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Isolation of high purity tissues from developing barley seeds

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TITLE:

Isolation of high purity tissues from developing barley seeds

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Seed development, *Hordeum vulgare*, embryo, endosperm, seed maternal tissues, flow cytometry, RNA isolation

SUMMARY:

Here we present a protocol for high purity manual isolation and quality control of embryo, endosperm and seed maternal tissues during entire barley seed development.

ABSTRACT:

Understanding the mechanisms regulating the development of cereal seeds is essential for plant breeding and increasing yield. However, the analysis of cereal seeds is challenging owing to the minute size, the liquid character of some tissues, and the tight inter-tissue connections. Here, we demonstrate a detailed protocol for dissection of the embryo, endosperm, and seed maternal tissues at early, middle, and late stages of barley seed development. The protocol is based on a manual tissue dissection using fine-pointed tools and a binocular microscope, followed by ploidy analysis-based purity control. Seed maternal tissues and embryoes are diploid, while the endosperm is triploid tissue. This allows the monitoring of sample purity using flow cytometry. Additional measurements revealed the high quality of RNA isolated from such samples and their usability for high-sensitivity analysis. In conclusion, this protocol describes how to practically dissect pure tissues from developing grains of cultivated barley and potentially also other cereals.

INTRODUCTION:

Seeds are complex structures composed of several tissues of maternal and filial origin[1]. Cereal grains represent a special type of seed, with the largest part being formed by endosperm, a specialized triploid tissue that protects and nourishes the embryo. Cereals provide around 60% of global food resources and are the most valuable output from plant production[2]. The

knowledge of molecular processes controlling cereal seed development is important due to their economic prominence and central role in plant reproduction[1,3].

Cultivated barley (*Hordeum vulgare* subsp. *vulgare*; 2n = 2x = 14; 1C = 5.1 Gbp) is the fourth most important cereal crop worldwide. It is used for animal feed, food, and biotechnology[4]. Besides that, it is also a classical temperate zone cereal crop model species of growing importance[5]. Barley genomic resources include genetic maps, collections of cultivars, landraces and mutants, high-quality genome assemblies and annotations as well as transcriptomic data of the major developmental stages[5–7]. Also, barley genes are used for genetic improvements of other cereals. Resistance to abiotic stresses such as drought and salinity, specific pathogens, and high content of beneficial compounds (e.g., β-glucan) make barley a valuable source of traits for wheat breeding[8].

Seed development is initiated by fertilization on the day of pollination (DOP). DOP is defined by evaluation of the morphology of stigma and anthers according to the Waddington scale (W10.0)[9]. The spikes containing non-pollinated flowers were characterized by compact (unbranched) stigma and green anthers, whereas pollinated spikes contained extended spiklets, extended and widely branched stigma, swollen ovule, opened anthers and free pollen. The flowers at DOP represented an intermediate phenotype. The anthers had a yellow color, disrupted easily and then released pollen. Stigma had widely spread sigmatic branches of the pistil (**Figure 1C**).

Barley seed development includes three partially overlapping stages[1,10]. The stage I (0 – 6 days after pollination; DAP) is launched by double fertilization, typified by cell proliferation and the absence of starch synthesis; stage II (7 – 20 DAP) comprises differentiation and great biomass gain accompanied by the production of starch and protein storage molecules; stage III (after 21 DAP) corresponds to seed maturation, weight reduction by desiccation and the onset of dormancy. Alternatively, the phases are called early, middle and late, respectively[11].

Barley grain is covered by hulls, which consist of the lemma, palea, and glumes[12]. In most barley genotypes, the hulls tightly wrap dry seeds. The seed itself is formed by the embryo, endosperm and seed maternal tissues (**Figure 1A**). The diploid embryo originates from the fertilization of the egg cell by one sperm cell nucleus. In the fully developed seed, the embryo consists of the embryonic axis with the coleorhiza surrounding the radicle, the coleoptile enclosing the shoot meristem and primary leaves, and the scutellum (cotyledon)[1,10,13,14]. The triploid endosperm is the result of fertilization of the diploid central cell by the second sperm cell nucleus. The proliferation of endosperm begins with the syncytial (coenocyte) stage, where the dividing nuclei are pushed to the periphery by the central vacuole. At the end of the syncytial phase, microtubules form a radial network around the nuclei and indicate the anticlinal cell wall formation and the onset of endosperm cellularization. Endosperm differentiation occurs simultaneously with the cellularization and results in five major tissues: the starchy endosperm, the transfer cells, the aleurone and subaleurone layers, and the embryo surrounding region. Seed maternal tissues are a multi-layered diploid structure of maternal origin containing pericarp and seed coats[10,12]. Seed maternal tissues include a nucellar projection on the dorsal side of the grain that has a transport-related function, and becomes embedded in endosperm at later stages of

seed development[15].

[Place **Figure 1** here]

Recent progress in high-throughput genomics provides the tools for the study of individual seed tissue development. However, the major obstacle of this purpose is the compact structure and tight adhesion of the seed tissues[1]. We developed a protocol for high purity dissection of seed tissues from developing barley seeds with possibility to subsequent use for highly sensitive analyses, such as RNA-sequencing. In addition, the presented protocol can be easily adapted to other cereals.

PROTOCOL:

1. Growing plants

NOTE: Considering that a single barley plant usually has 5 to 6 tillers and only the middle 5 to 6 spikelets of each spike should be used for dissection, then a maximum yield per plant is 72 seeds for two-row and 216 seeds for six-row cultivars.

1.1. To germinate barley seeds, prepare a Petri dish padded with three layers of cellulose tissue paper covered with one layer of filter paper. Moisturize it with distilled water, so there is no excess water, put the seeds on the surface and close the Petri dish. Filtration paper avoids growing the roots through the cellulose tissue. Germinate the seeds for 3 days at 25 °C in the dark.

NOTE: Alternatively, germinate seeds by putting them directly in a wet soil mixture (see step 1.2).

1.2. Transfer germinated seeds with a visible radicle and shoot of about 5 cm into 5 cm x 5 cm peat pots with a mixture of soil and sand (3:1, v/v). Water regularly. After 10 days, transfer the plants into 12 cm x 12 cm pots filled with the same soil mixture.

1.3. Grow the plants in a climatic chamber under the controlled long-day regime (16 h day 20 °C, 8 h night 16 °C; light intensity 200 µmol m^{-2} s^{-1}; humidity 60%).

NOTE: Spring barley requires approximately 8-10 weeks from sowing to the beginning of anthesis, with no requirement for vernalization. Winter barley needs 7-8 weeks of vernalization (short-day conditions, 8 h day 4 °C, 16 h night 4 °C; light intensity 200 µmol m^{-2} s^{-1}; humidity 85%) to induce flowering.

2. Determination of pollination

NOTE: Precise determination of pollination is needed for proper estimation of developmental progression. Barley is a self-pollinating species. To define day of pollination (DOP), we monitored the day of self-pollination. This trait is cultivar specific, but starting protrusion of the awns from

the leaf sheath is a good indicator of approaching DOP (**Figure 1B**).

