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Title: Preparation of barley pollen mother cells for confocal and super resolution microscopy

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Summary

Recombination (crossing over) drives the release of genetic diversity in plant breeding programs. However, in barley, recombination is skewed towards the telomeric ends of its seven chromosomes, restricting the re-assortment of about 30% of the genes located in the centromeric regions of its large 5.1 Gb genome. A better understanding of meiosis and recombination could provide ways of modulating crossover distribution and frequency in barley as well as in other grasses, including wheat. While most research on recombination has been carried out in the model plant *Arabidopsis thaliana*, recent studies in barley have provided new insights into the control of crossing over in large genome species. A major achievement in these studies has been the use of cytological procedures to follow meiotic events. This protocol provides detailed practical steps required to perform immunostaining of barley meiocytes (developing pollen) for confocal or structured illumination microscopy.

Key words: barley, meiosis, immuno-cytochemistry, confocal, 3D-SIM, antibodies.

1 Introduction

Meiosis is a specialised cell division that underpins sexual reproduction by producing haploid male and female gametes. When the parent plants are heterozygous, each gamete will carry a unique set of alleles. During meiosis, the chromosomes need to pair and recombine in order to segregate properly **(1)**. Cytological and immunochemical methods have been used to study meiosis in model organisms for decades (**2-4**). In crops, *in situ* hybridization has been particularly helpful in both pre-breeding programs and academic research (**5-7**). Despite this, the use of immuno-cytochemistry in large genome crops, such as barley, has been limited and as such remains novel. The application of immuno-cytochemical methodologies has recently elucidated the timing of meiotic events and provided insight into the control of crossover formation (**8-10**). With the rapid progress in sequencing the barley genome (**11**), it is now possible to create custom tools such as antibodies and protocols to study meiosis and recombination in this large genome crop (**12-14**). Confocal microscopy is a standard method to analyse chromosome behaviour during cell division, and its application in barley is straightforward (**15**). The emergence of super resolution microscopy such as 3D Structured Illumination Microscopy (3D-SIM) has pushed our understanding of meiosis and has helped illustrate once more the differences between small and large genome species (**9, 16-18**). Here we describe a method to prepare barley pollen mother cells for investigating the progression of meiosis through analysis with confocal or 3D-SIM. We detail a protocol using custom barley antibodies to follow synapsis and recombination in male meiocytes and/or sections through developing anthers.

2 Material

All solutions should be sterile and prepared with ultrapure water and microscopy grade reagents. Prepare and store all reagents as indicated in the protocol. Wear the appropriate laboratory protective equipment and follow local waste disposal regulations. Local

suppliers or catalogue number can be used unless stated otherwise for specific reagent/equipment (e.g. slides).

2.1 Plant material

Barley *cv. Bowman* (*see* **Note 1**) plants were grown from seeds under controlled environment in growth cabinets (SANYO) under the following conditions: 16h of light at 18-20°C and 8h of dark at 14-16°C. The humidity was kept between 60-80%.

2.2 Lab equipment

- Orbital shaker
- 4°C incubator (or fridge)
- Dark wet chamber (*see* **Note 2**)
- Fine forceps and/or insulin needles
- Glass rod
- Glass Coplin staining jar
- Embryo Dish 30mm Diameter
- Cavity Slide Single Depression
- 1.5ml, 2ml and 50ml centrifuge tubes
- Cryostat CM1100 (Leica) (*see* **Note 3**)
- Glass Microscope slides (for staging)
- Microscope Polysine® slide or Superfrost® plus
- Glass coverslip, 22x50 mm, thickness No 1.5
- Glass coverslip, 18x18 (or 22x22)
- Dark Dropping bottle (for aceto-carmine)

- Aluminium slide tray, 20 slides capacity (Brunel Microscopes Ltd.)
- Razor blade
- Tissue paper
- Nail varnish
- PAP pen liquid blocker for immunostaining

2.3 Microscopes

- Stereomicroscope with 10x Wide Field eyepiece
- Light microscope with 10x, 40x Objectives (Optional: 60x objective)
- Confocal microscope LSM 710 (Carl Zeiss), or equivalent, equipped with but not limited to:
    - Objective (for Zeiss LSM 710): 60x APO C-apochromat 63x/1.20 W Korr M27 or equivalent
    - DAPI fluorescence (blue 405, UV lamp) (Hoechst33342)
    - Fitted 405-30 diode laser 405nm (for Hoechst33342/DAPI acquisition)
    - Fitted Argon laser 488nm (for Alexa Fluor® 488 acquisition)
    - Fitted DPSS 561-10 laser 561nm (for Alexa Fluor® 561 acquisition)
- Deltavision OMX (GE Healthcare group) or equivalent, equipped with 405, 488 and 561 laser lines.

