ABC monoclonal antibody staining of dissected larval and adult CNS

Ito, 20.1.97

For 1st-instar CNS, use PBS instead of PBT throughout the process (small specimen is too fragile for the detergent).

for larva / pupa : Dissect CNS in **PBS**
for adult : Put the animal one minute in **50-70% EtOH** (to wash oil on the body surface)
Transfer to **PBS** and dissect the CNS

Fix: put **100-200** µl **fixative** into Eppendorf tubes.
most cases: **4% Formaldehyde (FA) / PEM** (100µl 37% FA / 900µl PEM, 400 µl 10% FA / 600µl PEM)
using " EM grade ultra-pure FA" yields better results
Pool dissected tissues in the **fixative** for up to **10-20 min**
Change to **fresh fixative** and incubate **R.T.** further **50 min** (for 1st instar: 25 min)

Wash: **2** times **5 min** with **PBT** (0.3-0.5 % Triton X /PBS)
For keeping the specimen at 4°C (up to a few days), wash once more with **PBT**.

Block 1: with **0.3% H_2O_2 / Methanol** (100µl 30% H_2O_2 / 10 ml Methanol)
R.T. 30 min to kill endogenous peroxidase

Wash: **4** times **5 min** with **PBT**

Block 2: with **1.5% horse serum / PBT** (150µl / 10 ml PBT)
R.T. >1 hr to fill nonspecific antibody binding region

1st Ab: **monoclonal antibody** (mouse) (1:500 in 1.5% horse serum / PBT)
4°C Over Night or **R.T. >3 hr**

Retrieve: keep used antibody in 4°C (used antibodies works better; up to a few weeks or a few staining sessions)

Wash: **3** times **5 min** with **PBT**

2nd Ab: **Biotin-conjugated horse anti-mouse IgG** (Vectastain 1:500 in 1.5% horse serum / PBT)
R.T. >2 hr

Retrieve: keep used antibody in 4°C

Wash: **3** times **5 min** with **PBS** (to wash out Triton X before avidin-biotin reaction and DAB staining)

ABC: Mix **10**µl Solution **A**, **10**µl solution **B**, **500**µl **PBS** (Vectastain Elite)
keep **R.T. >30 min** before use
then incubate the specimen **R.T. >1 hr**
Wash: **4** times with **PBS**

Stain: **0.5-1 mg/ml DAB** + **0.03% H_2O_2** (1 µl 30% H_2O_2 / 1 ml DAB)

1.5% H_2O_2	1.3µl	6.7µl
1mg/ml DAB	200µl	1000µl

R.T. 5-20 min (check the staining under dissecting microscope) ***keep hazardous DAB waste !***

Wash: **2** times **5 min** with **PBS** ***keep hazardous DAB waste !***

Post Fix: **2% Glutaraldehyde (GA) / PBS** (80 µl 25% GA / 920 µl PBS) ***keep hazardous DAB waste !***
R.T. > 1 hr

Wash: **3** times **5 min** with **H_2O** ***keep hazardous DAB waste !***

Dehydrate: **30%, 50%, 70%, 90%** each **3 min** -> **100% 5 min** -> **100% 10 min** -> **Xylen 15 min** -> **Araldit 30 min**
change **Araldit** again

Mount: in **Araldit**

Sigma DAB Tetrahydrochloride Tablet (D5905, 100 tablets)

antibody / Fluorescent staining of dissected larval and adult CNS

Ito, 21.1.99

For 1st-instar CNS, use PBS instead of PBT throughout the process (small specimen is too fragile for detergent).

for Larva : Dissect CNS in **PBS**
for adult : Put the animal one minute in **50-70% EtOH** (to wash oil on the body surface)
Transfer to **PBS** and dissect the CNS

Fix: **4% Formaldehyde (FA) / PEM** (100µl 37% FA / 900µl PEM, 400 µl 10% FA / 600µl PEM)
R.T. 50 min (1st instar: 25 min) *with gentle agitation*

