Antibody staining of Butterfly larval and pupal wing discs

Material and chemicals needed:

Silicone plates
PBS(4°C)
Surgical scissors
Pair of forceps
Fix buffer (4°C)
Ice bucket with ice
Block buffer (4°C)
Wash buffer (4°C)
Primary antibodies (Keep stock at -20°C and make aliquots that are kept at 4°C)
Secondary antibodies (1:500 dilution)
Aluminium foil

Freeze anesthetize the larvae and pupae for 20 mins before dissection.

Wing disk extraction and antibody staining:

1. Pin the larva/pupa in a silicone petri dish and cold PBS. Make sure that the larva/pupa is submerged in PBS.

   Dissect the wings based on protocol mentioned in: Banerjee, T. Das, & Monteiro, A. (2020). Dissection of Larval and Pupal Wings of Bicyclus anynana Butterflies. Methods and Protocols, 3(1), 5. https://doi.org/10.3390/mps3010005

2. For larval wings, as soon as you remove a disc place it in cold fix buffer on ice (500 µl buffer for small wells; 750 µl or 900 µl buffer for large wells). When you have dissected all the wing discs, add formaldehyde (see table below for volumes). Fix for 30 mins in ice.

   For pupal wings, place the wing disc in fix buffer (500 µl buffer) + 4% formaldehyde in room temperature to prevent the folding of the wings. After the fixation transfer the wing on ice. Fix each wing for 30 mins followed by washes as mentioned below.

If you added this much fix buffer…	**….Add this much 37% formaldehyde**
500 µl	55 µl

3. Rinse the discs in 500 µl cold 1X PBS on ice.

   Wash with 1X PBS 4 times: i) Quick
   ii) 5 min
   iii) 5 min

iv) 5 min

4. Place discs in block buffer (500 µl buffer) overnight at 4°C (in refrigerator).

5. Incubate discs in 200 µl primary antibody diluted in wash buffer for 17-24 hours at 4°C. Cover against drying. The number of discs you can place in a single well varies with the size of the discs. Place no more than 4-6 late fifth instar or 10-12 early fifth instar discs per well or 2 pupal wings per well. If you are working with much larger discs like the Monarch you should use bigger wells.

6. Wash the discs 4 times for 20 min each in wash buffer at 4°C (500 µl).

7. Incubate discs in 100-200 µl secondary antibody (1:500 dilution) diluted in Wash buffer for overnight at 4°C. Once the fluorescent secondary is added keep the discs in the dark as much as possible (cover in aluminium foil)

8. Wash the discs 4 times for 15 min each in wash buffer at 4°C (500 µl buffer). Keep disc in final wash buffer until mounting.

9. Place drop of mounting media on slide (10-20 µl, depending on wing disc size) and add wing disc(s) to this drop. If disc(s) to this drop. If discs cannot be mounted immediately, they may be incubated for up to 1 hour in mounting media at 4° C. Be sure that mounting media completely cover slip slowly and add nail polish around coverslip. Wait an hour before imagining.

10. For long term storage keep in -80°C.

Stock solutions

Buffer	**Chemical (Stock Concentration)**	**Add**	**Half volume**
Fix buffer (PEM)† 30ml	0.1 M PIPES pH 6.9 (500 mM)	6 ml	3ml
	1 mM EGTA pH 6.9 (500mM)	60 µl	30µl
	1.0 % Triton x-100 (20 %)	1.5 ml	0.75ml
	2 mM $MgSO_4$ (1M)	60 µl	30µl
	1.5 – 2 % Formaldehyde (added just prior to the addition of the wing discs) (37%)	*	*
	H_2O	22.4ml	11.2 ml
Block buffer† 40ml	50 mM Tris pH 6.8 (1 M)	2ml	1ml
	150 mM NaCl (5 M)	1.2ml	0.6ml
	0.5% IGEPAL (NP40) (20%)	1ml	0.5ml
	5 mg/ml BSA	0.2 gr	0.1gr
	H2O	35.8ml	17.9 ml
Wash buffer† 200ml	50mM Tris pH 6.8 (1 M) 150 mM NaCl (5 M) 0.5% IGEPAL (20 %) 1 mg/ml BSA H_2O	10ml 6ml 5ml 0.2 gr 179 ml	5ml 3ml 2.5ml 0.1gr 89.5ml

† Make fix, block and Wash buffers fresh every two weeks and store at 4°C.
*For fix buffer, formaldehyde is not added to buffer solution, but is added directly to wells following wing disc direction (see step 4, above)

10x PBS

	Final volume	
Chemical	500 ml	1 l
K_2HPO_4	5.34 g (30 mmoles)	10.68 g (60 mmoles)
KH_2PO_4	2.64 g (20 mmoles)	5.28 g (40 mmoles)
NaCl	40.9 g (0.7 moles)	81.8 g (1.4 moles)
H_2O	To 500 ml	To 1 l

After adding water to final volume, autoclave to sterilize.
After diluting to 1x, adjust pH to 7.0 with 1 N NaOH.

PIPES pH 6.9, 500mM

Molecular weight 302.37

	Final Volume	
Chemicals	30 ml	60 ml
PIPES	4.53g (60 mmoles)	9.06 g (30 mmoles)
H_2O	To 20 ml	To 40 ml

Dissolve PIPES in milliQ, bring volume up to around 20/40 ml.

Adjust pH to 7.0 with 4N NaOH. Then bring final volume to 30/60 ml.

WARNING: Avoid inhalation, contact with eyes, skin and clothing. Avoid prolonged repeated exposure. Keep tightly closed, store in a cool dry place and wash thoroughly after handling.

EGTA pH 6.9, 500mM

Molecular weight 380.4

Dissolve 1.902 gram EGTA in very small amount of milliQ water, bring volume up to 10ml (with NaOH 5N). Adjust pH to 6.9.

Igepal CA 630 20%

Chemically indistinguishable from Nonidet P-40 (NP-40), which is no longer commercially available

For 100ml of 20% stock solution, add 80ml milliQ to 20ml Igepal CA 630.

Note: May develop turbidity or sediment on storage; a clear liquid can be obtained on heating to 40°C.. Store at Room temperature.

Tris pH 6.8, 1 M.

Molecular weight 121.14.

Dissolve 30.2 gram Tris in milliQ and bring up volume to 250 ml.
Adjust pH to 6.8 with…. And sterilize by autoclaving.

NaCl, 5M (saturated)

Molecular weight 58.44

Dissolve 29.22 gram NaCl in milliQ and bring up volume to 100 ml.

MgSO4, 1M

Molecular weight 246.48.

Dissolve 12.32 gram $MgSO_4$ in milliQ and bring up volume to 50ml.

Mounting Media

Mounting media	Tris-HCl (pH 8.0)	20 mM
	N-propyl gallate	0.5%
	Glycerol	60%

Modified on 5th March 2022 (Tirtha)