2.4 Solutions

1. **Aceto-carmine:** In a fume hood, bring a solution of 45% acetic acid (45ml glacial acetic acid/55ml of distilled water) to boiling and add 0.5g of Carmine. Stir under heat for 15-20 minutes, let cool and store at 4°C overnight. Filter to remove any precipitates and store in a dark bottle (*see* **Note 4**).

2. **PBS (Phosphate Buffered Saline):** For 10X stock solution; Dissolve 80g of NaCl, 2.0g of KCl, 14.4g of Na_2HPO_4 and 2.4g of KH_2PO_4 in 800ml dH_2O; First, adjust pH to 7.4 and then adjust the volume to 1L with additional dH_2O. Sterilize by autoclaving. Dilute with dH_2O to make 2X PBS or 1X PBS working solution. Add 5ml of Triton™ X100 and 5ml of Tween® 20 to make 1L working solution of 1X PBS, 0.5% Triton™ X100 , 0.5% Tween® 20.
3. **SSC (Saline Sodium Citrate):** Dissolve 175.3g of NaCl, 88.2g of Trisodium citrate dehydrate ($Na_3C_6H_5O_7$) in 800ml of distilled H_2O. Adjust the pH to 7.0 with 1M HCl then adjust the volume to 1L with additional distilled H_2O. Sterilize by autoclaving. Dilute in distilled water to make 1L of 4X SSC solution and add 5ml of Tween® 20.
4. **Fixative:** In the fume hood, dissolve 1g of paraformaldehyde in 12.5ml of water, add 2-3 drops of 5M NaOH and bring to 60°C (do not boil), stirring. Once the solution is clear (it takes about 5-10 minutes), remove from the heat and let cool for 20 minutes (or 5 minutes on ice). Then add 12.5ml of 2X PBS. The solution is ready to use and can be stored in 50ml tube in the fridge for 1 week and up to 2 weeks, (*see* **Note 5**).
5. **Blocking solution:** 1X PBS, 0.5% Triton™ X100, 0.5% Tween® 20, containing 5% Goat Serum Albumin and 5% Donkey Serum Albumin. Make it fresh.
6. **Antifading agent:** Vectashield antifade mounting medium containing DAPI (Vector Laboratories, catalogue number: H-1200) (*see* **Note 6**).
7. **Primary antibodies:** Custom antibodies or commercial antibodies with good reactivity for plant tissue raised in different hosts (rabbit, rat, etc.). Dilute the primary antibody in blocking solution. The dilution needs to be worked out empirically for each antibody and generally ranges from 1:200 to 1:1000.

8. **Secondary antibodies:** We use a combination of IgG (H+L) Secondary Antibody, Alexa Fluor® conjugate (Thermo Fisher Scientific) listed in Table 1 (*see* **Note 7**).
9. **Tissue Freezing Medium**: Embedding medium for cryosection. Although we use Jung tissue freezing medium, it is possible to use other alternatives from Leica, Tissue Tek or Electron Microscopy Sciences (eg. TFM^{TM}, catalogue number 72592).
10. **Counterstaining solution:** (optional) Hoechst 33342 (10mg/ml); Dilute in dH_2O to obtain a working solution at 1µg/ml. Store in the fridge and protect from light.