Wash: **4** times **5 min** with **PBT**

Block: with **10% goat serum / PBT**
R.T. >1 hr to fill nonspecific antibody binding region

1st Ab: **antibody** in **10% goat serum / PBT**
4°C Over Night or **R.T. >3 hr**
Retrieve: keep used antibody in 4°C (used antibodies works better; up to a few weeks or a few staining sessions)
Wash: **3** times **5 min** with **PBT**

2nd Ab: **Cy3-conjugated anti IgG** (Jackson, **1:100** in **10% goat serum / PBT**)
(anti mouse IgG for monoclonal, anti rabbit IgG for most polyclonal)
R.T. >2 hr
during incubation, put cover onto the Eppendorf tubes to keep specimen in the dark (in all the steps)
Wash: **3** times **5 min** with **PBS**

Dehydrate: in **50% Glycerol / PBS** for 1 hour
in **50% antifade Glycerol / PBS** for 30 min
Mount: in **50% antifade Glycerol / PBS**

Store: keep in refrigerator until observation (up to 1 week)

antifade 0.25 % n-propyl gallate 50% Glycerol, PBS
dissolve 25 mg n-propyl gallate in 5 ml PBS
add 5 ml Glycerol
adjust pH to 8.0-8.6 with 1 N NaOH (50-80 µl)

1st :	Cappel Rabbit polyclonal anti ß-gal	Cosmo Bio: 55693 (3712-0081)
	Promega Mouse monoclonal anti ß-gal	Z 3781
	Sigma Mouse monoclonal anti bovine-tau	T5530
	Sigma Rabbit polyclonal anti-WGA	T4144
2nd:	Jackson AffinitiPure Goat Anti-Mouse IgG (H+L) Cy3-conjugated	115-165-146
	Jackson AffinitiPure Goat Anti-Rabbit IgG (H+L) Cy3-conjugated	115-165-144
	Cappel: affinity purified goat anti rabbit IgG (H+L) - FITC conjugated (whole)	Cosmo Bio: 55662 (1612-0081)
	Zymed: affinity purified goat anti rabbit IgG (H+L) - FITC conjugated (whole)	Cosmo Bio: 62-6111

Preabsorbtion: If background is high, incubate the diluted antibody for >2 hrs with fixed embryos (dechorionate with breach for 4 min, fix with heptan / 10% FA for 30 min , deviterinize with heptan/methanol, wash with methanol and then with PBS)

GFP preparation of dissected larval and adult CNS

Ito, 20.1.99

for Larva : Dissect CNS in **PBS**

for adult : Put the animal one minute in **50-70% EtOH** (to wash oil on the body surface)
Transfer to **PBS** and dissect the CNS

Fix: put **100-200** µl **4% Formaldehyde (FA) / PEM** into Eppendorf tubes.
(100µl 37% FA / 900µl PEM, or 400 µl 10% FA / 600µl PEM)
Pool dissected tissues in the **fixative** for up to **10-20 min**
Change the **fresh fixative** and incubate **R.T.** further for **50 min** *with gentle agitation*

during incubation, put cover onto the Eppendorf tubes to keep specimen in the dark (in all the steps)

Wash: **3** times **5 min** with **PBS**

Dehydrate: in **50% Glycerol / PBS** for 1 hour
in **50% antifade Glycerol / PBS** for 30 min

Mount: in **50% antifade Glycerol / PBS**

Store: keep in refrigerator until observation (up to 1 week)

antifade 0.25 % n-propyl gallate 50% Glycerol, PBS
dissolve 25 mg n-propyl gallate in 5 ml PBS
add 5 ml Glycerol
adjust pH to 8.0-8.6 with 1 N NaOH (50-80 µl)

Anti *ß-gal* DAB staining of dissected larval and adult CNS

Ito, 4.6.95

For 1st-instar CNS, use PBS instead of PBT throughout the process (small specimen is too fragile for detergent).

for Larva : Dissect CNS in **PBS**
for adult : Put the animal one minute in **50-70% EtOH** (to wash oil on the body surface)
Transfer to **PBS** and dissect the CNS