2.1. Open the leaf-sheath covering the spike. Use fine-pointed tweezers to check anthers and the ovary inside the spikelets in the central part of the spike. Spikelets with yellow anthers and "fluffy" stigma will pollinate within few hours[16] and are considered as DOP (**Figure 1B**).

2.2. Clip off the spike near the tip of the last spikelet, and remove the flag leaf and the upper part of the awns. Then, clip off the top 1/3 of hulls in each spikelet. This dries the anthers and leads to their more synchronized opening and release of pollen.

2.3. Cover the spike with a glassine bag with the spike ID, plant number and defined DOP date. This also prevents cross-pollination, which may compromise specific experiments.

2.4. Note the information to a tabular editor. Use the following formula to calculate the day after pollination (DAP) when tissue isolation should take place.

$$xDAP = DOP + x$$

x = expected DAP

NOTE: The values should have 'Data' format.

2.5. For seed tissues dissection, collect the spikes at DAP according to the prepared tabular calendar.

3. Dissection of the seed tissues

NOTE: The following steps should be performed using a stereomicroscope. Remove the hulls before dissection using tweezers. Note that hulls become drier and more adherent from around 16 DAP. To keep physiological conditions and avoid drying of the plant materials during dissection, moisten the samples by putting them into a drop of 1x PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH = 7.4). Use a new seed for dissection of each tissue to avoid DNA, RNA, or protein degradation due to extended sample collection time. For RNA isolation from dissected material, use only RNase-free materials and chemicals. Do not exceed the total dissection time 15 minutes for one sample consisting typically from tissues dissected from 5-10 seeds to minimize RNA degradation.

3.1. Remove the rest of the hulls using fine-pointed tweezers before tissue dissection. Moisturizing with 1× PBS for 1 minute helps to remove dry residues of the spikelet.

3.2. Place the peeled seed on a Petri dish with a drop of 1× PBS and dissect individual parts using fine-pointed tweezers, fine-needle, and microcapillary pipette. A slightly different strategy is applied for dissection of individual tissues: the seed maternal tissues (step 3.3), the embryo (step 3.4) and the endosperm (step 3.5).

3.3. Dissection of seed maternal tissues

3.3.1. Dissection from seeds up to 8 DAP

3.3.1.1. Place a seed on the dorsal side and gently cut the seed along a longitudinal axis, and peel off seed maternal tissues except the last layer bordering endosperm from the apical to the basal part using tweezers.

3.3.1.2. Collect seed maternal tissues from 5 to 10 seeds into a 1.5 mL tube with 50 µL of 1× PBS, discard the buffer using a pipette, rinse the tissue twice with 100 µL of PBS, remove excessive buffer by pipetting and close the tube and freeze in liquid nitrogen or use directly for flow cytometric ploidy measurement. The amount of material is sufficient for typically one downstream application (e.g., RNA isolation or flow cytometric ploidy measurement).

3.3.2. Dissection from seeds after 8 DAP

3.3.2.1. Place a seed on the dorsal side, gently cut in the middle of the ventral side of seed maternal tissues and gradually peel off the tissue around whole seed including nucellar projection. For each downstream application collect and wash the tissue from 5 to 10 seeds as described in step 3.3.1.

3.4. Dissection of embryo

3.4.1. Dissection from seeds at 8 DAP and younger

3.4.1.1. Place a seed on the dorsal side and cut basal 1/3 of the seed. Carefully split separated part in half and release the embryo. For each downstream application collect and wash the embryos from 10 to 20 seeds as described in 3.3.1.

3.4.2. Dissection from seeds after 8 DAP

3.4.2.1. Place a seed on the dorsal side and remove seed maternal tissues from the basal part of the ventral side. Gently disturb the thin layer of endosperm around the perimeter of the embryo by fine-needle or fine-pointed tweezers and peel out the embryo. For each downstream application collect and wash embryos from up to 5 seeds as described in 3.3.1.

3.5. Dissection of endosperm

3.5.1. Dissection of syncitial endosperm from 4 DAP seeds.

3.5.1.1. Place a seed on the dorsal side, and remove seed maternal tissues except the last layer of cells bordering endosperm. Gently puncture layer in the middle of the ventral side by a thin needle, and suck the syncytial endosperm by capillary action using a microcapillary pipette.

3.5.1.2. For each downstream application collect liquid endosperms from 10 to 15 seeds into a new 1.5 mL tube with buffer suitable for the planned downstream analysis (i.e., 1x PBS, RNA isolation buffer, flow cytometry buffer). Buffer volume should reflect the protocol for the planned downstream aplication. Freeze in liquid nitrogen.

3.5.2. Dissection of celullarizing endosperm from 5 to 8 DAP seeds

3.5.2.1. Place a seed on the dorsal side, and remove all seed maternal tissues and embryo. For each downstream application collect and wash the endosperm from 10 to 15 seeds into a new 1.5 mL tube with 1x PBS and freeze in liquid nitrogen.

3.5.3. Dissection of celullarized endosperm from seed after 8 DAP

3.5.3.1. Place a seed on the dorsal side, remove all seed maternal tissues and embryo. For each downstream application collect and wash endosperm from a single seed per tube as described in step 3.3.1.

3.6. Store the tubes with isolated material at – 80 °C until use.

NOTE: The protocol can be paused here.

4. Control of tissue purity using flow cytometry

NOTE: The sample purity can be checked using flow cytometry before RNA isolation. Proper instrument calibration is critical for the biological sample analysis. The flow cytometer/ploidy analyzer optics should be adjusted using calibration beads (fluorescently stained polystyrene microspheres highly uniform with respect to their size and fluorescence intensity) until the maximal peak sharpness, typically reaching the coefficient of variation (CV) < 2%. Cereal seed tissues contain mainly populations of G1, G2 and endoreduplicated nuclei; therefore, using a logarithmic scale is recommended. Start with a leaf tissue that contains mostly G1 nuclei and serves as a basal ploidy control.

4.1. Use freshly prepared samples kept on ice (see step 3.3.1) or frozen tissue as described[17].

NOTE: Because the whole < 8 DAP sample is used for flow cytometry, this represents only an indirect control. We recommend researchers performing multiple isolations and measurements until reaching high proportion of pure samples (>90%) before proceeding to RNA isolation with < 8 DAP samples.

4.2. Release the nuclei from the 4 and 8 DAP embryo samples (for other samples see step 4.4) by homogenizing the tissues by 5 to 10 turns of the plastic pestle in 1.5 mL tube containing 300 µL of Otto I buffer (0.1 M citric acid monohydrate, 0.5% (v/v) Tween 20, filtered through a 0.22 µm filter)[18].

4.3. Filter the crude suspension through 50 µm nylon mesh into a flow-cytometry analysis tube and add 600 µL of Otto II buffer (0.4 M $Na_2HPO_4 \cdot 12H_2O$) containing 2 µg mL^{-1} DAPI (4′,6-diamidino-2-phenylindole)[18] to stain DNA.

