**Organelles and inter-organellar communication**

Equipment List Experiment carried out in pairs (example: 35 pairs)

Equipment for Practical

12x microfuge tubes

P1000 Gilson

Blue Tips

Minimum 4x Slides and Cover Slips

Eppendorf Rack

Fine Marker Pen

Forceps

Water bath 37°C

Heat block 65°C

Microscope

Stereomicroscope

Computers?

Foil?

Solutions for Practical

3ml Fixative

3ml EDTA

4ml GUS Reaction Buffer

14ml Ethanol

14ml H2O

50% Glycerol for mounting. Can use water, but glycerol is better as it hardly evaporates

Samples

2 Agar plates each containing the 4 Arabidopsis lines;

Seed types Alternative name and availability (from Nottingham Arabid. Stock Ctr.)

1) *pLhcb1::GUS*  pOCA108, [N9400](https://arabidopsis.info/StockInfo?NASC_id=9400)

2) *p35S::GUS*  Various available. Contact if required

3) *arc6*  [N286](https://arabidopsis.info/StockInfo?NASC_id=286)

4) Wild type *ARC6* Ws, [N3115](https://arabidopsis.info/StockInfo?NASC_id=3115)

5) Wild type for *pLH::GUS* Bensheim, [N76345](https://arabidopsis.info/StockInfo?NASC_id=76345)

One plate contains ordinary MS medium, the other contains MS supplemented with 0.5mM Lincomycin.

Pre-stained seedlings (5 of each seedling, type 1, 2 and 4, for each condition (+/-Lin) (6 tubes))

Growing the Arabidopsis seedlings

Seeds grown in Petri Plates, ideally 1.5 cm tall (Cellstar) with approximately 30ml of agar.

Sterilising the Filter paper 7cm

Label the filter paper with pencil. Autoclave the filter papers in a glass petri dish covered in foil, then dry in the oven.

Wild Type

Agar plates

1x MS salts and micronutrients

1x Gamborg’s vitamins (1µl of 1000x stock solution) To tell the truth, dispensable, never noticed a difference

2% Sucrose (20g/litre)

MES 0.5g/L

Make up to 1 litre with H2O and pH to 5.7 with KOH (better than NaOH)

Add agar at 0.8% (8g/litre)

Autoclave and cool to 55°C (i.e in water bath)

NB add stir bar to bottles that will have the addition of Lincomycin

At this point add 5ml/litre Lincomycin stock to required bottles

Lincomycin (100mM stock) – Dissolve 0.44g in 10 ml H2O and filter sterilise

Sterilising the seeds

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Sowing the seeds

Place the filter paper on top of the agar (all seeds to be first grown on MS medium only). Sow approximately 30 seeds of each type per quadrant. Then place the plates at 4°C for 3 days (approx depending on timing). The plates are then moved to an incubator at 21°C with light for around 40 hours (no more than this! i.e move to incubator at 5pm day 1 and then transfer to Lin+ plates 9am day 3). After this time half of the seedlings can be moved to the lincomycin plates. The seedlings are then left to grow for a further 7 days.**Chemicals Required**

MS salts and micronutrients (Duchefa, Melford Laboratory Supplies

Gamborg’s vitamins (dispensable)

Sucrose

Plant agar or micoagar (Duchefa)

Lincomycin 0.5g 1,000,000 units (Sigma, L6004) £21.50

Potassium Ferricyanide (Potassium hexacyanoferrate (III))

 Potassium Ferrocyanide (Potassium hexacyanoferrate (II))

 5-Bromo-4-chloro-3-indolyl-β-D-glucoronide (X-Gluc CHA salt) (Melford Labs MB1021)

Ethanol

Glutaraldehyde or Formaldehyde

EDTA

NaCl

Tris

Triton X-100

Dimethyl Formamide

**Solutions for GUS Reaction Buffer**

1) 2x TBS GUS Buffer = Tris 0.2M (2.422g/100ml), NaCl 0.1M (0.584g/100ml) pH7.5

2) Potassium Ferricyanide 0.1M (3.293g/100ml) (Potassium hexacyanoferrate (III))

3) Potassium Ferrocyanide 0.1M (4.224g/100ml) (Potassium hexacyanoferrate (II))

4) 5-Bromo-4-chloro-3-indolyl-β-D-glucoronide (X-Gluc CHA salt) 50mg dissolved in 1ml of Dimethyl Formamide and kept in the fridge)

Autoclave the first three solutions, they’ll last long time at room temperature. Cover ferri/ferro from direct sunlight. X-Gluc will last also months at least in DMF in fridge. It does not need to be CHA salt.

The ferri/ferrocyanide components can be omitted.

**GUS Reaction Buffer - 100ml**

1) 2x TBS GUS Buffer 50ml

 10% Triton X-100 0.5ml

2) Pot Ferricyanide 0.1M 0.5ml

3) Pot ferrocyanide 0.1M 0.5ml

4) X-Gluc 50mg/ml 2ml

 H2O 46.5ml

Can be frozen

**Fixative**

4% Glutaraldehyde (or formaldehyde) in 1x TBS GUS Buffer (32ml of 25% glutaraldehyde + 168ml of 1x TBS GUS Buffer).

**EDTA 0.1M**

EDTA Acid 3.26g in 100ml H2O adjust to pH9 with NaOH or

EDTA Na2 Salt 3.72g in 100ml H2O adjust to pH9 with Hcl

**Plan for 22 Feb and 35 pairs of students (example)**

Mon 7th – Sterilise tips and 100x filter paper. Make 100 MS agar plates (3.5 litres) and 50 MS + Lincomycin agar plates (2 litres) and pour plates. 35 student plates +/- Lin, 12 +/- Lin for harvesting day prior to practical and 3 +/- Lin as spares.

Wed 9th – Sterilise seeds and place on the filter paper in agar plates and put at 4°C for 3 days. Approx 30 seeds per quadrant for student plates and 60 (only types 1,2 and 4) for the harvesting plates.

Sat 12th – pm: Move the seedlings from 4°C to 21°C in the light.

Mon 14th – Transfer half of the seedlings (seeds on filter paper, just germinated) to Lincomycin plates under sterile conditions.

Mon 21st –

Make 270ml GUS reaction Buffer

Make >120ml Fixative

Make >120ml EDTA 0.1M

Harvest. 33 sets (Type 1, 2 and 4 +/- Lin) x 6 samples = 198 tubes. 5 seedlings per type +/- Lin into microfuge tubes then add 0.5ml GUS reaction buffer at 37°C for 24 hours prior to practical.