The Beetle Book

"As Gregor Samsa awoke one morning from uneasy dreams

he found himself transformed in his bed

into a gigantic flour beetle."

almost Franz Kafka

version 1-2

edited by Gregor Bucher 2009

Index:

General information, culturing and stockkeeping

Tribolium life parameter table

Temp. (°C)	egg (d)	hatched (%)	larva (d)	% larval mortality	pupa (d)	total dev. time (d)
40.0	2.7	86	23.6	65	4.4	31
37.5	2.6	77	13.7	13	3.9	20
35.0	2.7	92	12.9	4	4.5	21
32.5	2.9	75	14.6	7	4.6	22
30.0	3.6	88	17.2	3	5.5	27
27.5	4.7	84	24.3	13	7.5	37
25.0	6.8	87	31.2	4	10.2	48
22.5	9.3	88	51.0	17	13.4	74
20.0	13.9	77	109.3	60	24.4	148
17.5	...	0	...			

larval instars:

- 5-9 larval instars, depending on growth conditions
- Normal conditions: 6 instars

reproductive onset

females: - egg production at 25°C: 7d (29°C: 4d) after eclosion (they have to feed on full grain flour before they start laying eggs)
- fertilization immediately after eclosion

males: - mature at 2nd day (29°C)

fertility:

- single females lay up to 20 eggs/day (29C)
- average egg rate 25°C: 7 eggs in 3 days over 100d, then decline during the next 100 d; high yield only during the first three months
- males may be fertile up to 1 year
- number of offspring in a vial depends more on the amount of flour than on the number of females.

life expectancy:

up to 3 years, average: ca. 200 d

Mean generation time in population conditions: 55d

food

- no water

for stock keeping:

- 1 kg full grain flour (type 1700 = mg ash per kg flour) + 50g yeast powder + 5 g diluted Fumagilin
- presieve all components with 700 um mesh (in order to allow a good separation of beetles from flour using a 800 um mesh)

for egg collection

- 1 kg white flour (type 405) + 50g yeast + 5 g diluted Fumagilin
- presieve with 250 um (in order to allow a good separation of eggs from flour using a 300 um mesh)

Note: egg production decreases dramatically on white flour (within one day!). After half a day on full grain flour, fertility is up again. Egg production of freshly hatched beetles does not start until they had some full grain flour

for single egglays:

- instant white flour (larger grains, does not plug sieves as easily, but does not dissolve

completely in Clorix). (in Germany: Rosenmehl “Wiener Griessler” or “Doppelgriffiges Mehl” or “Instant Meh”l; Type 405)
- presieve with 250 um (in order to allow a good separation of eggs from flour using a 300 um mesh)

(order sieves at: http://www.retsch.de/index_en.php, Rheinische Strasse 36 42781 Haan,
e.g.:
Retsch Analysensieb 200x50mm, Maschenweite 800µm (DIN ISO 3310-1) No 60131000800
Retsch Analysensieb 200x50mm, Maschenweite 300µm (DIN ISO 3310-1) No 60131000300
...)

preparation of diluted Fumagilin:
grind 12 g of Fumagilin in a mortar and add to 200g pre-sieved white flour and mix well. Of this dilution use 5 g per 1kg flour

notes:
- Fumagilin/Fumidil is an antibiotic against sporozoans mainly used by bee people
- Fumagilin B is identical to Fumidil-B but better water soluable
- concentration of commercial Fumaglin/Fumidil: 500mg per 25g powder
- end concentration in flour should about 0.03% Fumagilin (i.e. 0.3g/kg)

(order online and pay with credit card at: Dadant & Sons, 550 E. Main, P.O.Box 385, Potterville, MI 48876, Phone (517)645-7629, Fax (517)645-0263)

Sterilization of food, tools
- against mites or other nasty beasts that might be hidden in supermarket flour: keep flour and yeast at -20°C for 48 h before use
- to avoid the spread of mites and mixing of different strains by using the same tools: keep sieves etc. at +60°C for at least 2 hours

Keeping beetles

large stock for egg / pupae collection

- plastic box with (appr. 15X15 cm square, 10 cm height are sufficient – look for Tupperware parties…), for air supply an opening must be present in the tap (appr. 4 cm diameter, secured by a 300 um mesh)
- depending on the amount of beetles fill the bottom with 1-2 cm flour
- 25°C: collect eggs two times a week and inoculate a new box every time. After some time (in about 12 boxes) all stages will be represented quite synchronously (as larvae, pupae and adults are not easily separated, this is very convenient)

Notes:

- too dense populations will become desynchronized
- too dense populations will produce respiration water and lead to ugly fungi infestations
- change flour before it turns red
- beetles do not like to fly – under starvation they do

intermediate stocks

- 20 ml?? fly vials with up to 5cm flour

single pair matings

- 5 ml?? fly vials with up to 2 cm flour

Note:

- beetles try to crawl up the vial, fall on their back and cannot get on their feet again. They will starve to death if there are not enough companions in the vial that pass by and help them turn back again. This is especially important for single pair matings. Either rock vials every week or add some large grains on top of the flour (e.g. boulgur)

Incubators:

Incubators need internal air circulation and some amount of fresh air supply. Sudden population downbreaks will occur in closed incubators.

