Plant Development

NST Part 1B Cellular and Developmental Biology Practical

This practical is a student-led exercise in data interpretation, hypothesis generation and experimental design. The practical runs over two weeks in the Teaching Laboratory in the Department of Plant Sciences. You should plan to attend the practical in either the morning session (starting at 11:00) or in the afternoon session (starting at 14:00). Please use the same groups that you have been assigned earlier in the year, as we need to follow Covid rules for spacing and numbers in the laboratory. The practical sessions are theoretical challenges, and lab coats are not required, however we still have a policy of wearing masks in this kind of crowded situation in the laboratory.

WEEK 1 timetable: Data-based experimental design

Introduction to Marchantia

A brief introduction to features of the Marchantia life cycle, production of gemmae and cellular features during germination and growth. After this presentation, the class will split into 4 working groups and locate to the corners of the laboratory. It would be helpful to nominate one or two spokespersons for each group.

Analysis of the effects of exogenous auxin on gemmae growth (20 min)

Each group will analyse the data provided in Section A, which shows the visible effects of application of exogenous auxin on germinating gemmae over time and at different concentrations. Each group should decide what they think are the main effects of auxin on growth. Are there particular features or cell types affected? What do you think this might imply for the natural role of auxin in this system?

Report back to the class

Each group will report back to the rest of the class to exchange views and help build a consensus. Then the groups will return to consider the next set of data.

Analysis of single cell gene expression data (20 min)

Each group will analyse the data provided in Section B, which shows the results of mRNA transcript analysis from cells within gemmae cells, 4 days after germination. The transcripts from each cell were uniquely bar-coded, sequenced and analysed to generate clusters of related cells that can be roughly mapped to different cell types in the gemmae. This allows one to estimate transcript levels of genes in different cell types. Values are provided for the set of Marchantia genes involved in auxin synthesis, transport and response. Can you build a hypothesis (or hypotheses) for how auxin might be working in this system?

Report back to the class

Each group will report back to the rest of the class to exchange views and help build a consensus. Then the groups will return to consider the next set of data.

Experimental design (20 min)

With your developing hypothesis in mind - are there experiments that you can design that would allow you to test your model, or look for correlated behaviour?

Summary session

Week 2: Analysis of data to test potential role of auxin in gemma patterning.

Auxin-mediated patterning in a simple plant system, *Marchantia polymorpha*

The selective traffic of auxin regulates cell polarity in plant tissues. A combination of influx and highly selective efflux transporters coordinate the flow of auxin within the plant and control cell fates and outgrowth. Further, auxin regulates the flux of its own traffic through plant tissues. Apical-basal polarity in plant embryos, outgrowth of the root and shoot meristem and initiation and maintenance of the plant vascular system are all dependent on this precise feedback-regulated traffic of auxin.

Current models for auxin based regulation of plant cell growth and differentiation rely on polar traffic of auxin, via specifically localised efflux carriers throughout the plant. Some form of positive feedback between efflux carrier and auxin flux results in long-distance pathways for auxin traffic. This mechanism provides a route for local cell interactions with different cells primed for auxin production, transport and response to auxin.

Introduction to *Marchantia polymorpha*

In this practical, we introduce the simple model plant *Marchantia polymorpha*. Marchantia is the best-characterised liverwort, member of a class of bryophytes that diverged from higher plants about 500 million years ago. Its ease of use, open form of development, haploid genetics and reduced gene set have generated great interest in its use as a new model plant.

One of the features of the Marchantia system is that it naturally produces prolific vegetative propagules, named gemmae (Figs. 1-3), that can he harvested and grown in sterile culture. Germination and subsequent development occur in the open, and can be monitored directly by microscopy.