Fix: put **100-200** µl **fixative** into Eppendorf tubes.
most cases: **4% Formaldehyde (FA) / PEM** (100µl 37% FA / 900µl PEM, 400 µl 10% FA / 600µl PEM)
some cases: **1% Glutaraldehyde (GA) / PBS** (40µl 25% GA / 960µl PBS)
GA works well with anti *ß-gal*, but some antibodies don't work with GA at all.
Pool dissected tissues in the **fixative** up to **10-20 min**
Change the **fresh fixative** and incubate **R.T.** further
FA: **50 min**, FA for 1st instar: 25 min, GA: 10 min

Wash: **2** times **5 min** with **PBT** (0.3-0.5 % Triton X /PBS)
For keeping the specimen at 4°C (up to a few days), wash once more with **PBT**.

Block 1: with **0.3% H_2O_2 / Methanol** (1/100 volume of 30% H_2O_2)
R.T. 30 min to kill endogenous peroxidase
Wash: **4** times **5 min** with **PBT**
Block 2: with **10% goat serum / PBT**
R.T. >1 hr to fill nonspecific antibody binding region

1st Ab: **rabbit polyclonal anti *ß-gal*** (Cappel 1:1000 in 10% goat serum / PBT)
4°C Over Night or **R.T. >4 hr**
Retrieve: keep used antibody in 4°C (used antibodies works better; up to a few weeks or a few staining sessions)
Wash: **3** times **5 min** with **PBT**

2nd Ab: for HRP: **Peroxidase-conjugated anti rabbit IgG** (Cappel, 1:500 in 10% goat serum / PBT)
for ABC: **Biotin-conjugated anti rabbit IgG** (Vectastain 1:500 in 10% goat serum / PBT)
R.T. >2 hr
Retrieve: keep used antibody in 4°C
Wash: **3** times **5 min** with **PBS** (to wash out Triton X before avidin-biotin reaction and DAB staining)

HRP: goto **Stain**
ABC: Mix **10**µl Solution **A**, **10**µl solution **B**, **500**µl **PBS** (Vectastain Elite)
keep **R.T. >30 min** before use
then incubate the specimen **R.T. >1 hr**
Wash: **4** times with **PBS**

Stain: **0.5-1 mg/ml DAB** + **0.03% H_2O_2** (1 µl 30% H_2O_2 / 1 ml DAB)

1.5% H_2O_2	1.3µl	6.7µl
1mg/ml DAB	200µl	1000µl

R.T. 5-20 min (check the staining under dissecting microscope) ***keep hazardous DAB waste !***
Wash: once **5 min** with **PBS** ***keep hazardous DAB waste !***

Post Fix: **2% Glutaraldehyde (GA) / PBS** (80 µl 25% GA / 920 µl PBS) ***keep hazardous DAB waste !***
R.T. > 1 hr
Wash: **3** times **5 min** with **H_2O** ***keep hazardous DAB waste !***

Dehydrate: in **50% Glycerol / H_2O** for 1 hour
in **80% Glycerol / H_2O** for 30 min
Mount: in **80% Glycerol / H_2O**

or:
For plastic mount:
Dehydrate: **30%, 50%, 70%, 90%** each **3 min** -> **100% 5 min** -> **100% 10 min** -> **Xylen 15 min** ->
Mount: in **Araldit**

Stock

1st :	Cappel Rabbit polyclonal anti ß-gal	Cosmo Bio: 55693 (3712-0081)
2nd	affinity purified goat anti rabbit IgG (H+L) (whole)	
	-- peroxidase conjugated : Cappel : Cosmo Bio: 55689 (3612-0081), Zymed : Cosmo Bio: 62-6120	
	-- biotinilated (whole) Cappel : Cosmo Bio: 55699 (8612-3711), Vector : Vectastain Elite	
DAB tablet:	Sigma DAB Tetrahydrochloride Tablet	Sigma D-5905
Goat Serum:	CMN Chemicon	Cosmo Bio S-26 017

