

Aug 09, 2024

Version 1

An improved whole-mount immunofluorescence protocol for juvenile *Amphimedon queenslandica* V.1

DOI

<https://dx.doi.org/10.17504/protocols.io.e6nvw1o2wlmk/v1>

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Protocol Citation: Bin Yang, Bernard M. Degnan, Sandie M Degnan 2024. An improved whole-mount immunofluorescence protocol for juvenile *Amphimedon queenslandica*. **protocols.io** <https://dx.doi.org/10.17504/protocols.io.e6nvw1o2wlmk/v1>

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Protocol status: Working

We use this protocol and it's working

Created: July 25, 2024

Last Modified: August 09, 2024

Protocol Integer ID: 104038

Keywords: whole-mount immunofluorescence, sponge, *Amphimedon queenslandica*, interferon regulatory factor 2, mount immunofluorescence protocol for juvenile *amphimedon queenslandica* sponge, low abundance proteins in other sponge, sponge species, immunofluorescence protocols for *amphimedon queenslandica*, juvenile *amphimedon queenslandica* sponge, underdeveloped in sponge, other sponge, juvenile sponge, multiple sponge, existing immunofluorescence protocol, sponge, immunofluorescence, mount immunofluorescence protocol, immunostaining pattern, subcellular detection of irf2, subcellular localisation of the low abundance transcription factor, detecting low abundance protein, phylogenetic position in the animal kingdom, subcellular detection, subcellular localisation, antigen masking, tailored antibody, immunostaining step, low abundance transcription factor, phylogenetic position, interferon regulatory factor, cellular processes in animal model, unique perspective on animal evolution, animal evolution

Funders Acknowledgements:

Australian Research Council

Grant ID: DP190102521

Australian Research Council

Grant ID: DP230102109

Gordon & Betty Moore Foundation

Grant ID: GMBF9352

Abstract

Sponges offer a unique perspective on animal evolution because of their simple body plan and phylogenetic position in the animal kingdom (Renard et al. 2018). However, established methods to investigate molecular and cellular processes in animal models are often underdeveloped in sponges. One such method is immunofluorescence, which has been successfully applied in multiple sponges but often restricted to specific commercial or tailored antibodies, sponge species and developmental contexts (e.g., Nakanishi et al. 2014; Schippers and Nichols 2018; Musser et al. 2021). Here we present an approach that improves on existing immunofluorescence protocols for *Amphimedon queenslandica* (Nakanishi et al. 2014; Sogabe et al. 2016) to detect subcellular localisation of the low abundance transcription factor interferon regulatory factor 2 (IRF2) in juvenile sponges; this detection could not be achieved using previously published methods nor using a range of other approaches that we trialled (Fig. 1). Specifically, we describe how sodium dodecyl sulfate (SDS) is introduced to reverse antigen masking that often appears after aldehyde-based fixation and to improve signal-to-noise ratios of immunostaining patterns (Shi et al. 2001; Salameh et al. 2018). A buffer containing PIPES, HEPES, EGTA and MgCl_2 (PHEM) is also introduced in rehydration and immunostaining steps to enhance morphological preservation and subcellular detection of IRF2. These modifications yielded significantly improved results (Fig. 1), and are likely to assist in detecting low abundance proteins in other sponges.

Materials

Materials

1. Costar tissue culture-treated 6 well plates. Note: it is necessary to use this specific brand to achieve successful larval settlement (Corning, catalog number: CLS3516)
2. Prolong glass antifade mountant (Thermo Fisher Scientific, catalog number: P36980)
3. Microscope slides (SAIL Brand, catalog number: 7103)
4. Coverslips (Carl Zeiss, catalog number: 474030-9000-000)
5. Transfer pipettes (Sigma-Aldrich, catalog number: Z135011)
6. 0.22 μm filtered artificial seawater (Tropic Marin Pro-Reef, 10 kg packages)
7. PFA+GA fixative: fresh (no more than 4 hours old) 4% paraformaldehyde and 0.05% glutaraldehyde in 0.22 μm filtered artificial seawater
8. 30%, 50% and 70% ethanol
9. PHEM buffer: 60 mM PIPES, 25 mM HEPES, 10 mM EGTA and 2 mM MgCl_2 ; pH 6.9
10. 1% sodium dodecyl sulfate (1% SDS)
11. Bovine serum albumin (BSA; Sigma-Aldrich, catalog number: A8022)
12. Blocking buffer: 1% BSA in PHEM buffer
13. AqlRF2 polyclonal antibody raised against TRSGSSADEQEPERPER peptide in rabbit (GenScript Biotech)
14. Goat anti-rabbit IgG (H+L) highly cross-adsorbed secondary antibody, Alexa Fluor 647 (Invitrogen, catalog number: A21245)
15. Hoechst 33342 (Thermo Fisher Scientific, catalog number: 62249)