Some salient facts are:

1. Gemmae are produced within cup-like structures ("splash cups") on the surface of the parent plant. The propagules are typically spread by water splash. As they land on a suitable substrate, they must quickly establish the correct dorsal-ventral polarity. Light and gravity provide cues for this, and auxin response is also required.
2. Day 1: Gemmae germinate immediately, and grow in stereotypical and staged fashion. Cells at apical notches on both ends of the gemmae undergo division. Cells in the central portion of the gemmae do not divide, but expand. The gemmae possess mirror symmetry at this stage.
3. Day 2: Long root hair-like rhizoids form, sometimes initially on both ventral and dorsal surfaces, but are eventually limited to the ventral side of the growing plant. Oil cells may also be apparent on the dorsal surface.
4. Day 4-5 The meristematic notches remain active, and distal cells under differentiation to produce air chambers. These can be recognised by characteristic air pore cell complexes. These will continue to form over the life of the plant and are specialised structures for photosynthesis and gas exchange.
5. Week 1: Secondary meristematic regions can result in the formation of additional "flaps" of tissue around the meristematic notches, as growth continues.
6. Week 2: First appearance of splash cups, the conical cup-like structures that produce new gemmae from a layer of somatic embryogenic cells at the base of the cup.

Fig. 1: *Marchantia splash cup - source of gemmae (image: Jim Haseloff)*

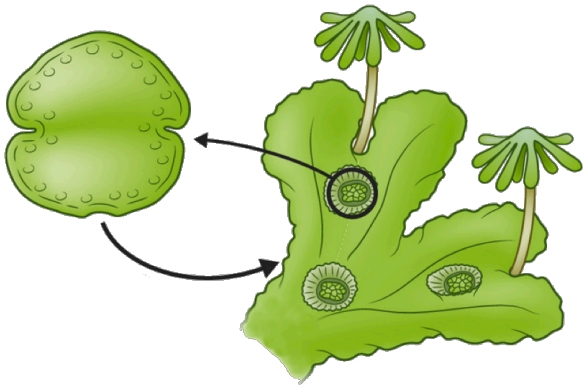
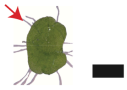
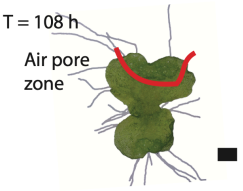
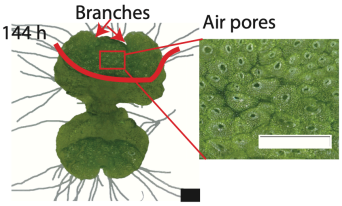
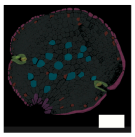
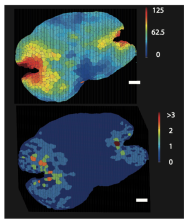


Fig. 2: *Vegetative life cycle - a gemma will grow to produce more propagules within 2-3 weeks.*

Fig. 3*: A Marchantia gemma removed from the splash cup (image: Jim Haseloff)*

T = 0 h 	**Stage 0 (T = 0 h, removal of gemmae from splash cups)** - Large central cells (1500-2500 µm^2 approximate surface area) - Small cells within the apical notches of the gemma (200-600 µm^2 approximate surface area) - Oil bodies observed
T = 48 h  Arrow: Rhizoid	**Stage 1 (From T= 12 h, thallus elongation, rhizoid elongation).** - Observed dorsal and ventral rhizoids elongating from the central zone of the gemma. The rhizoids elongate from the large central cells. - The thallus expands (Time to double the distance from AN -AN is 27-40 hours, measured on n = 19 samples)

T = 108 h Air pore zone 	**Stage 2 (From T = 4 days, air pore formation)** - The apices of the gemma contain a distinctive zone that forms air pores. - Air pores are not typically visible on the surface until T = 4-5 days onwards (some gemmae grow faster than others, this depends on the sample and growth conditions).
T = 144 h Branches Air pores 	**Stage 3: Dichotomous Branching (from T = 120 h)** - The air pore zone continues to grow - The apex forms two forks (branches) - arrow.

T = 0 h 	**Stage 0 (T = 0 h, removal of gemmae from splash cups)** - Dorsoventrally symmetrical top and bottom surfaces. - 5-6 cell layers. - Oil bodies on the edges of the gemma. - Parenchyma cells x5 larger in volume than epidermal cells.
T = 48 h 	**Stage 1 (From T= 12 h, thallus elongation, rhizoid elongation).** - Rhizoids elongate at a mean rate of 0.3 mm per day. - Nuclei in rhizoids polarise towards the tips - Cells within the vicinity of the apical notches experience strain rates of 100-125 % per day. - Cells within the vicinity of the apical notches divide faster than cells further away. - Central cells do not divide.