Antibody staining of Embryos (with Vectastain kit)

ITO Kei, 1995 April

collect eggs with a nylon mesh attached to the bottom of a funnel

Dechorionate: soak the eggs in **7.5% Bleach in H_2O** (50% Chrolox, 50% H_2O)
R.T. 4 min

Wash: with **H_2O**

Transfer into an Eppendorf tube containing

Fix: **FA / Heptan** (1 ml)
400 µl PBS, 100µl 37 % Formaldehyde, 500 µl n-Heptan
(or 100 µl PBS, 400 µl 10 % EM grade Formaldehyde, 500 µl n-Heptan)
R.T. 30-40 min

Deviterinyze: remove the FA+PBS phase (lower part)
add the same amount of **100% Methanol**
shake well by hand or by Vortex
remove the upper phase (Methanol)
add another volume of **Methanol**
shake well by hand or by Vortex
until the majority of the eggs go down to the bottom
remove heptan and Methanol solutions

Wash: **2** times with **Methanol**
until eggs become non-sticky

Keep embryos at this stage at 4°C (up to a few weeks)

Before staining, wash **4** times with **PBT** (0.3-0.5% Triton X-100 in PBS)

Block: with **10% goat serum / PBT**
R.T. >1 hr to fill nonspecific antibody binding region

1st Ab: **monoclonal antibody** (mouse) or **polyclonal antibody** (rat, rabbit, goat etc.)
(in **10% goat serum / PBT**) *preabsorbed*!
4°C Over Night or **R.T. >3 hr**

Retrieve: keep used antibody in 4°C (used antibodies works better; up to a few weeks or a few staining sessions)

Wash: **3** times with **PBT**

2nd Ab: Biotin-conjugated **antibody** (**1:500** in **10% goat serum / PBT**; Vectastain)
for monoclonal 1st: anti mouse Ig, for polyclonal 1st: anti rat, rabbit, goat,,, Ig
preabsorbed!
R.T. >2 hr

Retrieve: keep used antibody in 4°C

Wash: **3** times with **PBS** (to wash out Triton X before avidin-biotin reaction)

ABC: Mix **10**µl Solution **A**, **10**µl solution **B**, **500**µl **PBS** (Vectastain Elite)
keep **R.T. >30 min** before use
then incubate the specimen **R.T. >1 hr**

Wash: **4** times with **PBS**

Stain: **0.5-1mg/ml DAB** + **0.01% H_2O_2**

1.5% H_2O_2	1.3µl	6.7µl
1mg/ml DAB	200µl	1000µl

R.T. 5-20 min (check the staining under dissecting microscope) ***keep hazardous DAB waste !***

Wash: **2** times with **PBS** ***keep hazardous DAB waste !***

Wash: once with **H_2O** ***keep hazardous DAB waste !***

Dehydrate: in **50% Glycerol / H_2O** for 1 hour
in **80% Glycerol / H_2O** for 30 min

Mount: in **80% Glycerol / H_2O**

For plastic mount:

Dehydrate: **30%, 50%, 70%, 90%** each **3 min** -> **100% 5 min** -> **100% 10 min** -> **Xylen 15 min** ->

Mount: in **Araldit**

Preabsorbtion: incubate the diluted antibody for >2 hrs with fixed embryos (dechorionate with breach for 4 min, fix with heptan / 10% FA for 30 min , deviterinize with heptan/methanol, wash with methanol and then with PBS)

X-gal staining of embryo

Ito, 4.6.95

Dechorionate: put the eggs in **7.5% Bleach** (in **H_2O**)
R.T. 4 min
collect the eggs with a nylon mesh
Wash: with **H_2O**
transfer to an Eppendorf tube

Fix: **GDA / Heptan** (300-500 µl)
45 ml PBS, 5 ml 25% Glutardiardehid, 50 ml n-Heptan
store at 4°C, shake well just before use and take the upper phase
R.T. 15 min