4.4. Place all other tissues (including 16 DAP or older embryos) on a Petri dish containing 500 µL of Otto I buffer and homogenized by chopping with a razor blade. Filter the suspension as in step 4.3 and stain with 1 mL of Otto II buffer containing DAPI.

NOTE: Manipulation with a sharp double edge razor blade requires special attention. To reduce the risk of injury, there are a single edge razor blades or special blade holders available.

4.5. Estimate the nuclear DNA content of the sample using a flow cytometer. At least 2000 particles per sample are required for analyzing the sample purity.

5. RNA isolation and quality measurement

5.1. Use frozen tissue to prevent RNA degradation by endogenous ribonucleases. From seed maternal tissues and embryo samples isolate RNA using commercially available kits or TRIzol reagent[19]. Due to a high starch content in endosperm tissues, isolate total RNA from all samples using commercial on-column RNA extraction protocols for problematic tissues (e.g., Spectrum Plant Total RNA Kit) with an on-column DNase I treatment[20].

5.2. Measure RNA concentration and integrity using a dedicated protocol for RNA gel electrophoresis or Agilent 2100 Bioanalyzer.

NOTE: Intact total RNA has a clear 18S and 25S rRNA bands/peaks of size around 1.9 and 3.7 kb respectively. The 25S rRNA band should be approximately two times more intense than the 18S rRNA band.

REPRESENTATIVE RESULTS:

To perform a tissue-specific transcriptomic analysis of barley seed development, we established a protocol for high purity tissue isolation. The protocol is based on the manual dissection of embryo, endosperm and seed maternal tissues from peeled (after manual hull removal) grains (**Figure 1A**). The protocol was successfully used for isolating materials from several two- and six-row spring barley cultivars, and the spikes were harvested at a given DAP and directly used for extraction without fixation (**Figure 1D**).

The definition of DOP was a critical parameter to be estimated before dissection. Barley spikes mature from the middle towards the edges. Therefore, the middle flower was used for DOP evaluation. At most, six seeds from the middle of the spikes were used for tissue dissection. The success rate of self-pollination was close to 100%. In comparison, manual pollination had much lower success.

During the selected developmental window (4 to 24 DAP), the difficulty of tissue dissection

generally decreased over time. However, it has to be noted that the separation of tissues becomes again more difficult during and after seed dessication (> 24 DAP) due to tissue adherence. During the dissection of the tissues from 4 DAP seeds, a critical part was not to collapse the squashy syncytial endosperm (**Figure 2A**). Therefore, we removed seed maternal tissues by gentle cut and peel off. The embryo had to be protected against drying by adding buffer. At 8 DAP grain, the isolation strategy was analogous to younger seeds, but the nucellar projection (a part of seed maternal tissues on the dorsal side of the seed) appeared at this stage and its careful removal was required to avoid contamination of endosperm tissues. Conversely, this tissue is an important part of seed maternal tissues and should not be forgotten during isolation of this tissue. At later stages (16 and 24 DAP) seed maternal tissues were more cohesive. Our practical experience was that seed maternal tissues can be dissected and harvested in strips without damaging the endosperm (**Figure 2C**). The perimeter of the embryo should have a clear round shape and its original position in the seed should be clean of any rests of embryo tissues (**Figure 2C**).

[Place **Figure 2** here]

To test the purity of isolated tissues, we estimated nuclear DNA content using flow cytometry (**Figure 2B and D**). We used fresh barley leave to establish the position of the peak corresponding to diploid (2C) nuclei (**Figure 3A**). This tissue contained > 95% nuclei with 2C and 4C DNA content, corresponding to G0/G1 and G2 phases of the cell cycle, respectively and < 5% nuclei with 8C and 16C DNA content, corresponding to endoreduplicated nuclei. Next, all subsequent seed-tissue samples were measured with the same flow cytometer settings. Flow cytometric histograms of the whole seeds contained C-value peaks for diploid seed tissue (a mixture of an embryo and seed maternal tissues; 2C, 4C, 8C and 16C) and triploid endosperm tissues (3C, 6C, 12C and 24C). In properly dissected seed tissues, only C-value peaks for either the diploid or the triploid tissues were present (**Figure 2B and D**). Samples mixing tissues were identified based on the presence of contaminant diploid or triploid peaks (**Figure 3B and C**).

[Place **Figure 3** here]

As an example of downstream use of the samples, we isolated RNA from separated seed tissues using either the commercial RNA isolation kits or TRIzol reagent. However, due to high starch content in endosperm of older seeds (after 16 DAP), we used a commercial column-based kit for RNA isolation from problematic tissues. RNA isolation from endosperm samples older than 16 DAP using TRIzol resulted in unsuficient RNA quality and high level of protein contamination. To remove a residual DNA contamination, we performed on-column DNase I treatment that is an optional step in the commercial kits. The amount of isolated total RNA per sample was 200 – 3,000 ng for endosperm, 600 – 15,000 ng for embryo and 1,500 – 15 000,ng for seed maternal tissues. Next, we assessed the quality of isolated RNA using a bioanalyzer. Although the pattern can differ between tissues, two sharp peaks/bands representing the large and the small ribosomal RNA subunits should be normally present in the spectra/gel (**Figure 4**). The presence of additional peaks and high background in the fast and the inter-region indicates RNA degradation, whereas the 5S rRNA region includes various types of small rRNAs and those peaks

do not affect the quality of the isolated RNA. A signal in the precursor region can indicate residual genomic DNA contamination. The samples with RNA integrity number (RIN) ≥ 7 are considered of sufficient quality for analysis including reverse transcription PCR or RNA-sequencing.

[Place **Figure 4** here]

To test the purity of tissues prepared using this protocol at the molecular level, we performed RNA-sequencing (Kovacik, Nowicka and Pecinka, unpublished data) and analyzed transcript levels for several well known marker genes of embryo, endosperm and seed maternal tissues development (**Figure 5**). As embryo markers, we selected barley homologs of maize *LEAFY COTYLEDON 1* (*HvLEC1*; HORVU.MOREX.r2.6HG0506770) and *GLOBULIN 2* (*HvGLB2*; HORVU.MOREX.r2.5HG0430450), which are important genes for embryogenesis and production of storage protein, respectively[21, 22]. *HvLEC1* transcript was highly abundant in emryo tissues at 8 DAP and its amount strongly decreased at 16 DAP and was absent at 24 DAP embryo and all stages on other analyzed tissues. In contrast, *HvGLB2* transcript started low at 8 DAP embryo, but greatly increased at 16 and 24 DAP embryos. *HvGLB2* showed also low level of transcript in endospem and seed maternal tissues. Endosperm marker genes were represented by barley homologs of maize *ALEURONE 9* (*HvAL9*; HORVU.MOREX.r2.1HG0010310) and *GLUTENIN SUBUNIT* (*HvGS*; HORVU.MOREX.r2.1HG0001010) which are related to aleurone differentiation[23–25] and energy storage, respectively. The transcripts were highly specific for endosperm tissues, and *HvAL9* peaked at 8 DAP while it was 16 and 24 DAP for *HvGS*, which is consistent with endosperm tissue differentiation and energy accumulation. Our markers for seed maternal tissues were represented by barley *BETA AMYLASE* (*HvBA*; HORVU.MOREX.r2.2HG0113950) and *CHLOROPHYLL A/B BINDING PROTEIN* (*HvCAB*; HORVU.MOREX.r2.1HG0073450). BETA AMYLASE is connected with utilization of first storage protein deposition in seed maternal tissues at early seed development[1]. As the seed maternal tissues are the only green tissue in the seed, CAB proteins are important for photosynthesis. Highly tissue-specific profiles of all selected marker genes demonstrate that our protocol has potential for genetating samples without or with only minimal contaminoation from surrounding tissues.