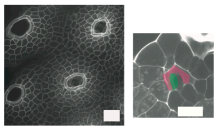
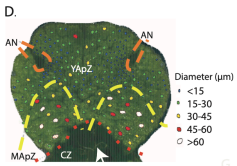
T = 108 h 	**Stage 2 (From T = 2 days - intercellular spaces) (From T = 4 days -- air pores)** - Intercelluar spaces observed increase from 1-15 between T = 2-6 days. - Air spaces develop in four phases: initial schizogenous aperture, aperture enlargement, development of photosynthetic filaments, development of air pores.
T = 144 h 	**Stage 3: Dichotomous Branching (from T = 120 h)** - The gemma contains multiple apical notches. - Younger air pores are produced in the vicinity of the apical notches and pushed towards the central zone during gemma morphogenesis. - The air pore zone is sub-divided into a young and mature air pore zone. - The central zone does not have air pores.

***Table 1**: Stages of development during gemma growth after germination (Nuri Purswani)*

Experimental observations:

A. What happens to gemmae development after addition of auxin?

There is strong evidence that auxin plays a role in regulating growth and differentiation during gemma development in Marchantia. Examine the preliminary evidence show below, where germinating Marchantia gemmae have been exposed to levels of exogenous auxin (napthalene acetic acid, NAA)

1. Try to classify the discrete visible events that take place during normal gemma growth and differentiation.
2. Can you identify defects in these processes that are apparent when exogenous auxin is present? Make a list of these for discussion.