Standard temperatures:

32°C fast embryonic development (3 days), highest RNAi effects

25°C slow embryonic development (7days) / stock keeping – beetle transfer appr. every 3 months

23°C stock keeping – beetle transfer appr. every 5 months

18°C backup (embryos don´t develop, hatched larvae do develop to adults, adults survive but need one week on higher temperature to produce normal offspring again)

-20°C way to beetle heaven

Humidity:

The beetles need some degree of humidity. Especially in winter when relative humidity in climatized rooms drops significantly, their fertility decreases and they may even die. At 80% relative humidity they perform better than at 60% (Angeli, personal communication). However, at high relative humidity, mold may start to grow and compromise the cultures. A 40-60% have proven a good compromise (Beeman, Klingler, personal communication).

Molecular biology

DNA preparation

Extracting DNA from 15-30 pupae

(Bucher/98)

Solutions:

Homogenization buffer (Hom buffer):

80 mM EDTA pH8
100 mM Tris pH8
0,5% SDS
(autoclave)

Proteinase K (stock: 14.4 mg/ml)
RNAase (stock: 10 mg/ml), boiled (15 min 95C, let cool slowly to allow refolding of RNAse)
Phenol equilibrated with 0.5 M Tris pH 8
3 M NaAcetat pH6
TE buffer:(10 mM Tris pH8, 1 mM EDTA, pH8)

We use pupae because we hope to get less inhibitory substances in the DNA preparation compared with adult beetle (but adults work, too)

Homogenisation, cell-lysis, RNA-Degradation

1. Place 15 pupae with 1 ml Hom buffer in a 2 ml eppendorf tube (made of Phenol resistant material), and homogenize with a tightly fitting pestle (solution turns brown)

(alternative: homogenize pupae in N2-cooled morter and transfer still frozen homogenate into hom buffer; before use, morter has to be treated with 1M HCl overnight and backed at 80 degree Centigrade to remove contaminating DNAs)

2. Add **Proteinase K** to a final concentration of 100 ug/ml (7 ul of a14.4 mg/ml stock solution)
3. Incubate 2-3 h at 50C; rock from time to time
4. Spin down (5 min at 10 000 rpm) and transfer supernatant to a new tube (discard pellet)
5. Incubate 1 h at 65 C to inactivate ProteinaseK
6. Add **RNAse** to a final concentration of 20 ug/ml (2ul of a 10 mg/ml stock (boiled!))
7. Incubate 2 h at 37 C

Phenol-Chloroform-Extraction:

8. Add 500 ul Phenol(pH 8!) and 500 ul Chloroform
9. mix/vortex 5 min
10. centrifuge 5 min at 13 000 rpm (6000 works too), transfer the aqueous (upper) phase into a new eppi (avoid to transfer interphase-junk!)
11. (repeat 8-10 until aqueous phase is only slightly coloured (usually 2 times))
12. Add 1ml Chloroform; mix and centrifuge as before; transfer upper phase into a new eppi as before
13. Add 1/10 Vol of 3 M **NaAc** pH6
14. Add 2 Vol of ETOH 100% (room temperature! do not use cold ETOH because non-DNA substances will fall out)
15. Mix **gently**; part of the DNA will fall out and form a white tangle that can be transferred with a glass-hook into a new eppi containing 70% Ethanol (This DNA is very pure, but you´ll loose about half of DNA)
16. Centrifuge new eppi for 30 min at 6 000 rpm
17. Remove supernatant carefully,

    (If pellet is not solid but kind of viscious repeat step 11. (Chloroform))

18. Wash with 1 Vol ETOH 70%
20. Decant supernatant, aspirate remaining drops from the wall
21. Air dry to remove ETOH (pellet becomes transparent), but do not let the pellet dry out completely
22. Take up pellet in 200 ul **TE**

1ul on a gel should give a well visible high-molecular band (about 15 ng; this corresponds to a total yield of 3 ug). A low molecular band is probably leftover RNA.