[Place **Figure 5** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Developing barley seeds. (**A**) The schematic drawing of cereal grain at the sagittal plan with indicated seed maternal tissues (SMTs, green), endosperm (END, yellow), embryo (EMB, orange) and hulls (H, grey). (**B**) Morphology of barley spike close to the anthesis. Scale bar = 1 cm. (**C**) Morphology of stigma and anthers at the stages before, during and after pollination. Inset shows detail of the stigma with pollen grains (arrowheads). Scale bar = 5 mm, inset bar = 200 µm. (**D**) Sagittal and transverse sections of 4, 8, 16 and 24 DAP seeds. (NP, nucellar projection) Scale bar = 5 mm.

Figure 2: Flow cytometric estimation of the purity of dissected seed tissues. (**A**) Morphology of

dissected seed tissues from 4 DAP seeds. The groups correspond to the whole seeds (WS) photographed as hulled (left) and peeled from ventral and dorsal sides (middle and right, respectively); the next are dissected seed maternal tissues (SMTs), endosperm (END) and embryo (EMB). Scale bar = 5 mm, except for the inset to which 100 µm scale bar applies. (**B**) Representative histograms of nuclear DNA content obtained from described tissues. The histograms show marked C-value peaks for diploid EMB and/or seed maternal tissues (2C, 4C, 8C) and/or triploid END tissues (3C, 6C, 12C). The x-axis shows DNA content (relative fluorescence on $\log_3$ scale) and the y-axis the number of measured particles. NOTE: The scale on the y-axis should not be compared between the histograms as it varies depending on the starting amount of material and duration of the measurement. Only the presence/absence of a peak and relative height within one histogram should be evaluated. (**C, D**) Morphology and flow cytometric profiles of 16 DAP seeds. The figures are organized as in A and B. The flow cytometric measurement reveals also 16C and 24C nuclei, from diploid and triploid tissues, respectively. Scale bar = 5 mm.

Figure 3. Examples of control and contaminated seed tissue samples as revealed by flow cytometry. (**A**) Representative histogram of nuclear DNA content from 10 days old barley leaf representing somatic tissue control. (**B**) Example histogram of dissected 16 DAP endosperm (3C, 6C, 12C and 24C peaks) contaminated by a diploid tissue (2C peak – red-labeled). (**C**) Example histogram of 8 DAP dissected seed maternal tissues contaminated by endosperm tissues as indicated by the presence of 3C and 6C peaks (red-labeled). The x-axis shows DNA content (relative fluorescence on $\log_3$ scale) and the y-axis the number of measured particles. NOTE: The scale on the y-axis should not be compared between the histograms as it varies depending on the starting amount of material and duration of the measurement. Only the presence/absence of a peak and relative height within one histogram should be evaluated.

Figure 4. Quality control of isolated total RNA. (**A**) A representative spectrum of high quality (blue) and partially degraded (red) total RNA from 8 and 16 DAP seed maternal tissues respectively produced using Agilent 2100 Bioanalyzer with the RNA integrity number (RIN) 8.00 and 6.40 respectively. The graph shows the intensity of the peaks of the ribosomal RNA subunits: nuclear large-25S, small-18S and 5S RNA. nt = number of estimated nucleotides based on ladder; FU = relative fluorescence units. (**B**) The electrophoretic gel-like view of high quality (blue) and partially degraded (red) RNA indicating the subunit bands.

Figure 5. Examples of expression from marker genes. PolyA enriched mRNAs from barley seed tissues were sequenced in three biological replicates using Illumina platform. The graphs show an average fragments per kilobase per million reads (FPKM) at different days after pollination (DAP) in embryo (orange lines), endosperm (yellow lines) and seed maternal tissues (green lines). Standard deviation between biological triplicates is indicated by the gray field. Two examples of early (top row) and late (bottom row) marker genes are shown for (**A**) embryo, (**B**) endosperm and (**C**) seed maternal tissues.

DISCUSSION:

Here, we present a protocol that allows high purity isolation of barley seed tissues. Although it was developed and tested for barley, it can be easily adopted to other members of the *Triticeae*

tribe such as wheat, oat, rye or triticale[26]. The initial part of the protocol, focusing on seed tissue dissection, does not require any non-standard or expensive equipment and therefore should be accessible to many scientists. A highly specialized instrument such as a flow cytometer is required for the thorough quality control analysis. However, many plant research institutions have a flow cytometer or ploidy analyzer operated by a trained research staff.

For plant pollination, we make use of the barley's ability to self-pollinate and rely on a set of simple morphological parameters that define the exact day of natural pollination. Hence, the protocol avoids manual flower emasculation and pollination that is a common approach applied to many plant species. We have initially tried both methods and the manual pollination method resulted in much smaller rate of successfully developing seeds (< 40%). Although the monitoring of spontaneous pollination requires experience in estimating maturity of stigma and anthers, it can be very reliable method with a reduced hands-on time, and can produce higher numbers of seeds needed for dissections.

The difficulty of tissue dissection changes over the time of seed development. The most challenging is the isolation of tissues from the youngest (0 to 8 DAP) seeds, which are minute and easy to damage due to their soft texture and liquid character (endosperm). Therefore, fine tools are needed. Using the presented protocol, we were able to manually isolate seed maternal tissues and endosperm from 4 DAP or older seeds in sufficient amount and quality for complex assays. Dissection of embryo before 8 DAP was problematic and we were not able to collect sufficient amount of tissue for downstream analyses. We envisage that further improvements could be achieved with a micromanipulator. An alternative method could be tissue sectioning followed by a laser microdissection[27]. Although this method offers great resolution it usually brings a very small amount of material that needs to be extensively PCR amplified before analysis. This may introduce certain biases or redundancy. The presence of cell walls in plant tissues prevents separation of intact cells and their simple isolation by fluorescence-activated cell sorting (FACs) as performed for animal or fungal cell cultures. In plants, fluorescence-activated nuclei sorting (FANs) based on the nuclei C-values is feasible[28]. Although FANs is highly sensitive method, it represents only part of cellular information (e.g., cytoplasmic RNA and proteins are largely missing), generates small amounts of sample, and requires highly advanced instruments. It has to be emphasized that the protocol presented here provides relatively large amounts of material and does not involve PCR-based amplvification before library preparation. Dissection of tissues older than 8 DAP with a cellularized endosperm is considerably easier, but drying of seed parts at later stages may decrease tissue separability. A simple solution is moisturizing the tissues with a physiological buffer.