Equipment and software

1. Sunflower mini shaker (Grant-Bio, catalog number: PS-3D)
2. Fluorescence stereomicroscope (Nikon SM225)
3. Inverted fluorescence microscope (Nikon Eclipse Ti)
4. Confocal microscope (Zeiss LSM 900)
5. Fiji (Version 2.15.1)

Before start

Juvenile *Amphimedon queenslandica* are reared on coverslips to 4 days old in 6-well plates using filtered artificial seawater as previously described (Sogabe et al. 2016).

To achieve optimal preservation of the sponge samples, it is critical that PFA+GA fixative (see below) is prepared no more than 4 hours before being used for fixation.

Initial fixation

- 1 Remove all filtered artificial seawater from a 6-well plate containing juveniles reared on coverslips (Sogabe et al. 2016) and add 0.5 mL of PFA+GA fixative into each well. Note: other fixatives trialled under the same conditions are less effective in preserving *A. queenslandica* juvenile morphology (Fig. 1).

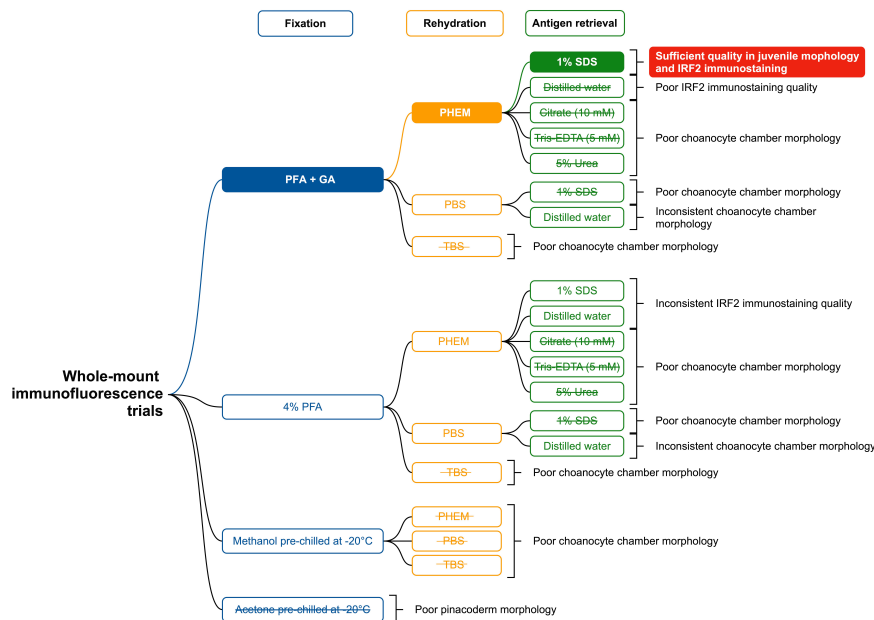


Fig. 1. Fixation, rehydration and antigen retrieval methods and reagents trialled during the development of an optimised immunofluorescence protocol for *Amphimedon queenslandica* juveniles. The steps described in this report are in the solid colour boxes. Strikethrough text represents reagents that produced insufficient or poor results and thus are not included in the final protocol.

- 2 Gently swirl the 6-well plate for 5 seconds and immediately replace the initial fixative with 1 mL of fresh PFA+GA fixative.
- 3 Incubate juveniles in PFA+GA fixative on a slow nutator (10 rotations per minute) for 60 minutes at room temperature. Note: all subsequent wash steps in sections Dehydration and rehydration, Antigen retrieval and Immunostaining are conducted on the nutator at this rotation speed.

Dehydration and rehydration

- 4 Remove all fixative from the wells and then wash and dehydrate fixed juveniles by stepping through 1 mL of 30%, 50% and 70% ethanol (each pre-chilled at -20 °C) for 5



minutes each. Note: dehydrated juveniles can be stored in 70% ethanol at -20 °C for up to 6 months.

- 5 Remove all ethanol from wells and then rehydrate dehydrated juveniles by washing them 4 times for 5 minutes each in 1 mL of PHEM buffer at room temperature. Note: other buffers either do not maintain morphology or are not compatible with the SDS antigen retrieval step (Fig. 1).