For PCR use 5 ng

Single Embryo DNA

Preparation of Embryos:- collect eggs (ca.2d) and bleach 2x 4' in 100% Klorix

- wash 3x with NTE+0.1% Tween
- use a cut- off tip to place them on a microscope slide
- place single embryos in 0.5 ml tubes
- freeze @ -20°

Extraction: - add 10 µl NTEK & macerate embryo with pipette tip

- incubate for 90' @ 42°
- spin shortly & denature ProteinaseK @ 95° for 3'
- spin 2' @ 14000 rpm
- transfer 8µl of supernatant in new tube (a pellet should be visible), this is the DNA solution, 4 µl make up a good template for a 20 µl standard PCR

Buffers&Solutions:

NTE:25 mM NaCl,
10 mM Tris pH 8,
1 mM EDTA;

NTEK: NTE + 200 µg/ml Proteinase K (prepare freshly)

RNAi

DsRNA from a PCR-template

(Gregor Bucher, Martin Klingler, 4/05)

For dsRNA use the following primers:

1) T7
2) T7-T3 (anneals to T3 promotor and has a T7 promotor sequence attached)
3) T7-SP6 (anneals to SP6 promotor and has a T7 promotor sequence attached)

4a) Use primer combination according to template plasmid used (bluescript: T7 & T7-T3, pZero: T7 & T7-SP6) or

1. PCR:

H2O + DNA 24,6 (Plasmids: just dip in 5mm with a tip, stir twice and inoculate reaction or: 0,2 ul of a 1:100 dilution - too much inhibits PCR!)

MgCl (25mM)	2,4	(1,5 mM endconcentration)
10Xpuffer	4	
dNTP (2mM)	4	
Primer super mix:	4	mix reaction (or 2 ul of each primer)
Taq Poly (Fermentas)	1	mix again
	------	
	40 ul (split into two PCR-vials 20ul each)	

PCR program:

3'	94°		
30"	94°	*	
30''	60°	*	30X
2'	72° (one minute per kb template length)	*	
3'	72°		
constant 8°			

2a with precipitation:

pool both reactions and add:

3M NaAc pH 5,2	4ul	
Glycogen (carrier)	2ul	mix (optional, recommended for short products <200)
EtOH 100%	80ul	mix

-20° for 1h; centrifuge 1h (max);
wash pellet with 500ul EtOH 70%, centrifuge 30', discard supernatant, air dry pellet
dissolve in 20ul H2O (pipetting up and down, wash also sides of eppi!), let evaporate residual EtOH by placing open eppi in 45°C heating block for 3-5 min.
determine concentration (usually 300-400 ng/ul).

2b without precipitation:

Ambion Kit: precipitate only if the template concentration is too low (100-500 ng PCR-template per in vitro transcription are ok)
estimate concentration of PCR product on a gel
use 1-8ul of PCR for dsRNA reaction

3. In vitro transcription for dsRNA (RNAi):

use the T7 Ambion Megascript Kit (since the template has T7 promotors at both ends, only one enzyme, i.e. T7, is required)

be careful to fully resuspend reaction buffer and rNTP solutions; assemble the reaction at room temperature to avoid precipitation of certain components of the buffer

template + water	8ul (PCR product: 300-500ng)	
10X buffer	2ul	
nucleotides each	2ul	
	2ul	
	2ul	
	2ul	mix
enzyme-Mix	2ul	mix

20ul keep at 37° for 4-5 hours (not over night)

4. LiCl-precipitation (Ambion kit):

add to reaction (which may look a little milky):

H2O	30ul	
LiCl-Lsg	25ul	(mix)

precipitate at -20° for 1h (freezes)

thaw, centrifuge for 30' at 15.000 rpm
remove supernatant (a milky pellet should be visible)
add 1ml 70% EtOH
centrifuge again for 30' at 15.000 rpm,
remove supernatant, let dry at air for 20-30 min.

resuspend in 20-40 ul injection puffer (H2o works also, but solution is less viscous in buffer – especially useful for eRNAi; repeated freezing and thawing may help bring the RNA into solution)

5. annealing

(this procedure changes the product as observed on the gel: the smear becomes less pronounced and the intensity focusses in a smaller region. This might indicate higher portion of dsRNA. We have not checked for changes in interference effect. Most preparations also work without annealing – we have not compared efficiency)

(prepare 100 ml boiling water, place Eppi for 2 minutes on 95° heating block, remove water from stove, place Eppi in the water bath until temperature is about 70° (15 minutes).)