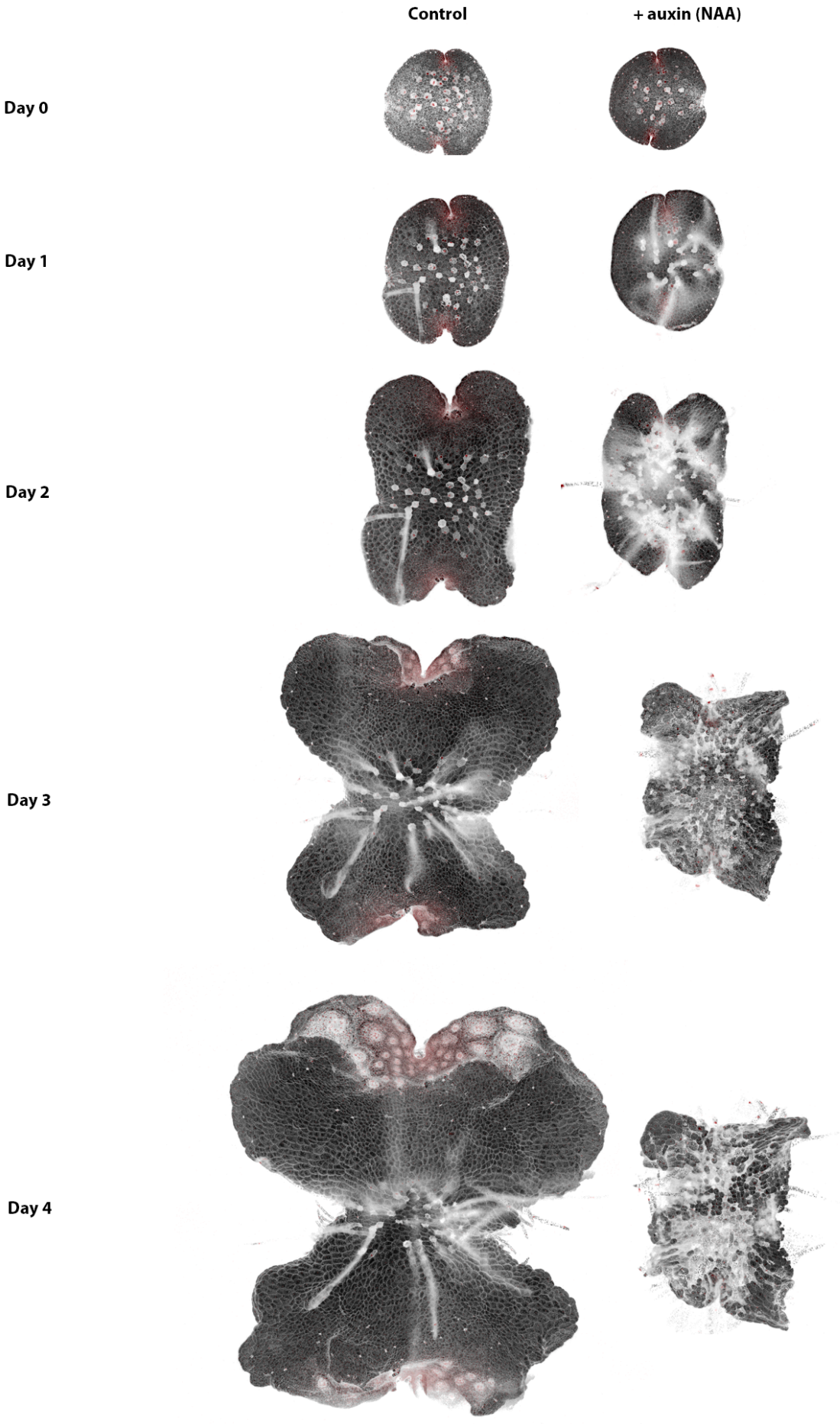


***Fig. 4**: Time course of gemmae growth, exposed to 100µg/L NAA auxin (images: Mihails Delmans)*

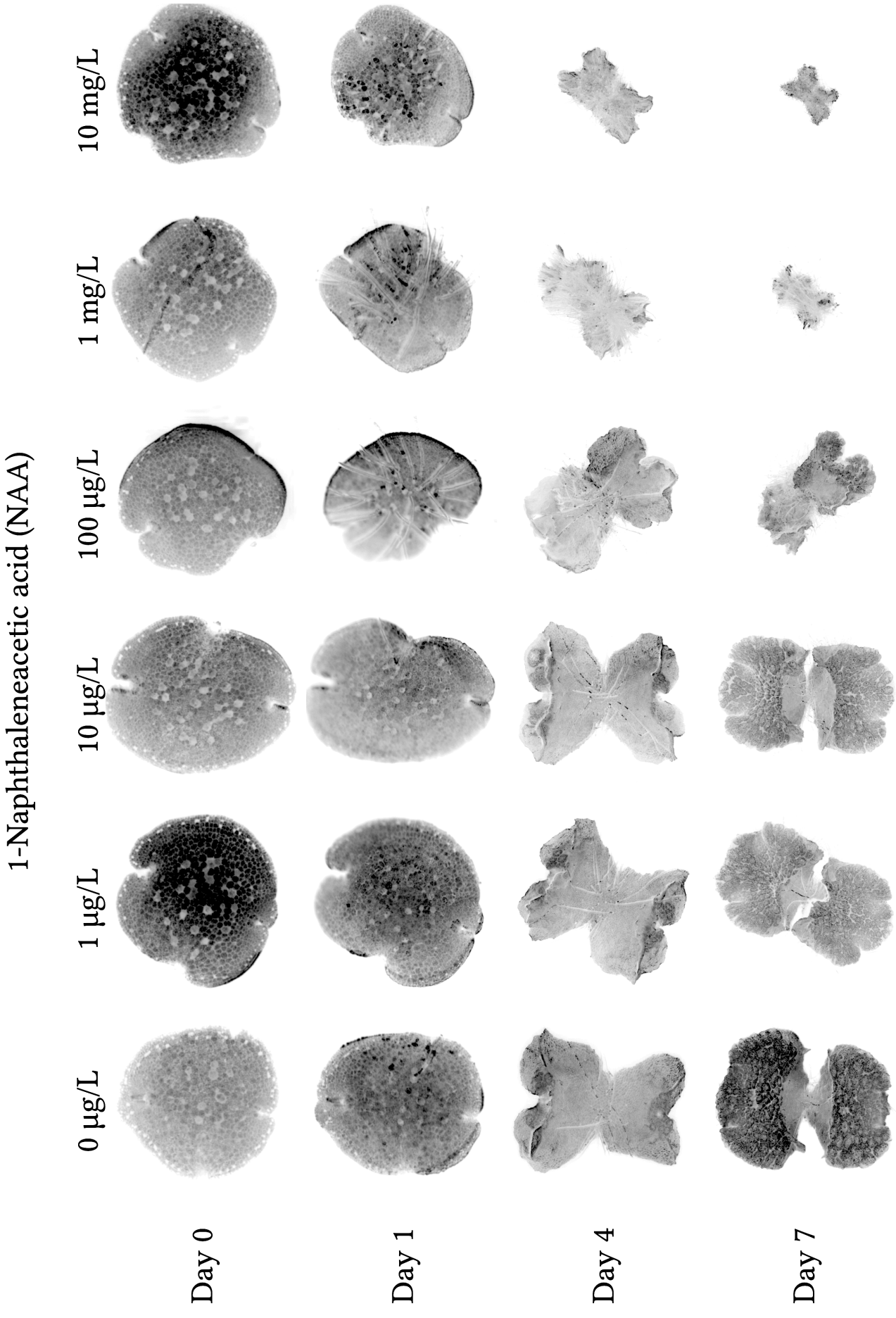


***Fig. 5**: Time course and dose response to NAA auxin for Marchantia gemmae grown on agar media. (images: Mihails Delmans)*

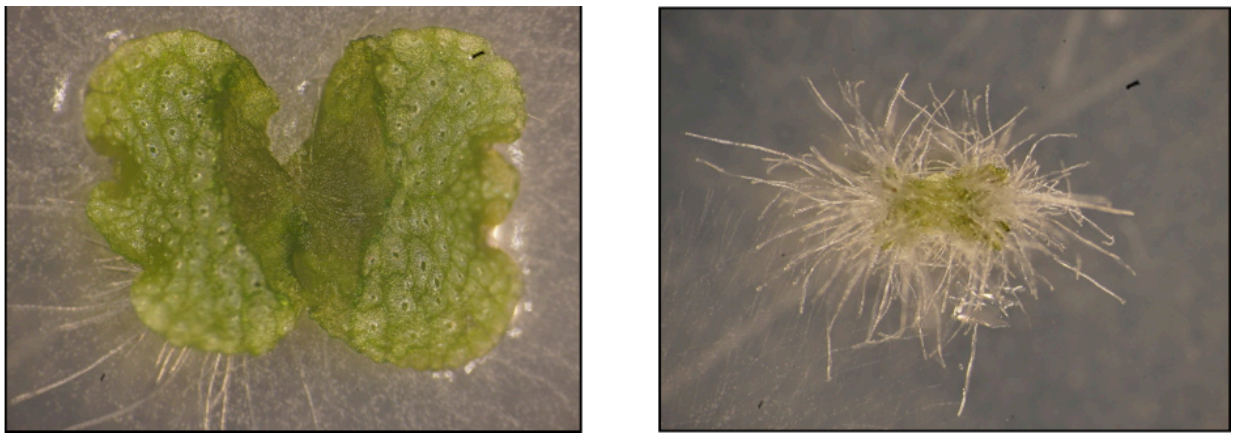


***Fig 6**. Gemmae 5 days after germination on nutrient agar (left) or grown on100µg/L NAA auxin (right). (images: Mihails Delmans)*

B. Where are the genes required for auxin response expressed?

In addition to the simple cellular architecture of Marchantia, the genetic pathways for auxin synthesis, transport and response are relatively simple. Unlike flowering plants, where analysis is complicated by the presence of multiple copies of gene variants, Marchantia has single genes at most steps in the pathway (Fig. 8).

