**STEM WORKSHOP on Pollen Biology**

**University of Massachusetts**

**Biochemistry and Molecular Biology Department**

**(April 6, 2013)**

**Presented by: Professor Alice Y. Cheung (acheung@biochem.umass.edu)**

**Professor Hen-Ming Wu**

**Dr. Yan-jiao Zou**

**Lederle Graduate Research Tower, 10th floor conference room**

**Sponsored by the NSF support Research Coordination Network on**

**Integrative pollen biology.**

[**http://pollennetwork.org/**](http://pollennetwork.org/)

**Summer internships for teachers available:**

**The RCN provides Supports for $750 per 40 internship hours for** **up to $3000 per summer (e.g. 20 hrs-week for**

**8 weeks); can be pro-rated up or down as budget allows**

**Contact Alice Cheung (**[**acheung@biochem.umass.edu**](mailto:acheung@biochem.umass.edu)**)**

**Continued interaction between teachers and sponsor is encouraged for consultation to set up a laboratory exercise, give a talk in your class, show @ tell, host student visits from your class, and to host you again in future summers or other continuing education opportunities.**

**General references for pollen and plant reproductive biology**

# Cheung et al., 2010. The pollen tube journey in the pistil and imaging the in vivo process by two-photon microscopy. J. Exp. Bot. 61, 1907-1915. <http://jxb.oxfordjournals.org/content/61/7/1907.full.pdf+html>

Marton and Dresselhaus. 2010. Female gametophyte-controlled pollen tube guidance. Biochemical Society Transactions 38, 627-630. <http://www.biochemsoctrans.org/bst/038/0627/0380627.pdf>

Cheung and Wu. 2007. Structural and functional compartmentalization in pollen tubes. J. Exp. Bot. 58, 75-82. <http://jxb.oxfordjournals.org/content/58/1/75.full.pdf+html>

**Agenda:**

**9:00 am** Brief introduction of the workshop’s agenda and experiments

**9:30 am** Get experiments started.

* Pollination, pollen tube growth in the pistil
* *In vitro* pollen germination,
* semi *in vivo* pollen tube growth

**9:45 am** Lecture, Introduction to pollen biology and plant reproduction

and on experimental procedures

Dr. Zou gets more experimental samples going for the group

**10:45am** Break and Q@A from teachers in the audience

**11:30-** Sample observations (separate into two groups, one stay in

Lecture hall, another goes to Cheung microscope room;

Exchange. Continue Q@A

**12:45 pm** Conclusion, teacher’s summer internship opportunity

**Protocols for experiments and demonstrations**

[These are from the protocol book prepared by RCN working group members Mark Johnson (Brown University) and Sheila McCormick (U.C.Berkeley) for a short course on pollen research methodology. The entire protocol book is on the RCN website]. These protocols work, but there are also many variations that could work comparably well.

**EXPT ONE. Aniline blue staining of pollen tube in the Arabidopsis pistil**

**Reference:** MORI, T., H. KUROIWA, T. HIGASHIYAMA and T. KUROIWA, 2006

GENERATIVE CELL SPECIFIC 1 is essential for angiosperm fertilization. Nat Cell Biol 8: 64-71.

**Solutions:**

Fixative Acetic acid/EtOH (1:3) solution

EtOH series 70%, 50% and 30% EtOH

Alkaline treatment solution (ATS) 8 M NaOH

Decolorized(\*) aniline blue solution (DABS) 0.1% (w/v) aniline blue in 108 mM K3PO4 (pH ~11)

\* Decolorization After preparation of the solution above, store it in the fridge at 4C

overnight.Prepare a funnel with filter paper and add a teaspoonful active carbon powder, then

filter the solution through the powder on the following day. Add glycerol to the filtrate so that its

final concentration becomes 2% (v/v). Store it in the fridge at 4C.

**Protocol Steps:**

1. Fixation Collect Arabidopsis pistils from one- or two-day-old flowers after flowering

and put them in a plastic tube of the fixative. Aspirate the air in the capped tube using a 50 mlsyringe

with 18 gauge until the specimen does not release any bubbles. Leave the tube for at

least 2 hr at room temperature (RT).

2. Exchange the fixative with 70% EtOH and leave for 10 min at RT. After that, do the

same treatment using 50, 30% EtOH and DW.

- 16 -

3. Alkaline treatment: Move the specimen into the small petri dish (96 well plates are

very efficient) of ATS carefully. Leave the dish with its lid overnight at RT.

4. Washing Exchange ATS to DW carefully because each pistil must be very softened.

At this time, you can observe the specimen clearing. Leave the dish for 10 min at RT.