6. determine conzentration:

nano-drop: 1ul
measure OD (dsRNA-settings are 45 (info by nano-drop))
concentration should be 1-5 ug/ul – at higher concentrations viscosity is too high for injection)

7. injections:

remove contaminating particles (which might clogg injection capillary) by centrifugation 1´ maximum (alternatively: use 45um Ultrafree-MC (Millipore) – but you will loose volume). For injection of pupae/adults you may omit this step – because the capillary is thicker

Inject as much as possible (2 - 5 ug/ul). Also lower concentrations (100-500 ng/ul) can result in phenotypes, but usually weaker

Primer sequences:

T7: 5' gaa ttg taa tac gac tca cta tag g 3'

T7-T3: 5' taa tac gac tca cta tag gaa tta acc ctc act aaa ggg 3'

T7-SP6: 5' taa tac gac tca cta tag gat tta ggt gac act ata ga 3'

T7-M13R: 5' taa tac gac tca cta tag gca gga aac agc tat gac 3'

for in situ ssRNA templates:

SP6: 5' gat tta ggt gac act ata ga 3'

T3: 5' aa tta acc ctc act aaa ggg 3'

M13R: 5' cag gaa aca gct atg ac 3'

injection buffer:

1,4 mM NaCl

0,07 mM Na2H PO4

0,03 mM KH2 PO4

4 mM KCL

Parental RNAi

(Gregor, 9/06)

Prepare injection aparatus and a binocular; the needle should be oriented in angle of approximately 60 degree.

The angle of the needle is approximately 60 degree, pressure is controlled by a 12 or 50 ml syringe.

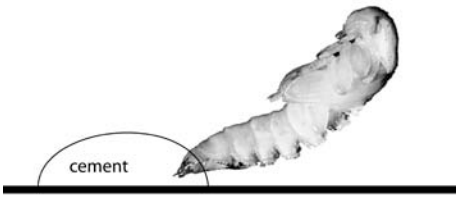
Select 10-20 female pupae; use old pupae (dark wings, eyes developed) because they survive treatment better (if you plan to fix the eggs, you may want to inject 50-200 pupae).

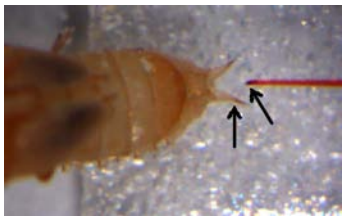
Fix double sided sticky tape along one rim of a microscope slide. (Alternative: Bring rubber based cement (Fixogum) onto a microscope slide)

Glue pupae with their posteriormost dorsal part of the abdomen to the glue (ventral side with wings is up). The region should not exceed the size of the last sternite. This is a critical step: gluing more anterior body parts will prevent them from hatching!

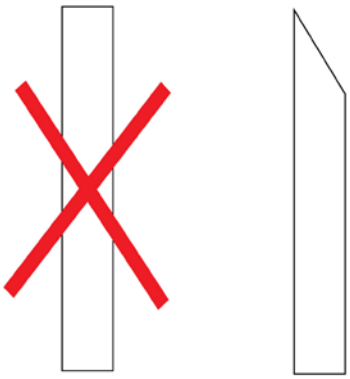
This is a pupae shortly before hatching (dark wings, legs sclerotized, eyes fully developed)

Side view of the slide and the rubber based cement. If you glue more of the abdomen to the cement (or sticky tape) the survival rate drops.





Break capillary such that the tip is approximately as thick as the sclerotized part of the urogomphi (see arrow). This is not very critical for survival but influences the handling. (Too much of the abdomen has been glued to the sticky tape – this pupa will not hatch)



It's important that the tip of the capillary is sharp to simplify the injection.

Pipet 5 ul of dsRNA solution onto an upside down Eppi-cap. Fill capillary through the tip by immersing it into the drop and applying slight underpressure. Be careful not to aspirate air – the dsRNA may suddenly be sucked into the capillary holder.

Inject laterally between abdominal segments 3 and 4 (just where the wing tips point to) (central injection leads to higher mortality). Go in by one fast movement (reaching appr. the middle of the pupa) then go back a bit. If fluid doesn't flow, move needle up and down a bit. Stop injecting when pupae are stretched and cannot move anymore because of raised turgor. When leaving the pupa you have to reduce the pressure at the same time (if not, the solution will leak out – if you reduce too much, solution will be sucked into the capillary holder…). Solution will initially extend anteriorly into the abdominal cavity and may be restricted to one half, but will distribute subsequently. The rule is: the more you inject the better.

The left pupa is not injected, the right one is stretched because of the filling (PhenolRed).

Place the injected pupae together with the slide upside down on full grain flour such that pupae inmerse in flour and the slide "covers" them. Add males or male pupae (5 males for 20 females). After 4-5 days at 32° Celsius remove the slide (you may have to free some beetles from the glue). Females need to feed on full grain flour before they can lay eggs and need appr. two days after hatching before they start to lay eggs.