3 Methods

3.1 Meiocyte preparation

1. Harvest the barley stems and keep them in water until use. For the Bowman cultivar, meiosis normally starts 6-7 weeks after sowing. You can estimatethe size of the spike by gently press the stem with two fingers up and down along the stem and feel the spike (Figure 1a). Meiosis progresses from the base of the barley spike (Figure 1b).
2. Open the stem to release the spike (the barley inflorescence) (Figure 1a, b, e, j). Barley produces three anthers in each developing spikelet (Figure 1f, h, k, m). Spikelets (individual barley flowers) are arranged either side of the central axis or rachis. Use a mounted insulin needle (or very fine forceps) to dissect out one of the three developing anthers from the centre of the developing spike (Figure 1e, j, arrow) and check under the microscope to determine the overall meiotic stage. To do this, place the anther on a microscope slide, add one drop of aceto-carmine (Figure 1c), place a coverslip (18x18mm) and tap very quickly but very gently to squeeze the meiocytes out of the anther. Look under 10X objective to locate the

anther and then to 40x (or 60x Objectives) to locate the meiocytes (Figure 1d, g, i, l, n). Use a filter paper to remove the excess staining solution if the cells are floating too much on the slide.

3. Keep the other two anthers in an embryo dish (or a cavity slide) in 1X PBS containing 0.5% Triton™ X100 and 0.5% Tween® 20 at room temperature (RT). Repeat for the other florets and pool the anthers at the same stage until there is enough material to fix (about 10-20 anthers).
4. In a fume hood, remove any liquid then add the fixative (*see* **Note 8**). Fix the samples for 15 to 20 minutes.
5. Wash the sample twice for 5 minutes with 1X PBS, 0.5% Triton X100, 0.5% Tween® 20. After the last wash, the sample is safe outside of the fume hood. Use it immediately.
6. Prepare a polysine (or superfrost) slide by making a well in the centre with the PAP pen and let it dry fully.
7. Remove most of the liquid (leave at least 5-10µl) and tap out the fixed anthers repeatedly with a glass rod to release the meiocytes (it takes about 5 minutes per sample). Add 30µl of 1X PBS, 0.5% Triton X100, 0.5% Tween-20, mix gently and transfer 25µl of the solution onto the prepared slide. Let it evaporate at RT slowly so the meiocytes fall at the bottom of the slides and stick to the polysine. Use the slide immediately.

3.2 Immunostaining protocol

1. Freshly dilute the primary antibodies in blocking solution. For most of our antibodies, we use a 1:500 dilution, but this depends on each antibody (commercial or custom) and the tissue. We recommend starting with 1:200 for a new antibody

and work out the best concentration by serial dilution. We used ASY1 at 1:2000 and ZYP1 at 1:500 to produce the images in Figure 2.

2. (Optional) Add 50-100µl of blocking solution onto the sample that is bound to the slide and leave to incubate for 30 minutes at RT. Remove the solution by tipping the slide over.
3. Add 30µl of the primary antibodies solution onto the slide and add a soft plastic coverslip (parafilm, or cut autoclave bags are suitable).
4. Leave for 30 minutes (up to 1h) at RT in the dark wet chamber. This step helps the solution to penetrate into the sample.
5. Move the dark wet chamber into a cold room or fridge (4-6°C) for 24 to 48h. The time of incubation also varies from one antibody to another and also on the type of tissue (e.g. meiocyte spread or section). We found that for meiocytes, 36h is the optimum time for most of our antibodies.
6. Remove the wet chamber from the fridge and leave to warm up at RT for at least 30 minutes (up to 1 hour).
7. Remove the plastic coverslip gently and wash the slides in 4X SSC, 0.5% Tween® 20 for 10 minutes and then in 1X PBS for 5 minutes, shaking gently. (*see* **Note 9**)
8. Apply 40µl of secondary antibody solution consisting of a 1:600 dilution of secondary antibodies (anti-rabbit, anti-rat) in blocking solution. Apply a soft plastic coverslip and leave to incubate at RT in the dark wet chamber for 90 minutes to 2 hours. (*see* **Note 10**)
9. Wash the slides as previously in step 7 (*see* **Note 11**).
10. Remove as much PBS without drying the slide and add one drop of Vectashield containing DAPI.
11. Add a full-length glass cover slip (Glass coverslip, 22x50 mm), press gently upside down on paper towel, and seal ends with nail varnish.

12. Leave the slide on an aluminium tray at RT to dry, and store in slide box, in the dark at 4°C for longer storage (*see* **Note 12**).
13. Proceed to either confocal or structured illumination microscopy (Figure 2).