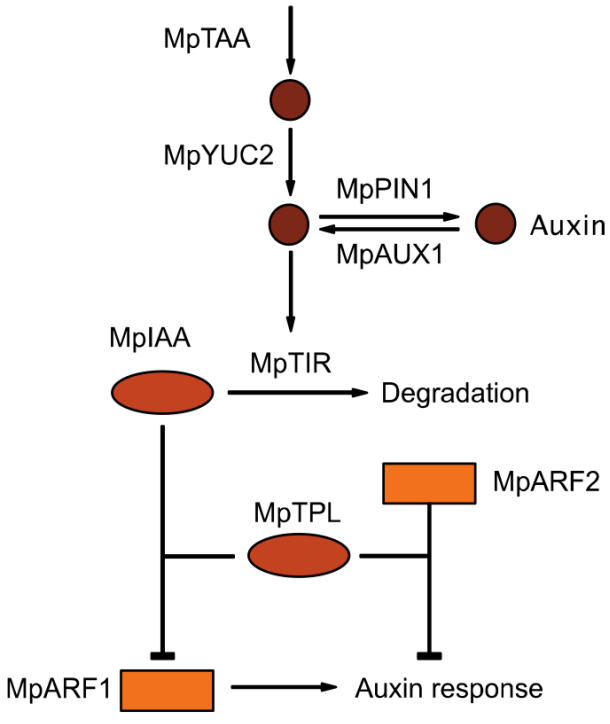


***Fig. 8**: Pathway for auxin response in Marchantia (image: Marius Rebmann)*

One recent and increasingly popular technique for mapping patterns of gene expression plants and animals - is to isolate single cells and to encapsulate these for the construction of individual cDNA libraries. One technique, commercialised by the company 10X (https://www.10xgenomics.com) is shown in Fig. 9. This has been applied to Marchantia gemmae at 4 days after germination. Gemmae were grown on agar media, incubated briefly with cell wall digesting enzymes and the resulting individual protoplasts were encapsulated in an emulsion with DNA barcodes and other reactants in a 10X microfluidics device. The mRNAs of individual cells were converted into separate sequence libraries, with a unique sequence barcode for each different cell. The sequence libraries were then pooled and subjected to next generation (NGS) Illumina sequencing methods. The resulting sequences were sorted into an expression matrix, and the collected sequences were tallied to create "signatures" for individual cells. The information was then analysed to reduce the dimensionality of the data, and to create a map of clustered cell types (Fig. 10).