Depending on the age of pupae, the first egglay can be taken 5-7 days post injection although maximal egg production is reached later. The portion and strength of phenotypes is high initially (up to 100%) but drops within days or weeks to zero (depending on gene and amount of injected dsRNA). Also the strength usually drops strongly with time. It is thus important to collect the first days.

DsRNA solution: use up to 1-5ug/ul (with higher concentrations, the solution gets too viscous for injection). The strongest effects are usually created by embryonic injections (up to 2ug/ul)!

Injection of adult beetles

(Markus Weber & Gregor Bucher, 2003)

Anesthetize about 10 adult females at one time with CO2 and affix them with their backs on a cover slide using Fixogum rubber cement.

Hold the affixed beetles once more over the CO2 plate. When they are dead drunk with CO2, they will relax and straddle their sclerites at the rear end and project their genitals. Then it is possible to inject the body cavity from behind. Be careful to inject as lateral as possible in order to avoid injuring the genitals, the ventral nervous system or the gut. As the ovary is located in the anterior part of the abdomen, we try to insert the needle quite a bit.

If the beetles retract their genitals, another CO2 treatment will make them willing again.

We used 1000 ng/ul dsRNA (Dll) diluted 1:1 with Pequlab transfectant (ask Alex). Results were not significantly different from injections without transfectant.

Egg laying is strongly reduced upon the treatment also with water injected controls (Gregor data with eagle, Dll, water) but recovers to some extend during the following days. Phenotypes can be observed one day after injection already.

Have fun!

Injecting Tribolium eggs for germline transformation

Andreas Berghammer & Martin Klingler 3/99

prepare
microscope slides
apple juice agar plates
use injection setup like for Drosophila transformation, prepare before line up
put the beetles on fresh Vollkornmehl for at least one night before using them
line up
approx1h egglay at 25°C in fine flour, let develop another hour (age strongly influences survival, the younger the more fragile)
dechorionate eggs carefully (1% Klorix in aprox 80ml;) in a large basked with large mesh (200um); move up and down until the flour gets off the vitelline membrane; change Klorix-solution once (total dechor. time: approx 2 X 40-60 sec); don't overdechorionate – strongly influences survival!
wash with deionized RT water by inmersing and moving up and down (do not wash too hard in order to avoid mechanical problems - if you wash too hard, some embryos will leak through the mesh - but you have enough for 1 hour line up anyway)
transfer embryos with a brush to a microscope slide with a 200 um sieve and some water,
remove excess water
with a Pinsel arrange embryos in a line along the edge of the slide; pointed end (=posterior) points towards outside
line up for approx 1-1,5 hours (don´t take too much time between dechorionating and injection)

injection under air
inject into the last third of the egg until you see the red colour between the yolk. Avoid leaking of too much yolk (injecting too much reduces hatching rate, injection directly into posterior or anterior tip leads to malformed larvae – probably because of interference with posterior patterning system)

place on apple juice agar plates (for humidity), put these plates into a closed plastic box
let develop at 32°C
when the first larvae hatched (day 3 in the afternoon at 32°) transfer slides into a dry box; transfer hatched larvae to flour (drying the eggs earlier will kill them, hatching in humid environment kills them as well...)
place slides such that embryos will fall into wheat when they hatch
DNA-Mix:

Andreas concentrations:	helper:	0,375 ug/ul
(endconcentrations)	DNA (6,5kb pBac):	0,5 ug/ul
	Phenolrot:	25%

fill up with water to a final volume of 20ul
clean by centrifuging in a 0,45um Ultrafree (red) for 5 min in a bench-centrifuge
place 2ul with a special plastic needle into a glass capillary, broken as distal as possible and schräg - bitte schön.

Comments
For best survival rate:
you need a good needle = thin and broken just like a medical syringe so that it will penetrate the eggs easily (with Roth´s puller: heat 8-10, manuell)
the more DNA you inject the less embryos will hatch (but transformation efficency might raise)
too much dechorionation is lethal to the eggs
the older the better they survive – inject eggs that are 2-3 hours old (time of injection!) .
, handle the eggs carefully and with RT-solutions and don't keep the eggs under water too long
Injecting under water does not change survival rate in comparison to injection under air - but visibility is better. Problems arise due to the use of heptan glue that will kill hatched larvae if not removed carefully and in time
the humid environment of an apple juice agar plate hinders some larvae to hatch successfully, if you dry them too early, they will die as well.

Apple juice agar plates

- mix 40 g Agar (Roth 5210.2) and 1 liter tap water in a 2 liter beaker, autoclave
- dissolve 33 g sucrose (supermarket)in 1/3 liter apple juice in a 60C water bath
- dissolve 2 g Nipagin (fungizide) in 8 ml ETOH

add dissolved Nipagin to hot apple juice, mix; then add hot apple juice to agar (take care to prevent formation of air bubbles) and mix using a magnetic stirrer
pour agar plates; when hardened, sealed into plastic bags, plates keep at 4C for many weeks.

