Francisco

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Protocol status: Working

We use this protocol and it's working

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Abstract

Immunofluorescence (IF) allows for the visualization of protein localization in fixed-cell samples. This protocol builds upon commonly used IF methods, with a focus on retaining and minimally damaging *S. rosetta*'s collar and flagella. It also introduces a new permeabilization buffer composed of LICOR Intercept blocking buffer for greater anti-ALFA tag nanobody specificity and signal. This protocol has been optimized for IF with the ALFA tag, but works well for common antibodies, such as DM1A and phalloidan. This protocol was developed with essential input from Flora Rutaganira and Alain Garcia de las Bayones

Protocol materials

⊗ Synthetic Seawater ASTM D 1141 **Ricca Chemical Company Catalog #8363**

⊗ Poly-D-Lysine Hydrobromide **Fisher Scientific Catalog #ICN10269410**

⊗ clear bottomed 96 well plates **MP Biomedicals Catalog #102694**

⊗ INTERCEPT blocking buffer, PBS **LI-COR Catalog #927-70001**



Grow cells

1 Seed cells at 10,000 cells/mL 24 hours in advance and grow at 22 °C

2 On the day of imaging, gently concentrate cells by centrifugation at

500 x g, 22°C, 00:05:00

5m

3 Carefully remove supernatant with a pipette, leaving the last 50-100 µL

Note

Leaving behind some supernatant is because the cell pellet is very delicate after the light spin and may not be visible.

4 Gently swirl the remaining supernatant and cells to resuspend the pellet. Do NOT vortex.

Adsorb cells to Glass-Bottom Dish

45m

5 Pipette 50 µL of 10 mg/mL Poly-D-Lysine onto the glass bottom of a glass-bottomed 96 well plate and incubate for 00:15:00 at Room temperature .

Poly-D-Lysine Hydrobromide **Fisher Scientific Catalog #ICN10269410**

clear bottomed 96 well plates **MP Biomedicals Catalog #102694**

6 Remove the excess liquid and add 50 µL filtered Synthetic Seawater (SSW) . Repeat twice more for a total of 3 washes.

Synthetic Seawater ASTM D 1141 **Ricca Chemical Company Catalog #8363**

7 Gently pipette 50 µL cells onto the surface of the glass. Use a wide-bore pipette tip (or cut a 1000 µl pipette tip) to decrease shear forces on cells. Let the cells settle on the

15m

surface for  00:15:00 .

Prepare buffers

- 8 Assemble The following buffers, and use all at room temperate

PFA fix buffer: Fixes the cells initially

A	B
100 μ L	10x CSB
187.5 μ L	16% paraformald ehyde
250 μ L	60% (w/v) sucrose
462.5 μ L	water
1 mL	total

This buffer is made fresh each time

- 9 **PFA/Tween fix buffer:** Continues the fixation process and the Tween addition very gently permeabilizes cells

A	B
100 μ L	10x CSB
187.5 μ L	16% paraformald ehyde
250 μ L	60% (w/v) sucrose
3.5 μ L	20% (v/v) Tween 20
459 μ L	water
1 mL	total

This buffer is made fresh each time

**10 Glycine wash buffer** : Quenches the remaining PFA from the previous fixation steps

A	B
500 μ L	10x CSB
1500 μ L	1M glycine, ph 6.1
1250 μ L	60% (w/v) sucrose
1750 μ L	water
5 mL	total

this can be made in bulk and stored at 4°C

11 PermB-meOH buffer: Permeabilizes the cells with a higher Tween concentration and methanol

A	B
870 μ L	LICOR Intercept blocking buffer, PBS
50 μ L	20% (v/v) Tween 20
80 μ L	methanol
1 mL	total

This buffer is made fresh each time

☒ INTERCEPT blocking buffer, PBS LI-COR Catalog #927-70001

12 PermB buffer: Permeabilization buffer without methanol for washing cells and diluting antibodies

A	B
950 μ L	LICOR Intercept blocking buffer, PBS
50 μ L	20% (v/v) Tween 20



	A	B
	1 mL	total

This buffer is made fresh each time

- 13 **PEM buffer** : For washing cells of excess antibodies and matching the reflective index of water immersion objectives

	A	B
	400 mL	1M PIPES, ph 6.1
	5 mL	1M EGTA
	5 mL	1M MgCl ₂
	1 L	total

This can be made in bulk and stored at room temperature

Cell Fixation

10m

- 14 Gently apply  50 µL PFA fix buffer and incubate for  00:05:00

5m

Note

How to gently apply buffers henceforth.

1. Using gel loading pipette tips only, slowly pipette 50 µl of buffer on the left side of the well.
2. Afterwards, slowly remove 50 µl from the right side of the dish.
3. ALWAYS leave the cells covered in the remaining 50 µl of liquid.
4. All steps are performed at  Room temperature .

- 15 Gently apply  50 µL PFA/Tween fix buffer and incubate for  00:05:00

5m

- 16 Gently apply  50 µL glycine wash buffer and proceed immediately to permeabilization

Permeabilization and Blocking

1h

17 Gently apply  50 µL PermB-meOH buffer and incubate for  00:15:00

15m

18 During the incubation, dilute antibodies in PermB buffer

	A	B	C	D	E	F	G
	Antigen	Source	Ex nm	Em nm	[Stock]	[Final]	Dilution
	alpha-Tubulin	Mouse (DM1A)			0.5 mg/ml	10 µg/ml	1/500
	Rosetteless	Rabbit			UNK	UNK	1/400
	Mouse IgG	Recombinant Nanobody	568	603			1/500
	Rabbit IgG	Recombinant Nanobody	647	665			
	DNA dye	POPO-1	434	456	0.1 mM	0.1 µM	1/1000
	F-Actin	Phalloidin	488	518	200 U/ml	0.8 U/ml	1/250
	ALFA tag	Recombinant Nanobody	650	670	5 µM	20 nM	1/250

Note

Henceforth keep the sample in the dark to protect from photobleaching!

19 Gently apply  50 µL PermB buffer

Note

Use PermB buffer WITHOUT antibodies, as this is only a wash step.

Apply Stains

1h 10m



- 20 Gently apply  50 μ L diluted antibodies . Repeat one more for a total of 2 applications to ensure the antibodies are not too dilute. Incubate for  01:00:00

1h

Note

Cover the plate/samples during this incubation to minimize photobleaching.

- 21 Gently apply  50 μ L PEM . Repeat one more for a total of 2 applications to properly wash the cells of unbound antibodies.

Image Samples

10m

- 22 With the cells in PEM buffer, proceed to imaging.

Protocol references

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