An important factor affecting all downstream analyses is the purity of extracted tissues. In highly sensitive experiments such as RNA-sequencing, tissue contamination results in decreased resolution and false information. Therefore, we implemented a flow cytometry-based purity control step in the protocol that is based on different ploidies of seed tissues. Distribution of nuclei within the tissue is not always homogenous and some parts can be absent of nuclei while other part of the same tissue may still contain nuclei (e.g., central starchy endosperm and aleurone layer, respectively). We were able to detect nuclei in each completely dissected tissue

in the selected developmental window. Based on their triploid nature, the endosperm tissues can be easily distinguished from the diploid seed maternal tissues and embryo. The most common type of contamination observed in the samples was between seed maternal tissues and endosperm, possibly originating from the nucellar projection. However, when the sample contained less than 5% of different ploidy level, this could not be visible as a separate ploidy peak. It is also obvious that the purity control cannot distinguish contamination between seed maternal tissues and embryo. However, this type of contamination is less likely as both tissues do not adhere to each other and can be easily manually separated. In addition, to reduce tissue contamination after dissections, we applied multiple washes using a physiological buffer.

Since RNA can be easily destroyed by the activity of endogenous RNases, the quality of dissected tissue was determined based on RNA degradation level. As an index of quality we used RIN calculated automatically by a bioanalyzer. Degradation is also a natural process caused by aging and together with unproper handling during dissection is the main factor affecting the quality of dissected tissues. This limitation did not allow isolation of good quality RNA from seed maternal tissues older than 24 DAP.

The isolated material is suitable for various types of downstream analyses including isolation of nucleic acids, proteins and other cellular compounds. We have already successfully used the tissues to isolate RNA and perform RNA-sequencing experiments. This is significant experimental improvement because until now, barley seed transcriptomic studies were done using either the mixture of embryo and endosperm[29] or even whole seeds[30]. Application of the optimized strategy will greatly increase the resolution and specificity of the RNA-sequencing data as also demonstrated on the expression values of several tissue-specific genes (**Figure 5**).

In conclusion, this protocol provides means for detailed analysis of individual seed tissues. This will help unravelling the mechanisms controlling seed development in barley and other cereals.

ACKNOWLEDGMENTS:

We thank Dr. Jan Vrána and Dr. Mahmoud Said for the maintenance of flow cytometers, Eva Jahnová for preparation of buffers Marie Seifertová for list of materials and Zdenka Bursová for plant care. This work was supported primarily from the Czech Science Foundation grant 18-12197S. A.P. was further supported by the J. E. Purkyně Fellowship from the Czech Academy of Sciences and the ERDF project "Plants as a tool for sustainable global development" (No. CZ.02.1.01/0.0/0.0/16_019/0000827).