Stainings

Whole mount in-situ hybridization for Tribolium embryos

(Martin Klingler 7/02)

A. FIXATIoN

1. Transfer embryos into egg basket (egg baskets are made from polyamide screen 0.10 mm mesh size, fused to a 1 cm section cut from a 15 ml falcon tube: fuse at 200 C on hot plate covered with aluminium foil; at this temperature the tube should melt but not the polyamide fiber)
2. Dechorionate (i.e. get rid of flour sticking to the eggs) by placing basket for 2 min into petri dish with 25% bleach (move basket with tweezers to stir the embryos)
3. Rinse well in deionized H_2O
4. Transfer with spatula into glass scintillation vial that contains 6ml heptane, 2ml PEMS, and 300ul formaldehyde 37%.
5. Fix for 30 min on shaking platform
6. Remove aqueous phase with pasteur pipette; devitellinize by adding 8ml MEOH and shaking vigorously for 30 sek
7. Transfer those embryos that fall to the bottom into an eppendorff vial. Embryos that remain at the interphase still have their vitellin membrane attached. Try to devitellinze these embryos by repeatedly aspirating and violently expelling the remaining fluid (both phases) in and out of the scintillation vial, using a syringe with 0.8 mm needle (19G needle). Transfer all embryos that fall to the bottom after this treatment into the eppendorf vial, discard those that remain at the interphase.

It is difficult to remove the vitellin membrane from post-gastrulation embryos since the serosa sticks tightly to the egg shell. Older stages usually only can be obtained as fragments, or as germ bands separated from yolk and serosa. **Care must be taken** during washing steps that these fragments are not aspirated with the supernatant, since they require more time to sediment than the intact blastoderm embryos

8. Rinse 2x with MEOH, store at -20^{o}C (indefinitely)

B. HYBRIDISATION AND STAINING

ul=microliter
All reactions and washes are done in Eppendorf vials; take 20-50ul settled embryos per vial
R = rinse: remove supernatant, add 1ml of new solution such that embryos are well mixed
W = wash: add 1ml of fresh solution, rock vial such that embryos are swirled and all vial surfaces washed. Let embryos settle down and then invert vial briefly to bring down embryos that stick to lid and walls.
W5: roll for 5 min. R R: rinse 2x.

Important for all washing steps!: The embyo mix consists of blastoderm stages (large) and germ bands (hardly visible). Be careful not to discard germ bands by rapid removal of supernatant – check against the light in order to detect floating germ bands!

6. R with 50% MEOH/PBT, let settle down, aspirate supernatant.
7. Post-fix in 1ml PBT + 140ul formaldehyde 37% for 15 min on wheel.
8. R R W W W with PBT
9. Incubate 5 min in 1ml PBT with 8 ug Proteinase K (Boehringer Mannheim, 15mg/ml, i.e. use 5 ul of fresh 1:10 dilution); (**don't overdigest**: rock vials for 4 min, then let embryos settle down for 1min. Stop by immediately proceeding with 10. Tribolium embryos tolerate only half as much protease than Drosophila embryos)
10. R R with PBT
11. Post-fix again in 1ml PBT + 140ul formaldehyde 37% for 15 min on wheel.
12. R R W R with PBT
13. Rinse with 250ul PBT + 250ul Hybe-B; replace by 250ul Hybe-B
  Replace with 250ul Hybe-A, preincubate for 1h at 65^{o}C.
14. Aspirate as much of Hybe-A as possible, add probe (ca. 1-5 ul) diluted in 30ul Hybe-A

---------- Hybridize over night in 65^{o}C waterbath ----------

15. add 500ul Hybe-B (prewarmed to RT), keep at 65^{o}C until embryos settle down; replace with 500ul Hybe-B, incubate at 65^{o}C for 15min.
16. proceed at room temperature: dilute by adding 500ul PBT;
then R W W15 with PBT
16b. W20 with PBT containing 0.5% **Boehringer blocking reagent**! (1 ml PBT + 200 ul 2.5% blocking reagent)
17. Rotate for 1h at RT with 1ml anti-Dig antibody on wheel (dilution 1:2000 in PBT; without blocking reagent!)
18. R R W W20, W30, W30 with PBT.
19. R W with staining buffer (freshly prepared)
20. Stain in 1ml staining buffer + 4.5ul NBT sol. + 3.5ul X-Phosphate sol. To avoid clumping of embryos, do not rock; just place vials horizontally and move gently from time to time. Staining may take 30min to several h or over night. 30 min to 1h is normal.