Deviterinyze: (divitelinize by hand works better)
remove **GDA / PBS** phase (lower part)
add the same amount of **80% EtOH** (in H_2O)
shake well by hand or by Vortex
(Although Ethanol deviterinization is not so effective as with Methanol, the loss of ß-galactosidase activity is smaller.)
remove the upper phase (EtOH)
add another **80% EtOH**
shake well by hand
until the majority of the eggs go down to the bottom
remove heptan and EtOH solutions
Wash: several times with **80% EtOH**
until eggs become non-sticky
wash 2 times with **PBT** (0.5% Triton X-100 in PBS)

Stain: heat **X-gal buffer** to **65°C**

buffer:		
	100 ml	0.1 M Na phosphate buffer
	860 mg	NaCl
	20.3 mg	$MgCl_2$
	109 mg	$K_3[Fe(III)CN_6]$
	140 mg	$K_4[Fe(II)CN_6]$

store at 4°C
add **1/100** volume of **20% X-gal**
200 mg X-gal in 1 ml DMF, store at 4°C
mix well and *gradually* cool down to room temperature
If precipitation appears, heat and mix the solution again.
apply the staining solution to the eggs
37°C 2 hr to **Over Night** (with gentle shaking)

Wash: **3** times **5 min** with **PBS**

Dehydrate: in **50% Glycerol / H_2O** for 1 hour
in **80% Glycerol / H_2O** for 30 min
Mount: in **80% Glycerol / H_2O**

or:
For plastic mount:
EtOH Dehydrate: **70%, 90%** each 5 min -> **100%** 5 min -> **100%** 10 min -> **Xylen** 15 min
Mount: in **Araldit**

X-gal staining of dissected larval and adult CNS

Ito, 4.6.95

for Larva : Dissect CNS in **PBS**

for adult : Put the animal **one** minute in **50-70% EtOH** (to wash oil on the body surface)
Transfer to **PBS** and dissect the CNS

Fix: put **100-200** µl **fixative** into Eppendorf tubes.
1% Glutaraldehyde (GA) / PBS (40µl 25% GA / 960µl PBS)
Pool dissected tissues in the **fixative** up to **10-20 min**
Change the **fresh fixative** and incubate **R.T. 15 min**

Wash: **2** times **5 min** with **PBT** (0.3-0.5 % Triton X /PBS)

Stain: heat **X-gal buffer** to **65°C**

buffer:

100 ml	0.1 M Na phosphate buffer
860 mg	NaCl
20.3 mg	$MgCl_2$
109 mg	$K_3[Fe(III)CN_6]$
140 mg	$K_4[Fe(II)CN_6]$

store at 4°C

add **1/400** volume of **20% X-gal**
200 mg X-gal in 1 ml DMF, store at 4°C
mix well and *gradually* cool down to room temperature
If precipitation appears, heat and mix the solution again.

apply the staining solution to the eggs
37°C 2 hr to **Over Night** (with gentle shaking)

Wash: **3** times **5 min** with **PBS**

Dehydrate: in **50% Glycerol / H_2O** for 1 hour
in **80% Glycerol / H_2O** for 30 min

Mount: in **80% Glycerol / H_2O**

or:
For plastic mount:
EtOH Dehydrate: **70%, 90%** each 5 min -> **100%** 5 min -> **100%** 10 min -> **Xylen** 15 min
Mount: in **Araldit**

Fixative

4 % Formaldehyde (FA) **good for the first trial**

10% Formalin / PBS

PBS Tablets pH7.4 (25 degree) (Sigma P4417)

4 % Ultra pure Formaldehyde (FA) **usually the good and most convenient**

40% EM grade Ultra pure Formaldehyde / PEM

Folmaldehyde, Methanol Free, 10%, Ultra pure EM grade (Polyscience, 04-1040-18)

4 % Paraformaldehyde (PFA) **good for fixing protein**

mix:	paraformaldehyde	4	g	1	g
	0.1 M Phosphate Buffer	90	ml	22	ml
warm to 60°C (ca. 20 min.)					
cool and mess up to		100	ml	25	ml
store at 4°C (*max 1 week*)					