3.3 Section Preparation

1. Collect stems and check one anther at the base, the middle and top of each spike to get an idea of the range of meiotic stages present in the spikes. Follow steps 1 and 2 from paragraph 2.1.
2. Leave the spike 15 minutes in 1X PBS, 0.5% Triton™ X100, 0.5% Tween®20, in a 2ml tube on ice.
3. In the fume hood, substitute the 1X PBS, 0.5% Triton™ X100, 0.5% Tween® solution with fresh fixative. Fix the sample for 35 minutes (*see* **Note 13**)
4. Wash the sample twice for 10-15 minutes each with 1X PBS. The best quality results will be achieved when preparing the slide immediately, even though, fixed spikes can be kept for 1-2 days in the fridge until use.
5. Start the Cryostat until the temperature reaches -20°C.
6. Prepare the Specimen disc by spreading a thin layer of Tissue freezing medium on the surface (Figure 3a), let if freeze and cut few slices to make the surface horizontal (Figure 3b,c).
7. If the spike is very small (less than 1cm), cut the whole spike longitudinally. For longer spikes, use transverse sections of single florets.
8. Mount the tissue on the flat freezing medium surface (Figure 3d), cover it with more medium, and leave it to freeze fully (Figure 3e). Cut slices until you reach the sample (Figure 3f).
9. Cut slices (20µm thick) and stick them on the slide by approaching the RT slides near the sample (*see* **note 3** and Figure 4).

10. Leave at RT until almost dry and delimit the borders of the samples with a PAP pen.
11. Proceed to immunostaining as per paragraph 3.2.

4 Notes

1. Growing conditions such as temperature or humidity might affect growing time and therefore entry into meiosis. Although the cultivar Bowman was used in this study, other cultivars such as Golden Promise or Morex can be used, but spike sizes and developmental time may vary.
2. Dark wet chambers are commercially available or can be easily made (12).
3. The cryostat CM100 manual is available at this link: https://drp8p5tqcb2p5.cloudfront.net/fileadmin/downloads_lbs/Leica%20CM1100/User%20Manuals/Leica_CM1100_IFU_1v3B_en.pdf. Although this is a different model form the one we use, the approach to transfer sample onto the slide is similar that is described in this video at 2:45min: https://www.youtube.com/watch?v=z-WoPn2YLRQ&t=178s
4. Chromosomes are darker stained in iron aceto-carmine solution and this could be helpful for early meiotic stages. Simply add a "mordant" by soaking a piece of iron (for example an iron nail) in your solution until the solution gets darker. Alternatively, use the following protocol. Make two solutions of 100ml aceto-carmine but in one of the solution, add 5g of Ferric oxide. Add the Ferric oxide solution drop by drop to 50ml of the first Carmine solution until a precipitate starts to appear. Immediately add the remaining 50ml of first Carmine solution to the titrate mixture and filter to remove any precipitate.

5. Paraformaldehyde waste needs to be disposed of in appropriate bottles. All other material can be decontaminated overnight in a solution of sodium metabisulfite before rinsing with plenty of water.
6. Other anti-fading agents can be used but we recommend Vectashield for 3D-SIM because of its correct refraction index. If using an anti-fading agent without DAPI, it is possible to add an extra step by adding 50µl of DAPI or HOECHST33342 (1µg/ml) for 10 minutes in the dark.
7. We found that the Alexa dyes are quite robust but can be replaced by other alternatives (Dylight for example).
8. We use 40µl fixative for cavity slides, 200µl for embryo dish and 1.9ml for the whole spike (in a 2ml tube).
9. Washing steps can be conducted using a combination of buffers with different stringencies. We found that for 'easy' samples, 15 minutes in 1X PBS is usually sufficient. Slides are safe in 1X PBS for up to 2 hours at RT.
10. Longer incubation in secondary antibody or higher concentration can increase background.
11. This method is compatible with time course studies using EDU (5-ethynyl-2'-deoxyuridine). In this case, EDU is injected into the transpiration stream of the sample below the inflorescence and meiocytes are collected at different times (**9, 13**). Samples are then fixed and immunostained as described in this protocol. After the secondary antibody wash, EDU detection can be performed as per supplier's protocol and then slides can be washed and mounted.
12. Slides can be kept in the fridge for a few months (up to 1 year) without any degradation of the chromatin or immuno-fluorescence signal, even after a few confocal sessions. In general, after 2 or 3 SIM sessions, slides can only be kept up to 3-4 months.