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

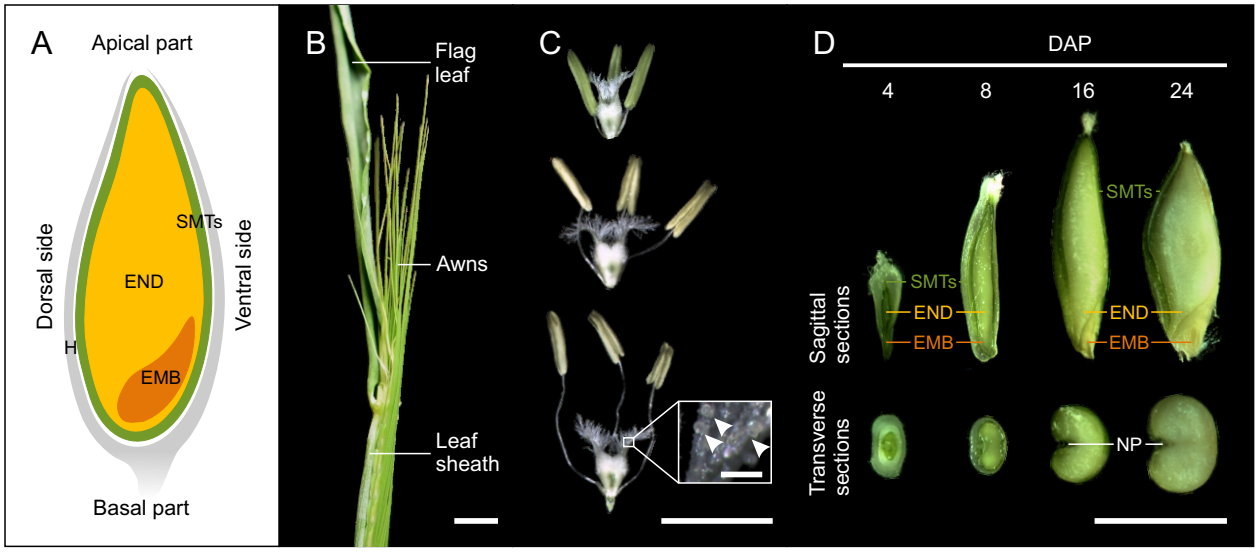


Figure 2

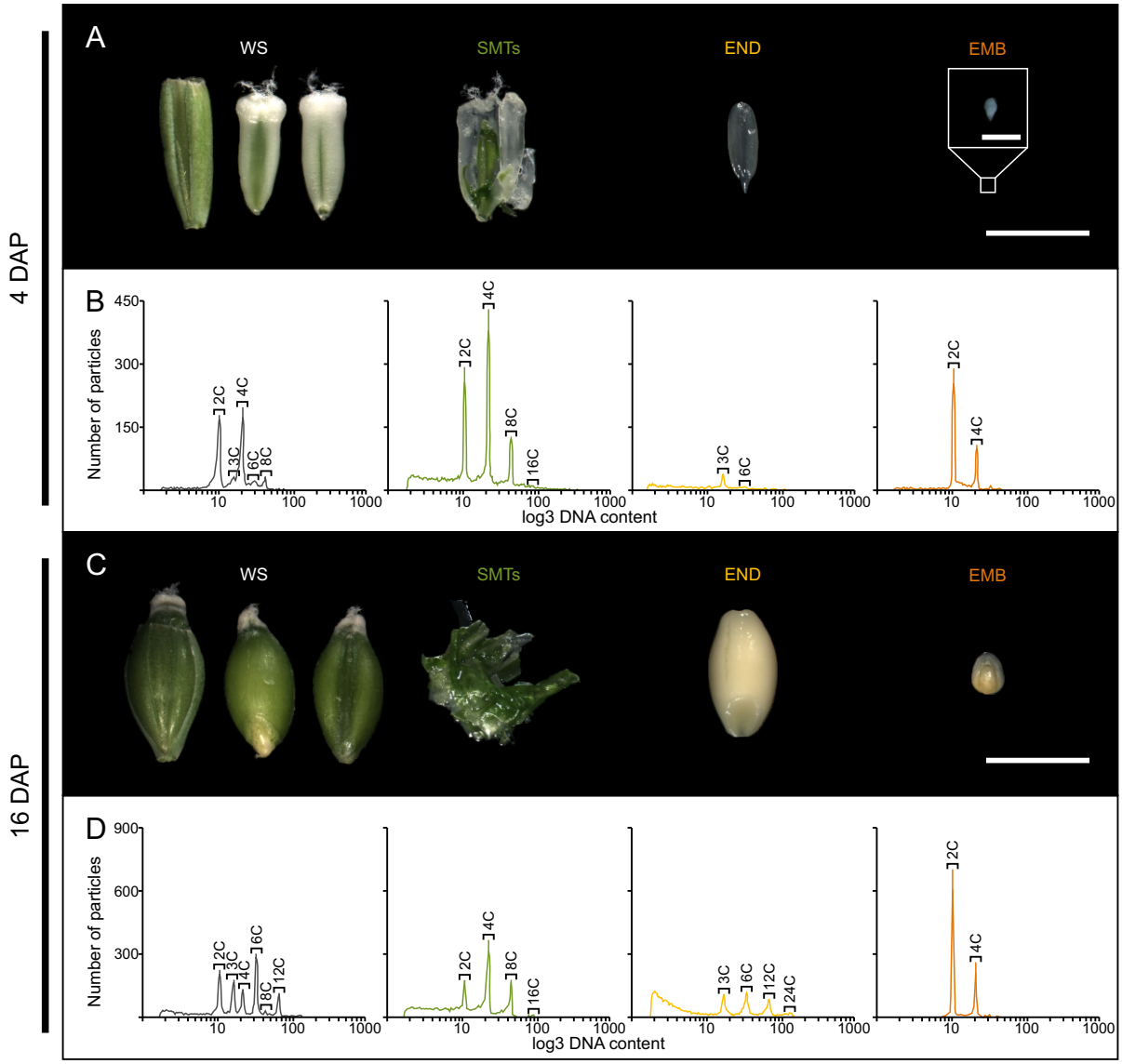


Figure 3

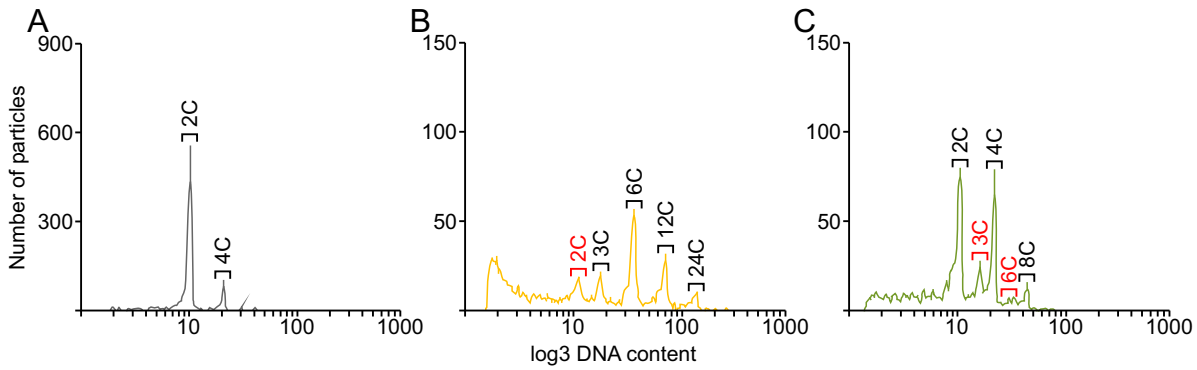


Figure 4

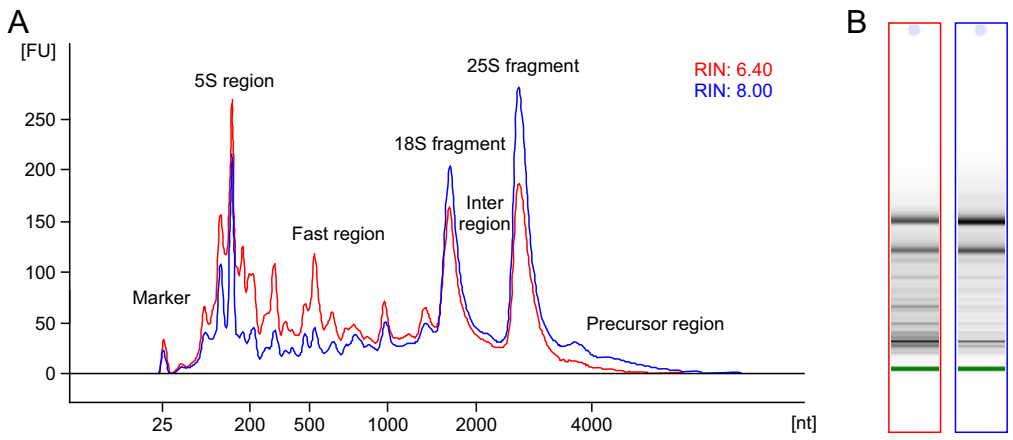
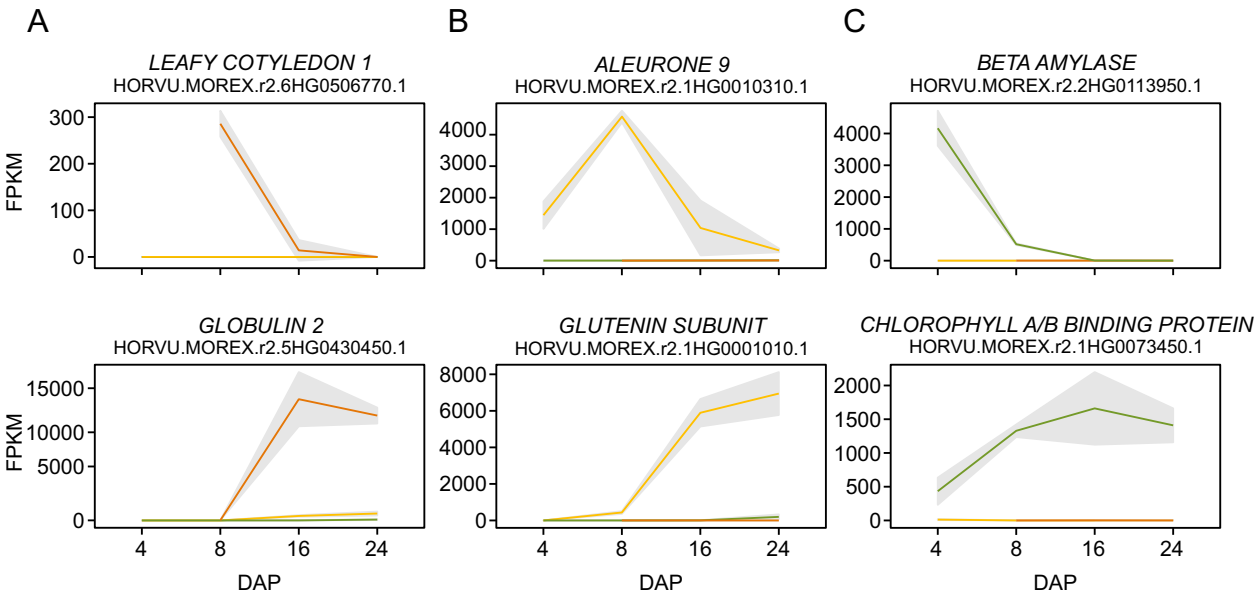