PLP (Periodate–Lysine–Paraformaldehyde) **PFA for protein, periodate-lysine for sugar**

Stock A:

mix:	Lysine–HCl	1.827 g	0.365 g	
	H_2O 50	ml	10 ml	
add	0.1 M Na_2HPO_4 to pH 7.4			
mess up with 0.1 M Phsphate Buffer to		100	ml 20	ml
store at 4°C (*max 10 days*)				

Stock B:

mix:	Paraformaldehyde (PFA)	8	g 0.2 g	
	H_2O 100	ml	5 ml	
warm to 60°C (ca. 20 min.)				
add 1N NaOH		a few drops to dissolve PFA		
filtrate				
store at 4°C (*max only a few days*)				

Just before use:

mix	Stock A	7.5 ml	9 ml	3 ml
	Stock B	2.5 ml	3 ml	1 ml
	$NaIO_4$	21.4 mg	25.68 mg	8.56 mg
total		*10 ml*	*12 ml*	*4 ml*
(final 0.01 M $NaIO_4$ – 0.075 M Phosphate – 2 % PFA			*pH. 6.2)*	

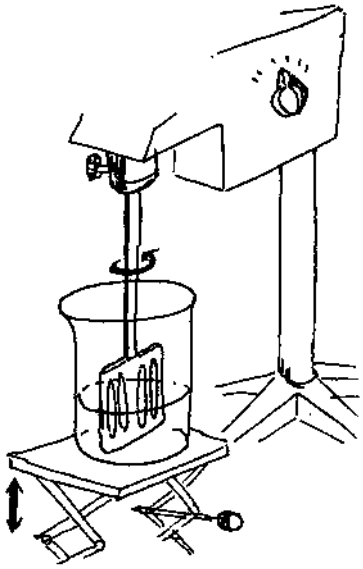
0.1 M Na-Phosphate Buffer			**for 1x soln.**	**for 10x soln.**
mix		$Na_2HPO_4 . H_2O$	10.22 g	51.1 g
	or	$Na_2HPO_4 . 2H_2O$	12.82 g	64.1 g
	or	$Na_2HPO_4 . 7H_2O$	19.30 g	96.5 g
	or	$Na_2HPO_4 . 10H_2O$	25.79 g	128.95 g
AND		$NaH_2PO_4 . H_2O$	3.86 g	19.3 g
	or	$NaH_2PO_4 . 2H_2O$	4.37 g	21.85 g
total			1 l	500 ml

PEM Buffer		**for 100 ml**	**for 500 ml**
mix	0.1 M PIPES (302.37)	3.0237 g	15.1185 g
	2 mM EGTA (380.35)	76.07 mg	380.35 mg
	1 mM $MgSO_4 . 7H_2O$ (246.48)	24.648 mg	123.24 mg

adjust pH to 6.95 with NaOH (or HCl)

Preparation of Araldit

Araldit CY 212	(Serva, #13824, 1 kg)
Araldit hardener HY 964	(Serva, #13826, 1 kg)
Araldit softener (Phtalic Acid Dibuthyl Ester: PADE)	(Serva, #32805, 250 ml)
Araldit Beschleuniger DY 964	(Serva, #13825, 100 g)



For semi-thin sectioning

100 ml Araldit CY 212 into a 500 ml glass cup.
Add **100 ml Araldit hardener HY 964** slowly.
Put the rotor of a low-speed electric mixer in it.
Let the mixer slowly rotate for at least **2 hrs.** (avoid making bubbles in the solution.)
Add **10 ml Softener PADE** very slowly (drop by drop) with the mixer rotating.
Adding as little as 500 ul at once will result in uneven solution,
which is not usable any more.
Mix for another **15 min.**
Add **5 ml Beschleuniger DY 964** very slowly (drop by drop).
Mix for another **30 min.**

Aliquot the Araldit in 10-20 ml disposable syringe.
Store frozen at -20°C.

