

GUS Protocol (fool-proof version)

Kirsten Bomblies, adapted from François Parcy's protocol
With alterations specific for franks lab 13 March 2007

Harvest tissue and place in cold 90% Acetone on ice. This should stay on ice until all samples are harvested. For sample containers, eppendorf tubes and glass scintillation vials work well. Microtiter plates are useful for large numbers of samples, but these have to be polystyrene, NOT polypropylene.

Pull vacuum for 10 minutes at room temp , then fix at room temperature for 20-30 minutes.

In the mean time make up staining buffer without X-Gluc (see below for recipe) on ice with cold solutions.

Remove acetone from the samples, and add staining buffer **without X-Gluc** on ice. Pull a vacuum for 10 minutes and then release

Prepare staining buffer with X-gluc in a new tube to a final concentration of 2mM - from a 100mM stock solution of X-Gluc in DMF- this must be kept in the dark at -20°C .

Remove staining buffer without X-gluc (wash buffer) from samples and add staining buffer with X-Gluc on ice. Infiltrate the samples under vacuum, on ice, for 15 to 20 minutes. Release the vacuum slowly and verify that all the samples sink. If they don't, infiltrate again until they all sink to the bottom when the vacuum is released. May require two or three vacuum/release cycles

Incubate at 37°C (I usually do it overnight, but it depends on transgene strength. It is not advisable from my experience to go to long (over two days) as the tissue seems to begin deteriorating during long incubations.

Remove samples from incubator and remove staining buffer. Go through an Ethanol series in which samples are incubated successively in 20%, 35% and 50% ethanol at room temperature for 30 minutes each.

Incubate in FAA (recipe below) for 30 minutes at room temperature to fix the tissue.

Remove FAA and add 70% ethanol. At this point the tissue can be stored at 4°C for long periods, or examined under the microscope.

Two additional choices at this point:

If the tissue is to be sectioned, leave in 70% ethanol for 30 minutes and continue below.

if you want to look at whole mounted cleared tissues then

incubate 30 min at 80%

incubate 30 min at 90% ETOH

then **clear in chloral hydrate solution** - see chloral hydrate protocol - solution is 2.5 grams of chloral hydrate added to 1 ml of 30% glycerol.

allow to clear overnight in chloral hydrate, then dissect and mount under coverslips as desired for nomarski DIC analysis.

Chloral hydrate clearing yeilds excellent results for whole mount viewing!!

GUS Staining Buffer - Franks lab:

Stock Solutions: 10% Triton X
100mM NaPO₄ Buffer (see below for recipe)
100mM Potassium Ferrocyanide – on fridge door
100mM Potassium Ferricyanide - on fridge door
100mM X-Gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide
cyclohexamine salt - dissolved in DMF – see below)

To make 100mM NaPO₄ Buffer pH 7.2:

28 mls 0.2M NaH₂PO₄
72 mls 0.2M Na₂HPO₄
100 mls ddH₂O

200 mls 0.1M PO₄ Buffer

Make staining buffer fresh every time.

For 10 mls GUS Staining Buffer:	Final Concentration:
5 mls 0.1M PO ₄ Buffer	50mM
0.2 mls 10% Triton X	0.2%
0.2 mls 100mM Ferro/Ferri	2mM
4.6 mls ddH ₂ O	

Add 0.2 ml 100mM X-Gluc to **10 mls** of Staining Buffer (2mM Final Concentration)
If using smaller volumes in eppendorf tubes use 25 ul of 100mM X-GLUC for 1.25 ml of staining buffer to still give a 2 mM final concentration.

note: higher ferri and ferrocyanide concentrations give lower overall staining level, but more specificity. 2mM works well for most applications, but the concentrations may need to be adjusted for certain needs.

to make 100mM X-GLUC stock

we usually order 100mg of X-GLUC

add 1.9 mL of DMF (N,N, dimethyl Formamide) to 100mg of X-GLUC.

(i.e. 0.19millimoles of x-GLUCbecause the Formula weight of X-GLUC is 522 grams per mole)

mix until dissolved

then aliquot into screw cap tubes and store in -20 freezer.

FAA (make up fresh every time)

50% Ethanol

3.7% Formaldehyde

5% acetic acid

water to volume

for 100 ml of FAA

50 ml ETOH

35 ml of ddH₂O

10 ml of 37% formaldehyde

5 ml of Acetic acid

Embedding for sectioning:

It is best to do the following series of steps in glass scintillation vials. Eppendorf tubes are too small for the paraplast changes.