13. Vacuum Infiltration can improve the fixation of large tissue such as spike. In this case, 5-10 minutes prior to fixation is enough (**19**).

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6 References

1. Zickler, D. and Kleckner, N. (1999) Meiotic chromosomes: Integrating structure and function. *Ann. Rev. Gen.* **33,** 603-754.
2. Baudat, F., Imai, Y., and de Massy, B. (2013) Meiotic recombination in mammals: localization and regulation. *Nat. Rev. Gen.* **14(11),** 794-806.
3. Mercier, R. and Grelon, M. (2008) Meiosis in plants: ten years of gene discovery. *Cytogenet Genome Res* **120(3-4),** 281-90.
4. Mercier, R., Mézard, C., Eric Jenczewski, E. et al. (2014) The Molecular Biology of Meiosis in Plants. *Annu. Rev. Plant Biol*. **66**,297-327
5. Bass, H.W. and Birchler, J. (2011) Plant cytogenetics : Genome Structure and Chromosome Function. Springer, New York.

6. Colas, I., Shaw, P., Prieto, P. et al. (2008) Effective chromosome pairing requires chromatin remodeling at the onset of meiosis. PNAS **105(16)**, 6075-6080.

7. Schwarzacher, T, Anamthawat-Jónsson, K., Harrison, G. E. et al. (1992) Genomic in situ hybridization to identify alien chromosomes and chromosome segments in wheat. Theor Appl Genet **84(7-8)**, 778-86.

8. Barakate, A., Higgins, J.D., Vivera, S., et al. (2014) The synaptonemal complex protein ZYP1 is required for imposition of meiotic crossovers in barley. *Plant Cell* **26(2)**, 729-40.

9. Colas, I., Macaulay, M., Higgins, J.D.et al. (2016) A spontaneous mutation in MutL-Homolog 3 (HvMLH3) affects synapsis and crossover resolution in the barley desynaptic mutant *des10*. *New Phyt* **212(3)**, 693-707.

10. Phillips, D., Jenkins, G., Macaulay, M. et al. (2015) The effect of temperature on the male and female recombination landscape of barley. *New Phytol* **208(2)**, 421-429.

11. IBGSC, et al. (2012) A physical, genetic and functional sequence assembly of the barley genome. *Nature* **491(7426)**, 711-716.

12. Colas, I., Baker, K., and Flavell, A.J. (2016) Cytology and Microscopy Immunolocalization of Covalently Modified Histone Marks on Barley Mitotic Chromosomes. *Bio-protocol*, **6(12)** e1841.

13. Higgins, J.D. (2013) Analyzing meiosis in barley. In: Pawlowski, W., Grelon, M., Armstrong, S. (eds) Plant Meiosis: methods and Protocols, Methods in Molecular Biology, **990**, Springer, New York, pp.135-44.

14. Phillips, D., Wnetrzak, J., Nibau, C. et al. (2013) Quantitative high resolution mapping of HvMLH3 foci in barley pachytene nuclei reveals a strong distal bias and weak interference. *J. Exp. Bot.* **64(8)**, 2139-2154.

15. Ramsay, L., Colas, I., and Waugh, R. (2014) Modulation of Meiotic Recombination. In Kumlehn, J. and Stein, N. (Eds) Biotechnological Approaches to Barley Improvement , Biotechnology in Agriculture and Forestry **69**, Springer, Verlag Berlin Heidelberg, pp. 311-329.

16. Golubovskaya, I.N., Wang, C.J., Timofejeva, L. et al. (2011) Maize meiotic mutants with improper or non-homologous synapsis due to problems in pairing or synaptonemal complex formation. *J. Exp. Bot.* **62(5)**, 1533-1544.

17. Pawlowski, W.P., Wang, C.J., Golubovskaya,I.N. et al., (2009) Maize AMEIOTIC1 is essential for multiple early meiotic processes and likely required for the initiation of meiosis. *PNAS* **106(9)**, 3603-3608.