Antigen retrieval

- 6 To reverse antigen masking that appears to occur after aldehyde-based fixation, incubate rehydrated juveniles in 1 mL of 1% SDS for 5 minutes.
- 7 Remove residual SDS from the juvenile samples by washing them 3 times for 5 minutes each in 1 mL PHEM buffer at room temperature. This maintains juvenile morphology, as evidenced by the arrangement of choanocytes and choanocyte chambers (Fig. 2).

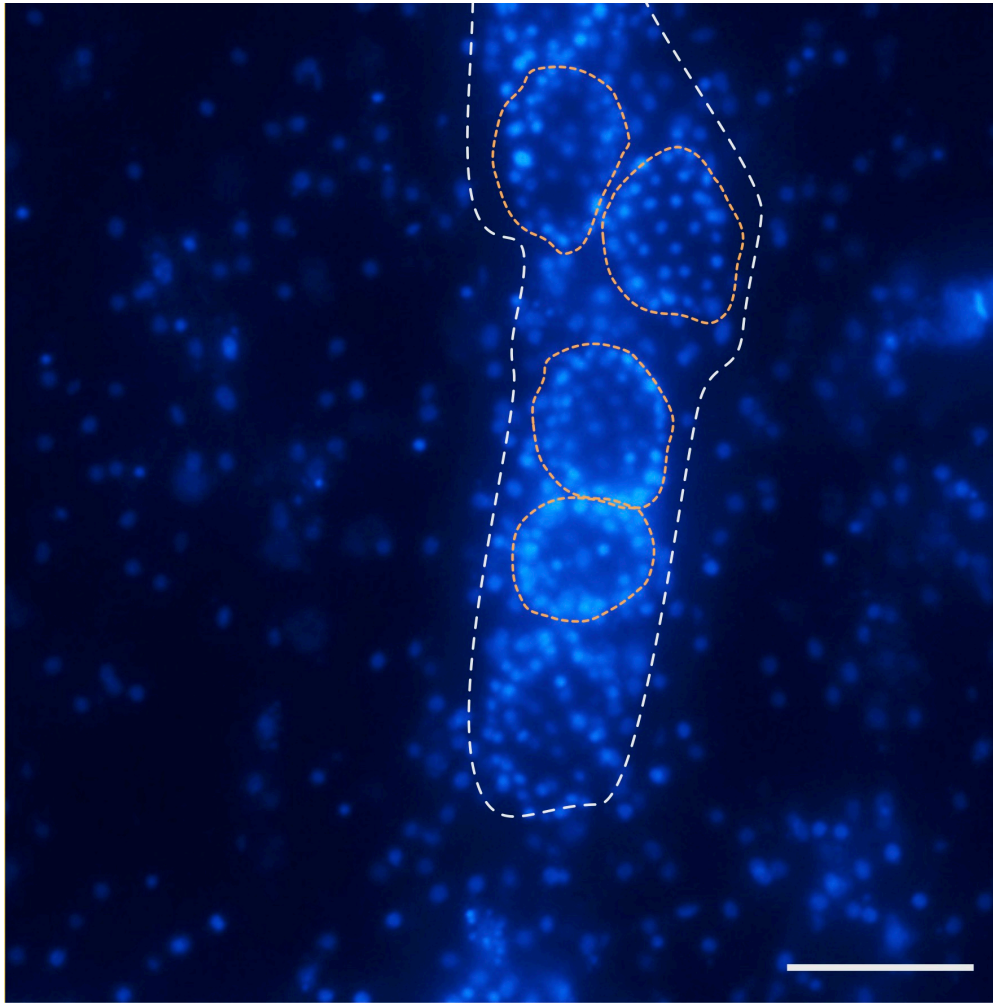


Fig. 2. Choanocyte chambers in juveniles fixed with GA+PFA fixative, rehydrated with PHEM and then subjected to SDS antigen retrieval treatment. Spherical patterns of nuclei (blue) of choanocyte chambers (outlined by yellow dashed lines) in the internal cell layer of Hoechst-stained juveniles (outlined by white dashed lines). Photomicrograph from a Nikon fluorescence stereomicroscope. Scale bar, 30 μ m.

Immunostaining

- 8 Incubate juveniles in 1 mL of blocking buffer for 1 hour at room temperature on the bench without rotating.
- 9 Gently remove all the blocking buffer and then incubate juveniles in 0.5 mL of fresh blocking buffer containing 1 μ L of the primary antibody (anti-AqIRF2 rabbit antiserum) at 4 °C overnight in the dark without rotating.
- 10 Wash immunolabelled juveniles in 1 ml of PHEM buffer 3 times for 5 minutes at room temperature on the nutator.