Figure 5



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.22 um filter	Merck	SLGSV255F	
1.5 ml Eppendorf tube	Sarstedt	72.690.001	
4',6-diamididno-2-phenylindole	Invitrogen	D21490	
50 um nylon mesh	Silk a Progrers	uhelon 120 T	
Agilent 2100 Bioanalyzer	Agilent	G2939BA	
Bulb Assembly	Drummond Scientific Company	1-000-9000	
Calibration beads	Invitrogen	A16502	
Cellulose tissue paper			
Citric acid monohydrate	Penta	13830-31000	
Climatic chamber	Weiss Gallenkamp		
DNase I	Sigma Aldrich	DNASE70	
Filter paper	Fagron		
Fine-pointed tweezers	Fine Science Tools	11254-20	
Flow cytometer	Sysmex-Partec		
Flow cytometry tube	Sarstedt	55.484	
Freezer			
Glassine bag			
KCl	Lachner	30076-AP0	
KH2PO4	Litolab	100109	
Liquid nitrogen	Linde		
Microcapillary pipette	Fivephoton Biochemicals	MGM 1C-20-30	
Minutien Pins	Fine Science Tools	26002-20	
Na2HPO4	Lachema		
Na2HPO4.12H2O	Lachner	30061-AP0	
NaCl	Lachner	30093-AP0	
Peat pots	Jiffy		5x5 cm
Petri dish			
Pin Holder	Fine Science Tools	26016-12	
Plastic pestle	p-Lab	A199001	
Pots			12x12 cm
Razor blade	Gillette		
RNAse zap	Invitrogen	AM9780	
Sand			
Scissors	Fine Science Tools	14060-11	
Soil			
Spectrum Plant Total RNA Kit	Sigma Aldrich	STRN50	
Stereomicroscope	Olympus		
Tween 20	Sigma Aldrich	P2287	
TRIzol reagent	Invitrogen	15596026	

RNA 6000 Pico Kit	Agilent	5067-1513

Olomouc, September 2nd 2020

Dear Editor,

thank you and the editors for careful evaluation of our manuscript and constructive critics. We have considered and (with very few exceptions) also implemented all suggested modifications. We hope that you and the reviewers find the changes satisfactory. Please let us know in case there are still some open issues.

Looking forward to your response.

On behalf of all authors
Ales Pecinka

Point-by-point response to the reviwer's comments

Reviewer #1:
Manuscript Summary:
The Authors present in very clear and elegant way a procedure for the isolation of different tissues from barley seeds. The protocol will be of great help specially for the researchers applying advanced (and therefore expensive) molecular methods, were the precision of selection the right tissue is a key for obtaining reliable results.

Major Concerns:
none

Minor Concerns:
1. Line 59: it is not clear what the Authors mean by "In addition, barley is used for genetic improvements of other cereals." Do you mean that barley is a model species for the cereals and results of the research performed on this species can be apply to other cereals? Or some barley genes are used to improve other cereals? Please, precise.
Response: We added "genes" to make this clear.

2. Lines 100-102: please, precise what the humidity of filter paper is optimal. For unexperienced in seed biology researchers "moisturize" does not mean much and if they add too much water the seed instead of germinate can rot.
Response: We added more information on this step to the protocol.

3. Line 106: please, precise what do you mean by "germinated". With visible radicle? With the radicle of a certain length?
Response: Emergence of the radicle and shoot. The information was added to the protocol.

4. Line 113: "germination" is not precise. From sowing? From seedling emergence?
Response: Yes, from sowing. Corrected.

5. Line 149: There is also not clear what "moist" means. How, for how long?
Response: We added more information on this step to the protocol.

6. Line 150: Please explain, why it is recommended to use a new seed for dissection of each tissue.
Response: We added following explanation: “Use a new seed for dissection of each tissue to avoid DNA, RNA and protein degradation due to extended sample collection time.”

7. Line 166: Since you recommend to isolate tissue from 5-10 seeds, please add information how many are needed for flow cytometry and how many for RNA analysis.
Response: For each downstream application one sample with mentioned amount of tissue is needed. We now added following information: The amount of material is sufficient for typically one downstream application (e.g. RNA isolation or flow cytometric ploidy measurement).

8. Line 195: "one endosperm" sounds awkwardly. "From a single seed"?
Response: Corrected

9. Line 206: Actually, any logarithmic scale is appropriate. Since in not all cytometers there is a choice of different log, to make it more universal please rephrase: "using a logarithmic scale (preferably log3)"
Response: Corrected

10. Line 209: De-freezing the sample, even slow, can cause the damage of some nuclei, thus to obtain histograms of high quality it is better to prepare samples directly from frozen material (see Staszak et al., Functional Plant Biology, 2019, 46, 152-164). It would be better to recommend "…and prepare samples from frozen material (preferably) or slowly defrost the samples."
Response: We have now recommended to use freshly prepared samples for ploidy measurement or prepare samples directly from frozen material (with reference to Staszak et al. 2019).

11. Line 213: Please add a reference to Otto buffer.
Response: Corrected

12. Line 216: there is no need to recommend 3.5 mL tubes; the volume is typical for the certain cytometer.
Response: Corrected

13. Line 232: from frozen tissue?
Response: RNA isolation has to be done on frozen tissue to prevent RNA degradation by endogenous enzymes. We have added this information into protocol.

14. Line 280: Authors' description for using leaves as a control can be not clear for unexperienced flow cytometry users. Perhaps it would sound more understandable if "as somatic tissue control" is replaced by "to establish the position of the peak corresponding to diploid (2C) nuclei"
Response: Thank you for this suggestion. We have modified the text accordingly.

15. Lines 281-282: Please, add the ploidy of remaining <5% of nuclei.
Response: Added. These are 8C and 16C endoreduplicated nuclei.

16. Line 281: 2C corresponds to G0/G1 and not only to G1 phase.
Response: Corrected.

17. Lines 291-305: There is only a reference to Fig. 4A and not to 4B.
Response: Corrected

18. Line 370: At the end of the first sentence please add "of seed development"
Response: Added.

19. Usually at the mature state of the seed the maternal tissues are dead, e.g. cells do not contain nuclei and therefore they cannot be detected by cytometry. Is it a case with barley? If so, after how many days after pollination? Did you still observed maternal tissue nuclei 24 DAP? It is not mentioned in the whole paper and in Fig. 2 only histograms of different tissues 4 DAP and 16 DAP are presented. This information is important for the protocol.
Response: Thank you for this point. In fact, we are able to detect some nuclei in each tissue even in dry seeds. While in majority of some tissues can end up without nuclei, other part of the same tissue may still contain nuclei (for example central starchy endosperm versus aleurone layer, which lose and keep their nuclei, respectively). We included short note on this in the discussion. We recently described this in detail in a manuscript that is currently being revised in a journal.