18. Phillips, D., Nibau, C., Wnetrzak, J. et al. (2012) High Resolution Analysis of Meiotic Chromosome Structure and Behaviour in Barley (Hordeum vulgare L.). *Plos One*, **7(6)**: e39539

19. Kuo, J. (2007) Processing plant tissues for ultrastructural study. *Methods Mol Biol* **369**, 35-45.

Table 1: Secondary antibodies used in this study

Reactivity	Alexa Fluor® dye	Catalogue number
Anti-Rabbit	568	A10042
	488	A21206
Anti-Rat	568	A11077
	488	A21208

Figure 1: Sample collection and meiosis staging: Spikes are located inside the barley stem (white box) (a). Meiosis progresses from the base of the barley spike towards the top (b). One anther is checked under aceto-carmine (c) and gently tapped to release the contents of the loculi (d). In 2cm spikes (e) it is possible to have meiocytes in early prophase at the base of the spike (f,g) and later prophase in the middle of the spike (h,i) – white arrow indicate anthers (f, k, h,m). In a 3cm spike (j), it is still possible to identify material in prophase in the 4th flower from the base (k,l), but most of the anthers will be at tetrad or pollen stages (m,n).

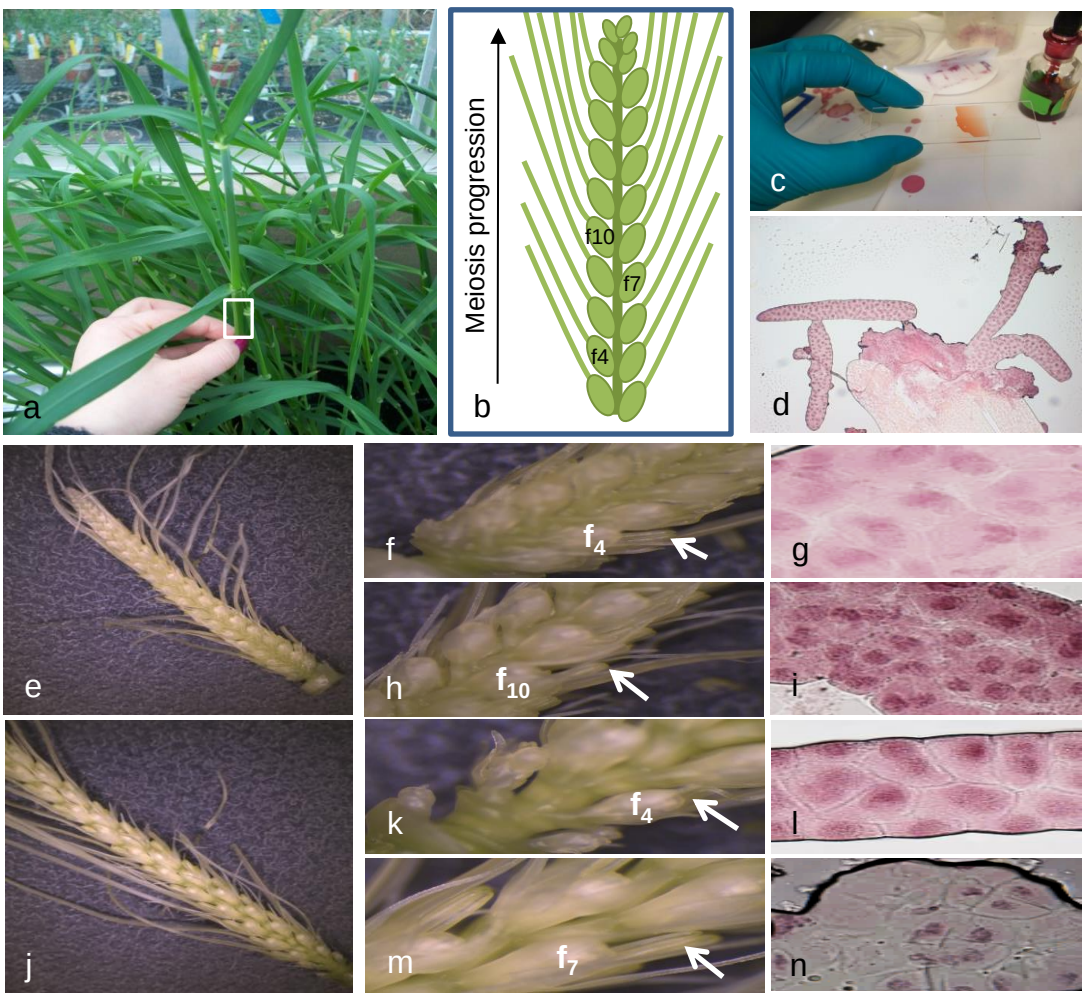

Figure 2: Following synapsis with confocal and SIM

The synaptonemal complex (a) is a tri-partite structure formed between the homologous chromosomes during synapsis. We can follow chromosome axis formation (a) and synapsis using antibodies against the protein ASY1 (green) and ZYP1 (magenta) respectively. The same slide preparation can be used for confocal (b-d) and 3D-SIM (e-j). At leptotene (b, e, h), chromatin is organized around the ASY1 axes (magenta). During zygotene (c, f, i), chromosomes synapse as the synaptonemal complex is formed between the homologues. At pachytene, (g, j), chromosomes (magenta) are aligned entirely and linked closely by ZYP1 (green). With 3D-SIM (e-j), is it possible to see the synaptonemal complex more clearly with ASY1 threads separated by polymerized ZYP1 (j). Scale bar 5µm.