Stop reaction by W with PBT (pH 7.4).

21. R W30 W30 with dH_2O. Store embryos in fridge, or in ETOH at room temp. (R with 50% ETOH, 70%ETOH, 2x 100%)

C. Solutions

PEMS: 0.1 M Pipes, 2mM $MgSO_4$, 1mM EDTA, pH 6.9.
(400ml: 12.08 g Pipes + 800 ul of 1M $MgSO_4$ + 800 ul of 0.5 M EDTA, to pH 6.9 with NaOH)

PBT: PBS plus 0.02% Tween 20; stirred with 0.5ml DEPC/l for 30min and autoclaved
(720ml dH2O + 80 ml 10x PBS + 0.8ml 20% Tween 20 + 0.4ml DEPC)
(10x PBS: 320g NaCl + 8g KCl + 8g KH_2PO_4 + 46g Na_2HPO_4, add dH_2O to 4l, pH=7.4)

Fixative: 10% paraformaldehyde in PBS, 50mM EGTA; (bring to boil, add PFA, stir, bring pH to 7 with HCl (pH paper), filtrate through Whatman 1 paper; store as 15ml aliquots at -20^{o}C)

Staining buffer (prepare freshly each time; mix after each addition to prevent precipitates):
20ml of 0.1M Tris pH 9.5
add 1ml of 1M $MgCl_2$, mix
add 400ul of 5M NaCl, mix
add 100ul of 20% Tween20

Hybe-A = Hybridisation solution (store at -20ºC for up to 1 week):
10 ml deionized formamide
5 ml of 20x SSC (Maniatis), make to pH 5.5 (yes!) with HCl
fill to 20 ml with dest. water.
add:
0.4 ml of 10 mg/ml of boiled sonicated salmon testis DNA (DNA aliquots at -20ºC; boil 10 min and chill in ETOH bath at -20ºC)
100 ul of 20 mg/ml tRNA (aliquots at -20ºC)
20 ul of 50 mg/ml heparin stock (-20ºC)

Hybe-B: just 50% formamide, 5xSSC (pH 5.5), water
Boehringer blocking reagent stock solution (2.5%): 1.25 g blocking reagent in 50 ml PBT (dissolve at 65ºC; bring pH to 7-7.5 with NaOH); store at -20ºC
NBT solution: 4-nitro blue tetrazolium chloride, 75mg/ml in 70% dimethylformamide (and water); store at -20ºC
X-phosphate solution: 5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt, 50mg/ml in 100% dimethylformamide (DMF); store at -20ºC

E. Trouble shooting

Good staining and good morphology are mutual exclusive. Hybridisation conditions (duration, pH, temperature) and protease treatment have to be balanced such that the embryos just do not fall apart.

embryos dissolve during hybridisation, or disintegrate, or clump together during washes after hybridisation or during staining:

- make sure hybridisation solution is pH 5; at 70^{o}C embryos fall apart if pH is 7.5.
- vary proteinase K treatment: too low concentration causes weak signal and high background. Too high concentration causes loss of signal and bad morphology. Variable results may be due to variations in room temperature, protease-lot, time of previous storage of embryos in ETOH (try less protease for fresh embryos).
- keep hybridization time at 70^{o}C below ca. 12h
- if clumping becomes apparent during washes after hybridisation, swirl embryos gently by moving the vials by hand from time to time, do not rock on shaking platform; make sure embryos are not deformed by surface tension when trapped between air bubble and plastic

High background/low signal

- negative controls: embryos with deletion for the gene in question for unspecific hybridisation & embryos without probe for antibody purity
- reduce amount of probe; check that probe recognizes insert of vector only
- thoroughly preabsorp the anti-Dig antibody (overdoing it results in reduced activity, though).
- increase protease concentration, duration of hybridisation, or vary pH of hybe-A (pH 5 - 6.5)
- increase temperature during hybridisation.
- for low abundant mRNA's (like hairy), DEPC treatment of PBT and sterile working might be important; gloves are not necessary if you take care not to touch inside of vials, lids
- RNA'se treatment after the hybridisation does not help: it reduces signal and increases background (even at 5ug/ml)

- store embedded preparation in the dark; light makes embryos pinkish; to avoid this, thorough washing of embryos after staining also very important

Precipitates form during staining reaction:

- colourless, amorphous precipitates form if staining buffer is contaminated with PBT.
- yellow/brown needles sometimes form when staining goes over night. Prepare staining buffer freshly, mix solution each time after another component added.
- little blue cristalls in embryos: thoroughly wash embryos after staining. Store stained embryos before embedding in 70% ETOH, at RT, or in water rather than in PBS

inefficient devitellinisation:

do not expose the embryos to bleach more than just necessary; if bleach fresh, dilute 1:4 or more; stop treatment in time, and wash very well afterwards;

Antibody staining of Tribolium embryos

(Klingler 3/99, based on a protocol from Sue Brown)

ul=microliter

All reactions and washes are done in Eppendorf vials; use 20-50ul settled embryos per vial

R = rinse: just swirl up the embryos with 1ml of new solution

W = wash: add 1ml of fresh solution, rock vial such that embryos are swirled and all vial surfaces washed. Let embryos settle down and then invert vial briefly to bring down those embryos that stick to lid and walls.