- 11 Incubate the juveniles in 0.5 mL of fresh blocking buffer containing 0.5 μ L of the secondary antibody (Alexa Fluor 647 goat anti-rabbit IgG) for 30 minutes at room temperature in the dark without rotating.
- 12 Wash immunostained juveniles in 1 mL of PHEM buffer 3 times for 5 minutes at room temperature on the nutator.
- 13 Counterstain nuclei in the juveniles with 500 μ L of 1 μ g/mL Hoechst 33342 in PHEM buffer for 20 minutes at room temperature in the dark without rotating.
- 14 Repeat the washes 3 times for 5 minutes each in 1 mL of PHEM on the nutator, then mount the juveniles in Prolong Glass Antifade Mountant on microscope slides.
- 15 Capture images of the cellular localisation of nuclei and the target protein in these juveniles using a confocal microscope, and analyze these images using Fiji software (Schindelin et al. 2012) (Fig. 3).

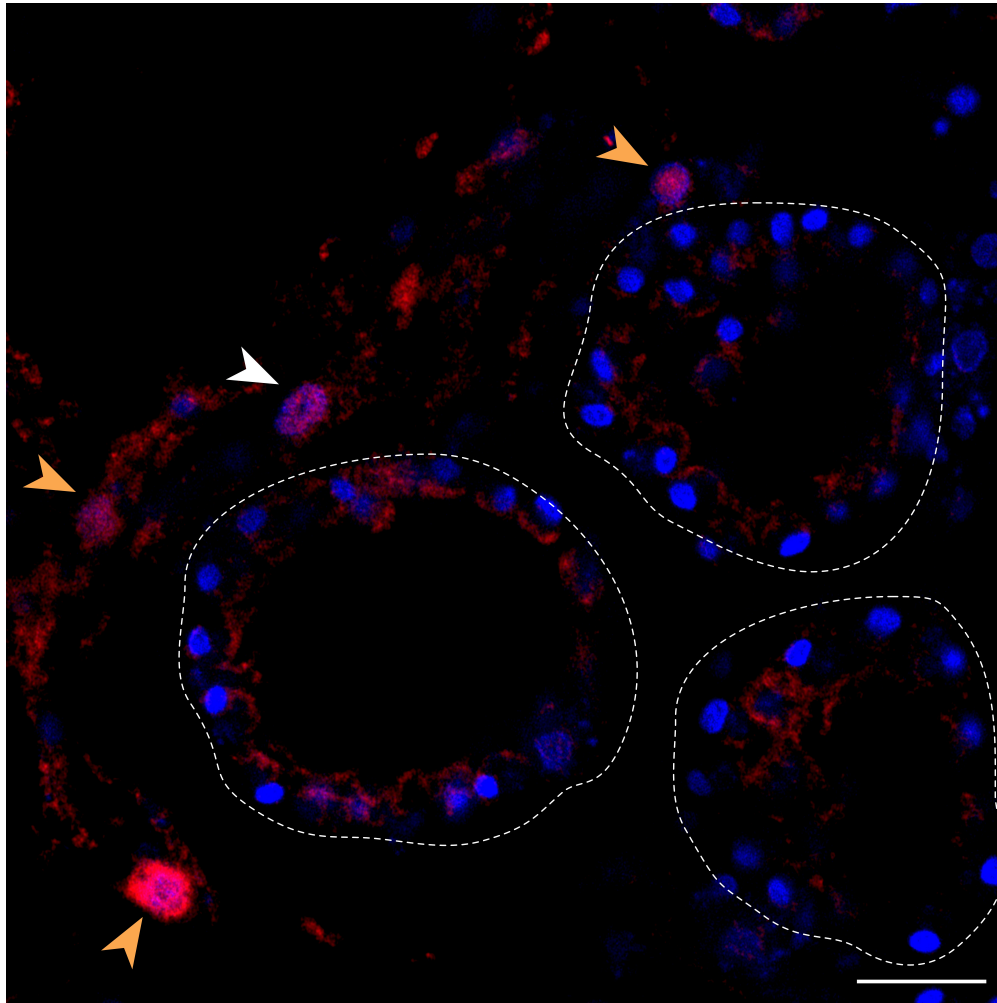


Fig. 3. IRF2 immunostaining pattern in juvenile cells. Immunolocalisation of IRF2 (red) in cells in and near choanocyte chambers (outlined by dashed lines) in *A. queenslandica* juveniles. Apical microvilli of individual choanocytes are labelled along with nuclei in amoebocytes (yellow arrowheads) and archaeocytes (white arrowheads). Photomicrograph from a Zeiss LSM 900 confocal microscope. Scale bar, 10 μ m.

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