Continue with an ethanol series by incubating in 80% and 90% ethanol for 30 minutes each at room temperature. Remove 90% ethanol and incubate overnight in 95% ethanol with 0.1-0.5% Eosin-Y.

Remove 95% ethanol the next day and incubate in 100% ethanol, shaking gently for 15 minutes at room temperature. Change the ethanol and incubate again for about 30 minutes.

Remove ethanol and add 25% tert-butanol in ethanol and incubate at room temperature for 30 minutes.

You must use tert-butanol and not xylene as the X-gluc precipitate is soluble in xylene.

Repeat with 50% tert-butanol. Remove this and add 100% tert-butanol and incubate at 60°C 6 hours or overnight. At this time, prepare 50% paraplast in tert-butanol by filling a bottle half-way with the tert-butanol and then doubling the liquid volume by adding paraplast chips. Place in a 58-60°C incubator to melt.

Pour out the tert-butanol and add 50% paraplast, filling the vial almost to the top. Incubate overnight at 60°C. At this time melt 100% paraplast chips in a new bottle (not the one that had tert-butanol in it).

Pour off the 50% paraplast the next day and add 100% paraplast. Incubate several hours at 60°C.

Swirl the vials and let the samples settle. It is easier to pour off the paraplast if the samples are sealed into the bottom by putting your hand against the bottom of the vial to let a thin layer solidify and seal the samples in. Then pour off the melted paraplast quickly. Add more melted paraplast and incubate overnight at 60°C.

The next day, do another paraplast change as described above and incubate for one or a few hours. Set up a water-ice slurry, boats for pouring samples (weigh boats work well, or containers can be constructed from foil), and a hot plate with a temperature gradient.

After the paraplast has melted again, swirl the vials to suspend the samples and pour quickly into boats on the hot end of a heat block. Adjust the samples quickly with a wooden stick and move the boats as quickly as possible to a cooler temperature to do fine adjustments as the paraplast hardens. Leaving the paraplast too long on the hot end causes bubbles to form on the tissue, which will cause problems during sectioning. Once the paraplast hardens around the base of the tissue, stop moving it and move the boat to a cool surface without agitating it too much. Once it solidifies a bit, move the boat to the water-ice slurry bath to solidify the rest of the way. Store the tissue at 4°C until sectioning.

Sectioning:

Mount the individual samples by cutting them from the blocks with a razor blade by first making a groove, and then breaking the block along the groove. Shave away extra paraplast and melt it on a spatula to mount the sample on a stub.

Adjust the angle of the sample and trim away excess paraplast with the microtome on trim. Then start sectioning. For GUS, 8 or 10 microns tends to give good results, but a variety of thicknesses should be OK. Help the ribbons along with a small paint brush so they don't curl up on themselves.

Place the ribbons on a slide loosely and examine under a dissecting scope or inverted scope to choose the sections you want to keep. Put these on black paper so they are easy to see and cut to size with a clean razor blade. Pipette water onto the slide on which you would like to mount the tissue (We use Fisherbrand Superfrost /Plus precleaned microscope slides) and pick up the ribbons gently with a wooden stick with the end slightly moistened with water by touching the ribbons lightly. Place ribbons on the water with the shiny side DOWN (if you look closely, the ribbons come off the microtome with the dull side up and shiny side down, this is the way they should be mounted, otherwise,

air bubbles get trapped under them). Place the slide with the water and ribbons on a 42°C slide warmer. Allow them to sit for about ten minutes and then remove excess water by tilting the slide to one corner and removing the drop with a pipette or a kimwipe, being careful not to touch any ribbons.

Leave the slides on the slide warmer at 42°C overnight.

If the slides are to be stored before they are deparafinized, they should be stored over drierite in a sealed container (but not touching the drierite).

Mounting:

Mounting GUS slides.

INCUBATION	TIME	TEMPERATURE
Tert Butanol	10 min	60 degree C
Tert Butanol	10 min	60 degree C
100 % EtoH	3 min	Room temp

After the 100% ETOH you have two choices.

Either proceed down through a graded etoh series until you get to ddH2O and then mount in poly aqua mount.

Or go from 100% ETOH to 100% xylene for 2 times one minute and then cover slip in cyto seal. I am not sure which method gives better results at this time.