W5: roll for 5 min. R R: rinse 2x.

1. Aspirate methanol, hydrate by R W10 W20 W20 with 1 ml PBT' each
   - In addition to the embryos to be stained, also process a falcon tube containing a similar total amount of embryos for **preabsorption** of the secondary antibody -
2. Aspirate and add 1 ml PBT' plus first antibody
   (anti-eve: 5 ul/ml; anti-invected (=anti-en): 50ul / ml PBT).
   In parallel, preabsorb secondary antibody (goat anti mouse: 1 ul/ml)
3. roll on wheel for 4h (room temperature) or over night (at 4°C).

---

4. save supernatant with secondary antibody for 5. (also can reuse primary antibody; in that case add serum to 5% in order to stabilize antibody)
5. washes: R W10 W20 W20 with PBT'.
6. Aspirate PBT' and add 1 ml of preabsorbed AP-conjugated 2nd antibody (goat anti mouse)
7. roll on wheel for 2h (RT).
8. R R W10, W20, W20, W30 with PBT'.
9. W W with staining buffer (remove all PBT' to avoid cristall formation during staining)

10. aspirate staining buffer and add 1 ml of staining solution (4.5ul NBT sol. + 3.5ul X-phosphate sol./1ml staining buffer).
    Mix and transfer into glass depression blocks for visual inspection under the stereo-microscope. Staining usually takes 30 min, but may be extended to several h.

11. To stop reaction, transfer back into eppendorff vial (using cut-off blue tip) and W with PBT' (pH 7.4 should inactivate alkaline phosphatase).

12. W10 W20 W30 with PBT'.

13. Embedd in glycerol (in glycerole, germ bands can be cleaned from yolk and vitelling membranes using tiny little needles; dehydrated embryos harden and can not be dissected)
    Glycerol: drop embryos onto slide, aspirate PBT' as well as possible, cover with 50% or 100% glycerol

Alcoholic Fuchsin-stain for Tribolium embryos

(Barbara Wigand / Martin Klingler 4/02; please cite Wigand & Klingler 1998, Tribolium Information Bulletin 38, pp 281-293)

- dechorionise Tribolium eggs by washing 2 x 4 min in bleach (Klorix)
- wash 2x with H_2O (in egg basket)
- transfer into glass vials and fix for 45 min in solution A (mix by shaking, let sit)
- discard lower phase
- add 10,0 ml methanol (MeOH), shake vigorously
- add another 5,0 ml MeOH zugeben and shake to dissolve the remaining heptane such that all embryos sink down
- tansfer embryos into eppendorf vial, discard supernatant
- wash 3 x with 1,0 ml MeOH
- fix for 1 h in sol. B at RT
- wash 4 x 20 min in 70% ethanol (EtOH)
- replace ETOH by 2 n HCl and keep for 10 min at 60°C (waterbath)
- wash 1 x with H_2O
- wash 2 x with 70% EtOH
- stain for 30 min with alkaline Fuchsin solution
- destain by washing with 95% EtOH
- dehydrate by washing 2 x with 100% EtOH
- remove supernatant
- add 1,0 ml sol. C, mix, wait until embryos settle down
- remove supernatant, add 1,0 ml Lsg. D

store at RT, analyse under stereomicroscope in glass block, or place on slide (in sol. D), using coverslide with plastilin-spacers

Solutions

A 3,0 ml PEMS
0,45 ml 37%igen Formaldehyd
5,0 ml Heptan

B 4,0 ml 95%igen EtOH

0,5 ml 100%ige Essigsre

0,2 ml 37%igen Formaldehyd

alk. Fuchsin solution 100 mg Pararosanilin (Sigma P-7632)

16 ml EtOH

4 ml H_2O dest.

0,2 ml conc. HCl

store in freezer

PEMS-buffer 12,08 g Pipes

800 ul 1 M MgSO4

800 ul 0,5 M EDTA

adjust with NaOH to pH 6.9

fill to 400 ml with Millipore-H_2O

C 100% EtOH : Lsg. D = 50 : 50

D Benzylbenzoat : Benzylalkohol = 4 : 1