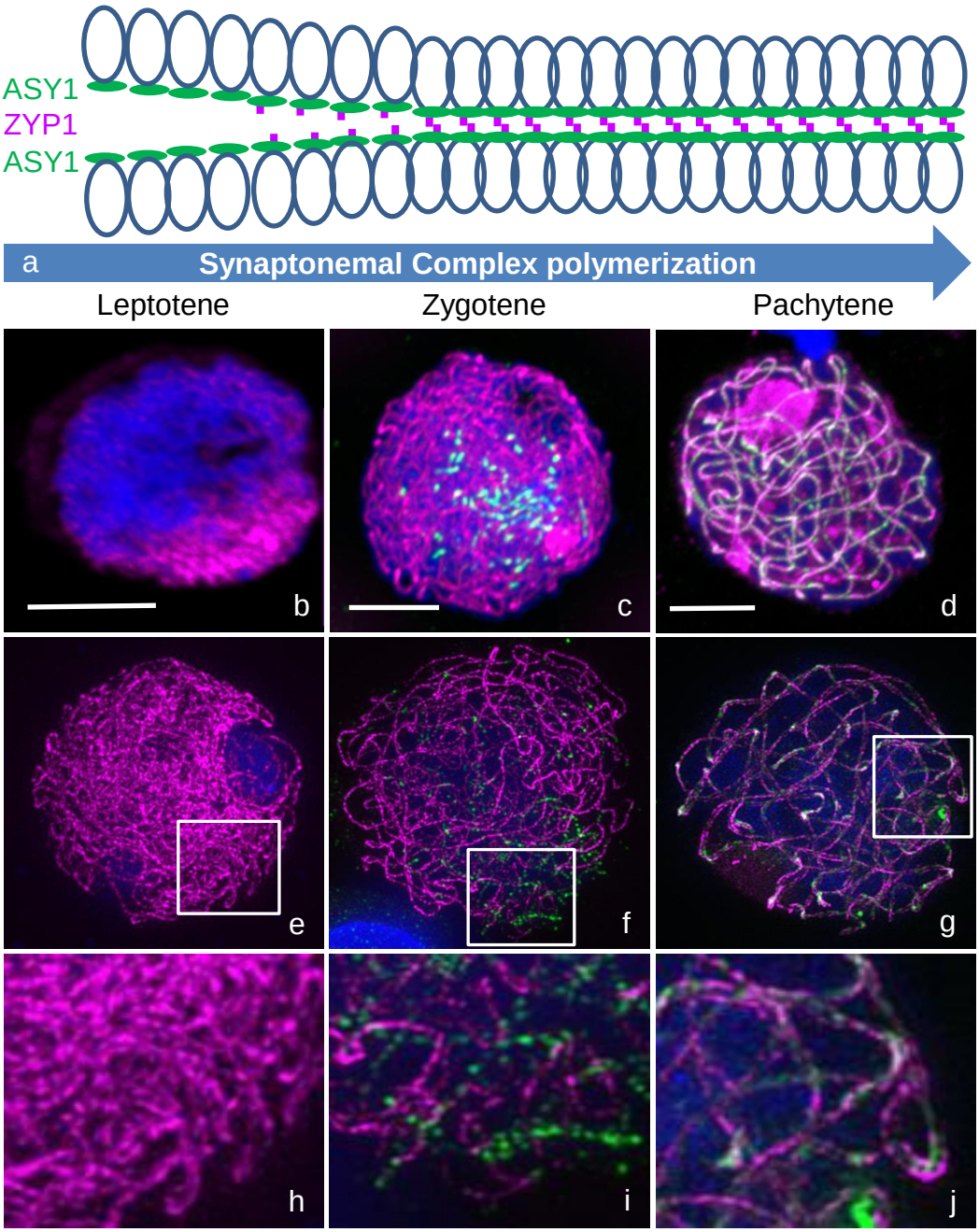

Figure 3: Mounting the sample for cryo sectioning
The grey is the Cryostat holder, blue shapes represent the freezing medium, dark green oval is for the sample and the double red arrows are the plane of sectioning. First, prepare the base (grey) with freezing medium (a) and slice it flat before placing the sample (b). Place the sample on the frozen surface (c), and add some freezing medium. Let it freeze and cut excess of medium around the sample in a trapezoid shape (d). Discard the first slices (e) until you reach your sample (f).

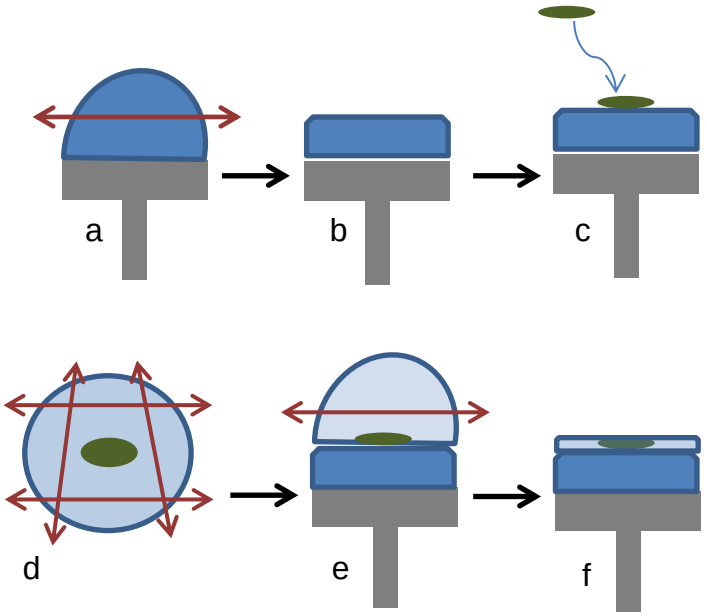

Figure 4: Section transfer on slide
Side view (a) of the cutting block (grey), that the 20µm slice (blue) is sticking to. Approach the slide (dark