5. DABS staining Exchange DW to DABS carefully and leave for at least 2 hr under dark

condition using a piece of aluminum foil at RT. You do not have to wash the specimen after this

treatment.

6. Observation. Put each pistil with extra DABS on the slide glass, and then put a cover

slip on it carefully from the end of pistil with avoiding bubble contamination. At this time,

you can see ovary walls are split by the weight of cover slip. After that, you only have to observe

pistils with a microscope under UV irradiation condition. If you would not like to disperse pollen

tubes so randomly, you had better increase the glycerol concentration in DABS.

**EXPT TWO: *In Vitro* Pollen Tube Growth**

**References**:

Temperature as a determinant factor for increased and reproducible *in vitro* pollen germination

in *Arabidopsis thaliana.* Leonor C. Boavida and Sheila McCormick. The Plant Journal (2007) **52**,

570-582 (link is <http://onlinelibrary.wiley.com/doi/10.1111/j.1365-313X.2007.03248.x/pdf>; if you have problem getting it, email me, I’ll send you a copy).

The protocol below is the Mark Johnson lab (Brown University) version of Boavida et al.

**Plant material required**

Flowering *Arabidopsis* from genotypes of interest

- 25 – 50 Flowers for each genotype is best.

-Younger plants are better, however the first few flowers on the primary bolt are often

infertile, look for flowers above siliques that have started to lengthen. Confirm anther

dehiscence on a dissecting scope before use.

-If the plants have been grown in a growth chamber, then avoid the flowers that have

been growing very close to the lights – these will have increased anthocyanin production and

will look purple or otherwise pigmented, heat stress can negatively affect pollen development

and dehiscence.

**Reagents Required**

Stock Solutions (at 100x) pre-autoclaved & stored at room temperature:

500mM KCl

500mM CaCl3

100mM MgSO4

1% H3BO3

Sucrose

Low-Melt Agarose

1M KOH (for adjusting pH)

MilliQ H20 or ddH20

**Equipment Required**

Microscope slides, Coverslips, ImmEdge Pen

Forceps

Pollen Growth Chamber

pH meter

65°C Water Bath

**Protocol Steps**

Making Pollen Growth Media (20ml):

1. Weigh out sucrose 10% for liquid media (2g), 20% for solid media (4g). Pour into a 50ml

Falcon conical tube.

2. Add 200μl of each stock solution to the sucrose to reach the final desired concentrations

(5mM KCl, 5mM CaCl3, 1mM MgSO4, and 0.01% H3BO3).

3. Add ddH20 to reach the final volume of 20ml and vortex until all solutes are completely

dissolved.

4. Add 1M KOH to reach pH 7.5. 4μl of 1M KOH is normally sufficient, however depending

on the pH of your ddH20 water source this is subject to variation. In the optimization

process it is best to determine this volume empirically. Once the PGM is at pH 7.5 it is

ready for us in liquid culture.

5. When making solid media add 1.5% to 2% low melt agarose to pollen growth media

containing 20% sucrose and incubate in a 65°C water bath until all the agarose is

completely dissolved.

6. When completely dissolved, this media can be poured into small (35 x 10mm) petri

dishes or onto a microscope slide (~100 !l).

Prepping microscope slides for liquid growth

1. Using the ImmEdge pen, trace 2 9mm x 9mm squares on each slide. Each slide

normally carries two independent replicates of each genotype of interest

2. Let the hydrophobic barrier completely dry before use.

3. Using a clean pair of forceps, apply the pollen genotype of interest to the center of the

square, either by dabbing whole flowers or anthers onto the glass, or by gently flicking

the flower across the tip of another pair of forceps, such that the square is densely

covered in pollen. When using healthy Arabidopsis flowers this normally takes between

4 to 8 flowers before the square is saturated. Alternatively pollen can be applied after

application of the growth media, both techniques can be used successfully.

4. Add 50μl of fresh pollen growth media onto each pollen-saturated square.

5. Gently flip the slide over so that the droplets are hanging from the surface of the slide.

The droplet will be held in place by the hydrophobic barrier laid down by the ImmEdge

pen. Be careful to not turn the slide over too slowly or too quickly, as the drop can fall off.

The pollen should quickly collect at the surface of the upside-down droplet.

6. Place the slides carefully into the pollen growth chamber. Make sure that the growth

chamber has a clean layer of water in the bottom to create the humid environment

needed for pollen tube growth.

7. Close the chamber when it is full of slides for pollen tube growth, and gently wrap the

chamber in tin foil as to block out light.