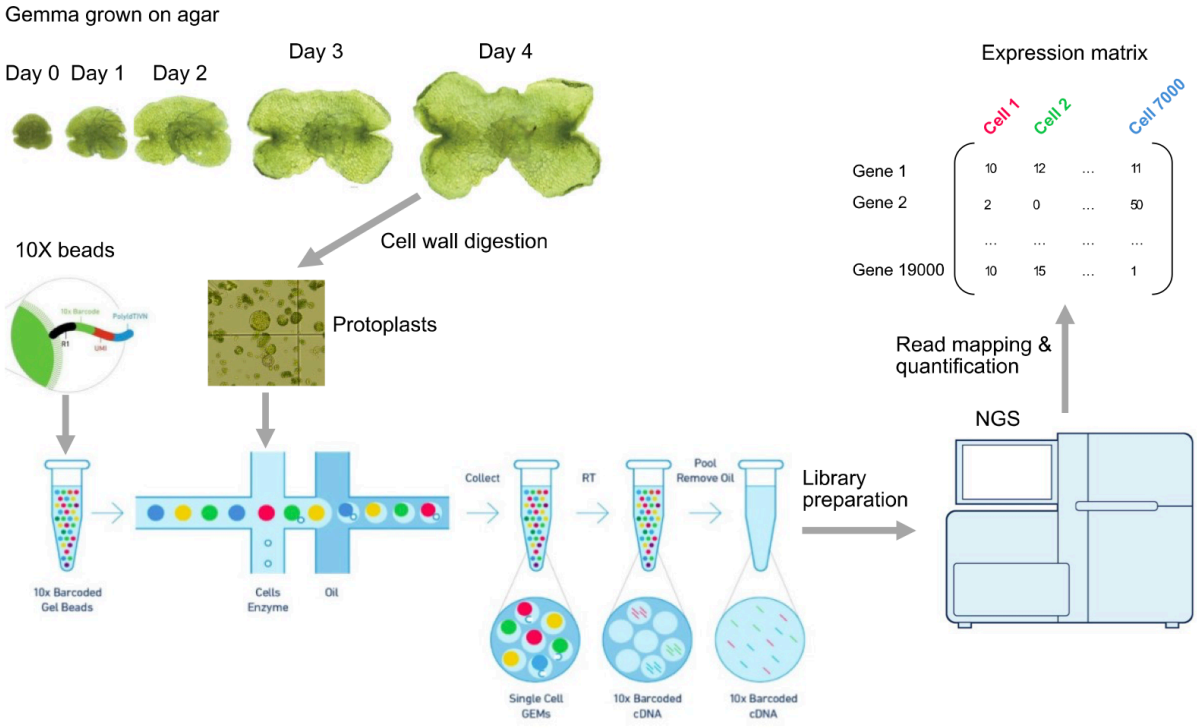


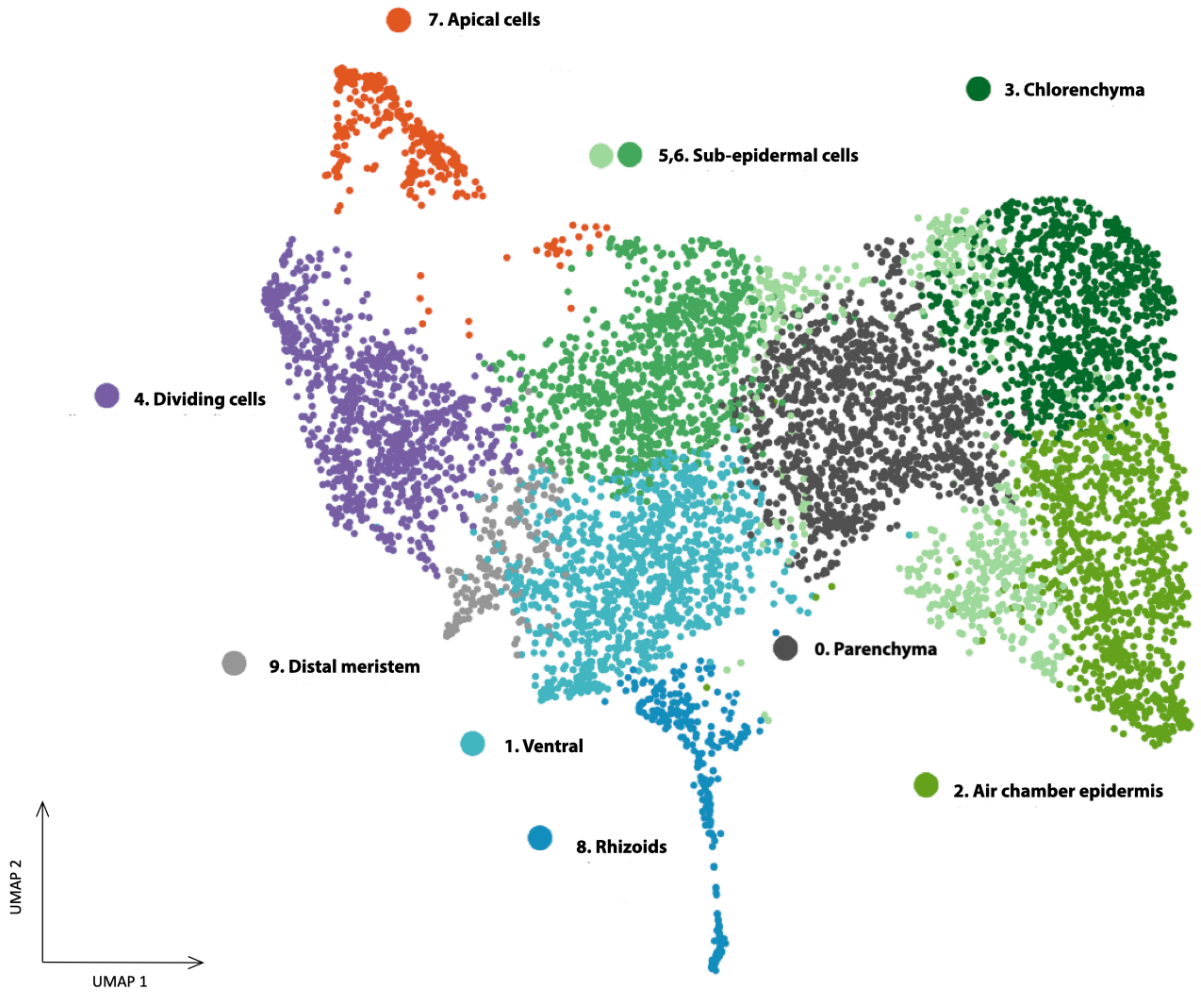
***Fig. 9**: Single-cell sequencing and mapping of gene transcripts in gemmae, 4-days after germination (image: Marius Rebmann)*