rectangle) carefully towards the sample slice and once the sample touches the slide (and defrosts), remove it quickly upside down to flatten the sample and let it dry. Front view (b) of the cutting block (grey), sample slice (blue) and slide (dark rectangle).

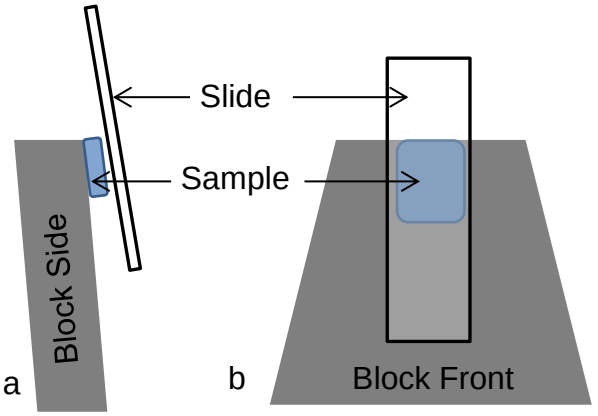

Figure 5: Immunostaining on section
Cross sections (a-c) of barley anthers sectioned with the cryostat (a) and DAPI stained (blue) (b). Close up of a cross-section with tapetal cells labelled with EDU (green – see note 11) around the meiocytes (arrows). Longitudinal section (d-f) of barley anthers showing cells labelled with EDU (green) and meiocytes labelled with ASY1 (magenta). Samples were collected 43.5 hours after EDU injection. Bars: 50µm (a,b e); 10µm (c); 200µm (d); 20µm (f)