8. Place the growth chamber in a temperature controlled chamber at 22°C for the desired

time of growth, typically a 6 hour time course is sufficient to have pollen tubes of an

average length of ~400 microns.

For solid media

1. After you have made fully solidified media slides, like with liquid media, gently dab or

flick the pollen onto the desired regions of the solid media slide.

2. Place these slides into a pollen growth chamber. If the slides are facing upright they run

the risk of precipitation from the humidity chamber falling onto them, so they are often

grown upside-down to avoid this.

3. Cover the growth chamber with tin foil to block light and place into a 22°C chamber for 6

hours.

Preparing pollen growth slides for imaging

1. Remove slides from the growth chamber and gently flip the slides back over. Pollen

growth should be visible under a dissecting scope.

2. If pollen is to be examined live (ie for DAPI, Fluorophore-tagged proteins, etc) remove

~30μl of the pollen growth media, and place a cover slip (24mm x 30mm) over the two

squares which each have 20μl of growth media left. (See Fluorescence-tagged protein

microscopy)

3. If pollen is to be imaged for length alone, completely remove the pollen growth media

and add 20-30 μl of 50% glycerol to each square (volume depends on cover-slip size).

Place a cover-slip over the squares. These can be stored long term by sealing with clear

nail polish.

**Measuring pollen tube length with ImageJ**

1) Open the ImageJ application

2) In Finder, locate the tif file of your image of interest.

3) Drag the file on top of the ImageJ button in the bottom scrollbar of your computer screen

(this will open the file using the ImageJ program)

4) To set your scale:

a. In the imageJ toolbar, right click on the button with the diagonal straight line (this is

the fifth button from the left). Set the setting to “straight” line

b. Trace your scale bar

c. Go to “analyze” - “set scale”

i. Type in your known distance (for example, if your scale bar represents 100

!m, type that in your known distance, and type in micrometer in the unit of length.

ii. Click the “global” button. This will keep your scale bar set for the entire time

you have ImageJ open.

5) Now go back to the imageJ toolbar and right click again on the button with the diagonal

straight line (the fifth button from the left). Now set the setting to “freehand”.

6) Trace your pollen tube from the edge of the pollen grain to the tip of the tube.

7) Command + M measures the length of the pollen tube. You can also get there by going to

“analyze à measure”.

8) Your measurements will show up in a new window called “Results”.

9) After measuring 50 pollen tubes for one rep, copy and paste your results into an excel

document for analysis.

**Fluorescence-tagged protein microscopy**

After pollen tube growth, the slides of genotypes bearing fluorophore-tagged protein constructs

should be mounted in pollen growth media and covered with a coverslip. If DAPI is to be added

to pollen tube growth DAPI should be added into the liquid pollen growth media, see protocol.

**EXPT THREE: Semi *In Vivo* Pollen Tube Growth**

**Reference:**Distinct short-range ovule signals attract or repel Arabidopsis thaliana pollen tubes in vitro.Palanivelu R, Preuss D. (2006) BMC Plant Biol **6**: 7. <http://www.biomedcentral.com/1471-2229/6/7>

**Plant Material Required**

*ms1* (*male sterile 1*) flowers

Flowering *Arabidopsis* from genotypes of interest

**Reagents Required**

Solid pollen growth media on plates and/or slides

**Equipment required**

Pollen growth humidity chamber

Microscope slides

Slide Coverslips

Forceps

Sharp surgical scissors (or a 27.5 gauge needle)

Petri dishes

Double-sided tape

**Protocol Steps**

1. Place a strip of double-sided tape onto the lid of a petri dish and place this under the

field of a dissecting microscope.

2. Pick several *ms1* flowers and place the whole pistil onto the double-sided tape.

3. Gently angle the pistil so the stigma is facing upwards and is amenable for pollination

with your pollen genotype of interest.

4. Using clean forceps gently pollinate the *ms1* pistil with your desired pollen donor.

5. Once the pistil is fully covered (or if in the case of limiting pollinations sparsely covered)

with pollen, use the surgical scissors (or a 27.5 gauge needle) to cut across the

shoulders of the style just below where they meet the stigmatic tissue. The aim is to

exclude *ms1* ovules, but to include the very beginning of the transmitting tract (see

following diagram).



6. Place the pollinated stigmas vertically onto the solid growth media such that the incision

is flush with the media. The stigma should be sticking up into the air, not allowing the

pollen to touch the media. Leave the stigmas standing for 1.5 – 1.75 hours. Keep the

dish/slide covered and humid whenever it is not being actively used.

7. Lay the *ms1* pistils that donated their stigmas flat onto the double-sided tape so that the

replum is in facing up with each carpel on either side.