The clustered cells indicated in Fig. 10 were tentatively identified by correlation with known marker genes, and by construction of expression vectors after fusion of candidate promoters with a fluorescent protein reporter gene. This provides a simple cell atlas based on the patterns of gene expression across the 4 day old gemmae. The clusters of related cells

The expression of Marchantia genes involved with auxin synthesis, transport and response is shown in Fig. 11. The numerical values are normalised, and correspond to the average numbers of sequence tags found in clusters of related cells in the gemmae. The highest detected amounts were classified as 1.00, and the remainder normalised with respect to this value. The table provides a (noise-prone) insight into levels of gene expression in different cell types. Using this table do you see any trends or differences that might provide clues about the potential arrangement of auxin sources and sinks within the gemmae?

We can see from the evidence in section A that exogenous auxin perturbs normal development in specific ways. Can you combine these observations with the measured distribution of gene expression patterns to build a hypothetical model, or set of alternative possibilities for auxin mediated synthesis and response in gemmae?

Can this be useful for designing further experiments?



Patterns of gene expression in 4 day old gemmae

	7	4	9	5	6	3	2	0	1	8	
Name	Apical cells	Dividing cells	Distal meristem	Dorsal 1	Dorsal 2	Chlorenchyma	Air chamber	Parenchyma	Ventral	Rhizoids	Function
MpTAA	0.17	0.00	0.03	0.06	0.10	1.00	0.28	0.63	0.11	0.05	Auxin synthesis
MpYUC2	0.90	0.24	0.22	0.09	0.07	1.00	0.23	0.36	0.15	0.00	Auxin synthesis
MpAUX1	0.77	0.53	0.39	0.12	0.66	1.00	0.48	0.30	0.17	0.00	Auxin influx
MpPIN1	1.00	0.07	0.00	0.02	0.10	0.33	0.23	0.07	0.04	0.00	Auxin efflux & polar transport
MpTIR	1.00	0.41	0.53	0.21	0.34	0.19	0.00	0.36	0.51	0.51	Auxin receptor
MpIAA	0.69	0.04	0.00	0.21	0.48	0.66	1.00	0.24	0.42	0.12	Aux/IAA protein
MpARF1	0.40	0.21	0.00	0.19	1.00	0.53	0.61	0.67	0.30	0.95	Auxin response factor A
MpARF2	0.46	0.37	0.24	0.00	0.24	0.28	1.00	0.30	0.67	0.76	Auxin response factor B
MpTPL	1.00	0.84	0.00	0.00	0.06	0.46	0.55	0.37	0.48	0.50	Repressor

***Fig. 11**: Normalised levels of auxin-related gene transcripts in gemmae cells (data: Marius Rebmann)*

C. Experimental design

Think about your model for the possible roles of auxin during growth. Analyse the evidence. Where is it produced in the gemmae, which cells are likely to be responding to auxin? How and where might auxin be transported?

Marchantia gemmae are very amenable to experimental manipulation:

(i) The plant can be readily transformed via Agrobacterium.

(ii) This can be used to introduce new genes or to disrupt existing gene targets via CRISPR/Cas9 generated knock-outs.

(iii) Bear in mind that Marchantia is a haploid organism, so gene deficiencies may be lethal.

(iv) Gemmae are highly robust, and it is possible to disrupt cells and tissues surgically or via laser ablation, and analyse the consequences under high resolution microscopy.

(v) It is possible to extract sequences from the genome of Marchantia and to create novel fusion genes for visualisation or perturbation of function in living plants.

Can you think of a plan to combine your model for auxin function with an experimental regime to further test this?