For whole-mount preparation

Softener PADE is not needed. Mix CY 212, HY 964, and DY 964 in this order.
Araldit for semi-thin sectioning is also applicable to whole-mount preparation, although it costs a bit more.

***
Araldit monomer is harmful. Use gloves while handling. Wipe out with acetone in case of an accident.
Beschleuniger is a highly volatile material; work in a draft chamber.

After use, wipe the glass cup and mixing rotor with acetone and keep them at 63°C over night until remaining araldit become polymerized.

Making Semi-thin Sections

Ito, 4.6.95

Larva, Pupa, Imago:

Dissect animals in Ringer or PBS

Embryo:

Prefix with 25% GDA / Heptan

R.T. 10 min

Wash 3 times with PBS (Remove Heptan)

Fixation 1:

Fix with 6% GDA (Glutardialdehyd)/PBS

4°C, 1-4 hr

Wash 2 times with PBS

Fixation 2:

Fix with 2% OsO_4/PBS

4°C, in the Dark, Over Night

CAUTION: AVOID SKIN and GAS CONTACT

Wash 3 times with PBS

Dehydrate and Embed:

30%, 50%, 70%, 90% each 3 min -> 100% 5 min -> 100% 10 min ->
Xylen 10 min 2 times ->
Araldit/Xylen (1:1) 6hr-Over Night in the Abzug (let Xylen evaporate) ->
Araldit 8-12 hr (let residual Xylen evaporate) ->
Araldit in the mold

Polymerize 45°C, 12 hr -> 65°C, 36-48 hr

Section

Staining

Richardson's Solution

1% Toluidine blue and 1% azur II - 1% Borax

60°C, 15-60 sec.

or TB Solution

1 ml 0.2% Toluidine blue / water
1 ml 1% Methylen blue / 1% Borax
18 ml water
60°C, 30 sec. - 2 min

Wash with running tap water
Wash with distilled water
Dry
Mount with Araldit

Anti ß-gal staining of larval CNS original in Mainz

Dissect CNS

Fix: 1% Glutardialdehyde (GDA) / PBS (40 µl 25% GDA / 960µl PBS)
R.T. 10 min
GDA does not work with other antibodies! Anti ß-gal works with Formaldehyde.
Use Formaldehyde (FA) when staining embryos.

Wash: 2 times with PBS

Block 1: with 0.3% H_2O_2 / Methanol
R.T. 30 min to kill endogenous peroxidase

Wash: 3 times with PBS

Block 2: with 10% lamb serum / PBS
R.T. >1 hr to fill nonspecific antibody binding region

1st Ab: anti ß-gal (rabbit polyclonal) (1:7000 in 10% lamb serum / PBS)
1:14 preabsorbed antibody solution (1:500)
4°C Over Night

Retrieve: keep used antibody in 4°C

Wash: 3 times with PBS

2nd Ab: Biotin conjugated anti rabbit IgG (1:500 in 10% lamb serum / PBS)
(Vector stain) preabsorbed!
R.T. 2 hr

Retrieve: keep used antibody in 4°C

Wash: 3 times with PBS

ABC: Mix 10µl Solution A, 10µl solution B, 500µl PBS
keep R.T. >30 min before use
then R.T. 1 hr with the specimen

Wash: 4 times with PBS

Stain: 1mg/ml DAB + 0.01% H_2O_2

1.5% H_2O_2	1.3µl	6.7µl
1mg/ml DAB	200µl	1000µl

R.T. 5-10 min

Wash: 2 times with PBS

Post Fix: 1% Glutardialdehyde (GDA) / PBS (40 µl 25% GDA / 960µl PBS)
R.T. 30 min

Wash: once with Water

Dehydrate: 30%, 50%, 70%, 90% each 3 min -> 100% 5 min
-> 100% 10 min -> Xylen 15 min -> 50% Araldit / Xylen O/N
-> Araldit 6 hr -> Araldit