8. Use a clean sharp hypodermic needle (27.5 gauge) to cut each carpel open, revealing

the ovules without damaging them.

9. Excise ovules from the dissected *ms1* pistils by gently rubbing the needle tip against the

funiculi of the ovules.

10. Transfer the ovules to the solid media in the desired arrangement. A single file line, or Ushaped

arrangement close to the base of the excised stigmas both work well. The

distance from the edge of the pistil explant is crucial, as you want the ovules to be within

~200-500 !m of the stigma explant.

11. After you have arranged the ovules, put the dish/slide into a humidity chamber. Plate this

at 22°C until the 1.5 hours is complete.

12. Take out the petri dish/slide after the initial 1.5 hours and gently push the stigma over so

that it is lying horizontally with the transmitting tract facing the ovules.

13. If targeting will be examined in real time take the dish to a dissecting scope equipped

with time-lapse capabilities. Otherwise, place the dish back into the humidity chamber

and grow at 22°C for another 6-18 hours to quantify tube growth.

**EXPT FOUR: *In Vivo* Analysis of pollen tube growth using LAT52:GUS (Blue Dot**

**Analysis)**

**References**:

-Arabidopsis *hapless* mutations define essential gametophytic functions. Johnson MA, von

Besser K, Zhou Q, Smith E, Aux G, et al. Genetics (2004) **168**: 971-982.

-Arabidopsis HAP2(GCS1) is a sperm-specific gene required for pollen tube guidance and

fertilization. von Besser K, Frank AC, Johnson MA, Preuss D. Development (2006) **133**: 4761-

4769.

**Plant Material Required**

Flowering *ms1* (male sterile 1) plants

Flowering *Arabidopsis* from mutant genotypes of interest bearing a LAT52:GUS transgene (i.e.

SAIL lines 1-456, 1052-1057, 1142-1205 & 1206(A-D))

Flowering Control LAT52:GUS line (76224)

**Reagents Required**

80% Acetone

X-Gluc Solution

- 13 -

(X-Gluc constituents final concentration: 5mM Potassium Ferrocyanide, 5mM Potassium

Ferriccyanide, 50mM NaPO4 pH7, 0.5mg/ml X-gluc (5-bromo-4-chloro-3-indolyl-"-D-glucoronic

acid, cyclohexylammonium salt)

50% Glycerol

**Equipment Required**

Dissection microscope

Double sided tape

Hypodermic needle (27.5 gauge)

Syringe

Forceps

Petri Dishes

Thread and/or Lab Tape

Scissors

Flat bottomed 96 well plate

Humidity chamber (Tupperware with water in bottom)

Microscope slides

Coverslips

**Protocol steps**

1. Position an *ms1* flower under a dissecting microscope, trim away older pistils with

scissors.

2. Tie a piece of looped thread around the pedicel of the flower or fold a labeled piece of

tape below flowers pollinated.

3. Using the forceps, fully pollinate the stigma of the *ms1* with the desired pollen genotype.

4. Allow the pollination to continue for 12-24 hours at normal growth conditions.

5. Place a strip of double-sided tape onto the lid of a petri dish and place this under the

field of a dissecting microscope.

6. Pick off the pollinated *ms1* pistils and lay them flat on the double-sided tape so that the

replum is facing up, and the two carpels are at either side.

7. Using a clean needle (27.5 gauge) attached to a hypodermic needle as a handle, gently

cut along the edge of each replum, and at the base and top of the carpel walls (see

figure in Johnson and Kost, 2010 – end of book).

8. Push the now loose carpel walls against the double-sided tape.

9. Gently cut the back wall of the carpels that is attached to the tape.

10. Carefully lift the pistil off of the tape and place it into a well in a 96 well plate filled with

200μl of 80% Acetone.

11. Allow the pistil to clear for 2 or more hours.

12. Remove the acetone and exchange for 100μl of X-Gluc solution.

13. Cover the 96 well plate and place into a humidity chamber.

14. Incubate at 37°C for 24 hours or longer. Depending on the strength of the LAT52:GUS

expression in the mutant allele, staining can be extended up to 7 days.

15. Remove the incubation chamber from 37°C and using forceps pull the stained pistils out

by the base of the pistil. Place the pistil on a microscope slide under a dissecting scope

and add 20μl of 50% glycerol to keep the pistil from drying out.

16. The pistil can be cut in half vertically for quantification of targeting events, or it can be

sealed as is by adding additional 50% glycerol and placing a cover slip over the pistils.

17. Image on a microscope, you should be able to see GUS precipitate in the ovules that

have been successfully targeted by LAT52:GUS containing pollen tubes.