Sep 03, 2024 Version 1

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

DOI
dx.doi.org/10.17504/protocols.io.5jyl85k38l2w/v1

Fredrick leon[1], David Booth[2]

[1]UCSF; [2]University of California, San Francisco

David Booth
University of California, San Francisco

DOI: dx.doi.org/10.17504/protocols.io.5jyl85k38l2w/v1

Protocol Citation: Fredrick leon, David Booth 2024. Immunofluorescence for detection of ALFA-tag in S. rosetta . **protocols.io** **https://dx.doi.org/10.17504/protocols.io.5jyl85k38l2w/v1**Version created by **David Booth**

Protocol status: Working
We use this protocol and it's working

Created: May 19, 2021

Last Modified: September 03, 2024

Protocol Integer ID: 104831

Keywords: choanoflagellate, immunofluorescence, ALFA-tag

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

Poly-D-Lysine Hydrobromide **Fisher Scientific Catalog #**ICN10269410	Step 5
clear bottomed 96 well plates **MP Biomedicals Catalog #**102694	Step 5
INTERCEPT blocking buffer, PBS **LI-COR Catalog #**927-70001	Step 11
Synthetic Seawater ASTM D 1141 **Ricca Chemical Company Catalog #**8363	Step 6

Grow cells

1 Seed cells at 10,000 cells/mL 24 hours 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 [M] 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 . 15m

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 surface for 15m

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 µL	10x CSB
	187.5 µL	16% paraform aldehyde
	250 µL	60% (w/v) suc rose
	462.5 µL	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 µL	10x CSB
	187.5 µL	16% paraform aldehyde
	250 µL	60% (w/v) suc rose
	3.5 µL	20% (v/v) Twe en 20
	459 µL	water
	1 mL	total

This buffer is made fresh each time

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

	A	B
	500 µL	10x CSB
	1500 µL	1M glycine, ph 6.1

	A	B
	1250 µL	60% (w/v) sucrose
	1750 µL	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 µL	LICOR Intercept blocking buffer, PBS
	50 µL	20% (v/v) Tween 20
	80 µL	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 µL	LICOR Intercept blocking buffer, PBS
	50 µL	20% (v/v) Tween 20
	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 MgCl2

	A	B
	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 (DM1 A)			0.5 mg/ml	10 µg/ml	1/500
	Rosetteless	Rabbit			UNK	UNK	1/400
	Mouse IgG	Recombinant	568	603			1/500

	A	B	C	D	E	F	G
		Nanobody					
	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 µL 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 µL 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

Götzke H, Kilisch M, Martínez-Carranza M, Sograte-Idrissi S, Rajavel A, Schlichthaerle T, Engels N, Jungmann R, Stenmark P, Opazo F, Frey S. The ALFA-tag is a highly versatile tool for nanobody-based bioscience applications. Nat Commun. 2019 Sep 27;10(1):4403. doi: 10.1038/s41467-019-12301-7. PMID: 31562305; PMCID: PMC6764986.