Reviewer #2:
Manuscript Summary:
The authors describe a protocol for dissecting tissues from developing barley grains and assessing their purity using ploidy analysis. The introduction gives a good background and context for why analysis of the developing grain is important, and how ploidy analysis would differentiate endosperm from other tissues.
This technique would benefit very much from a video demonstration and it is unfortunate that the manuscript is reviewed without access to the video. As written, the manuscript does not seem to contain sufficient detail to allow a user unfamiliar with grain dissection to complete the technique successfully.
The ploidy analysis provides a clever analysis for assessing contamination of seed maternal tissue (SMT) and embryo tissue with endosperm material. However, it does not provide a way of determining whether embryo and SMT are cross-contaminated. If the authors have performed RNA-seq experiments on their purified tissues, examining the expression of tissue-specific marker genes would be a much better way of assessing tissue purity from the dissection protocol.

Response: We would like to thank reviewer #2 for these insightful comments. Based on the journal policy, the video is made only if the manuscript successfully passes the review process. We have now added information that will hopefully make some critical steps clearer. In addition, we have included representative gene expression profiles from RNA-seq experiments run with RNA prepared from tissues described in this protocol (Figure 5).

Major Concerns:
1. The dissection part of the protocol seems to lack a lot of detail with materials and description. Line 158: "fine-pointed tweezers, fine-needle, and microcapillary pipette". Brand, model, size, etc?

Line 178: "Carefully split separated part by half and release the embryo" - what does this mean to someone who has never done it?
Response: Instrument brands, models and sizes are part of the list of materials specified in the Company and Catalog Number. We do not know whether this file was provided to the reviewers. We have now added more information about an embryo dissection in the protocol.

2. Line 126, also 255-258. Given how central the correct identification of the DOP is, a figure showing stigma and anther morphology before, at, and after anthesis would be helpful. Conversely, the equation (which is essentially x=0+x) is not necessary.
Response: We have now added a new section in Figure 1 showing morphology of stigma and anthers before, at and after DOP. Nevertheless, equation shows how we were counting DAP and thus it is methodically important.

3. Details of protocol lines 2.2 and 2.3 appear to refer to a self-fertilisation protocol that is not fully explained. It is not clear why is it necessary for the described protocol, and it certainly does not fit under the heading of "Determination of the anthesis and days after pollination". This aspect is referred to again in lines 262-263, 363, 366-368, but again, the authors do not explain why it is necessary for tissue dissection.
Response: We have now added information explaining this. In short, clipping of the top of the hulls leads to more synchronous pollination and covering spikes prevents cross-pollination.

4. Section 3: For each tissue, this section is currently divided into 4-8DAP and 16-24DAP. What happens if a researcher wishes to dissect tissue at 10DAP? What about 30DAP? This protocol would be more generally useful if there was a continuous timing, e.g., 4-10DAP, 11-25DAP. Perhaps the authors could also make a comment about the difficulties of dissection after 25DAP?
Response: Very good point. We have focused too much on the stages routinely used for our experiments. We now made clear that the two main phases correspond to the time before/after 8 DAP. At 8 DAP, endosperm undergoes cellularization, which represents a major developmental transition in seed development and of course tissue isolation strategy.

5. Line 150: If it is essential that a new grain is used for each tissue, it would be very helpful to emphasise this fact and explain why - especially since the first step for endosperm extraction is removal of maternal tissues. A time-poor, tissue-poor researcher would be very tempted to use the same grain for all three tissues.
Response: We have now added this information in the manuscript. Basically, tissue damage and extended time of preparation can lead to e.g. RNA degradation and thus compromise quality of the experiment.

6. Line 172: Keeping SMTs at room temperature while dissecting the remainder might explain why the RNA is so degraded. You've mentioned earlier that no tissue dissection should exceed 15 minutes (line 152), but here, you can have tissues hanging around at room temp for over 2 hours. A more rigorous protocol would be to wash the tissue isolated from each grain as soon as it is collected, then freeze immediately, i.e., in liquid nitrogen. This is true for solid SMT, embryo and endosperm from grain of all ages. Is explained on lines 403-406, but it could be a problem in the protocol, rather than solely a function of the older barley grain tissues.
Response: Thank you for this comment, our protocol was not clear at this step. In fact, total dissection time of 15 minutes corresponds to one sample containig tissues from 5-10 seeds, i.e. ca.

1.5-2 min per dissection of tissue from one seed. We have now provided this information in the NOTE for section 3.

7. Similarly, embryos (3.4.) and maternal tissues (3.3) are being harvested into PBS and being left at room temperature for 1-2h during collection. Not only will RNAses and other lytic enzymes start to act, but some soluble material may also start to be lost from the tissues. These caveats should be made clear when the authors claim that the tissues can be used for downstream applications.
Response: Please see the answer for point 6. The total dissection time for one sample (including tissues from multiple seeds) is 15 minutes.

8. Line 191: why are the liquid endosperms collected into PBS? Why not collect them into an eppendorf on a -80C cold block? If you harvest them into a buffer, you leave time for RNAses to act, and also dilute the endosperm contents for all downstream applications
Response: We have now added more information on this step in points 3.5.1 and 3.5.2.

9. Without the benefit of the video, it is very hard to envisage how the authors are successfully and replicably isolating 4DAP embryos. According to Fig 2, the 4DAP embryo is 30um, which with a standard stereomicroscope that does 40x mag, makes it 1.2mm to dissect - well under the size of the finest tweezers at that magnification. Not sure how you would rinse them in PBS etc, grind them, etc.
Response: Our protocol was not clear enough. We were able to dissect (more like remove) 4 DAP embryos, but we were not able to collect enough amount for downstream analyses. We corrected this in the protocol and discussed possible ways for dissection.

10. Line 188: Why do you need to remove SMT to extract endosperm with a needle?
Response: Thank you for pointing this out. Here our protocol was not clear enough. The trick is to remove SMT except for the last cell layer which holds together syncytial endosperm. This reduces contamination by disrupted cells during removing of the rest of SMTs and also makes it possible to work with endosperm syncytium, which would break otherwise. We have now added more information on this step.

11. Line 233-235: Commercial kits for problematic tissues don't always work for starchy tissues. For endosperm samples, an amylase step is best. Unless you have a kit that works well, in which case you should mention it specifically.
Response: We have added name of the kit that worked well in our hands for these tissues.

12. Line 265: "The difficulty of tissue extraction generally decreased with increasing DAP": only until a certain point, i.e., the 24 DAP that you chose. If you went on to 30, 35 or 40 DAP, it would become harder again. The authors should perhaps be more aware that they have selected only a subset of timepoints during grain development for their tissue isolation.
Response: Thank you for this comment. We have added this information in the text.

13. Line 268: " Embryo had to be protected against drying by adding buffer". What does this mean? Buffer wasn't added to any dissection as far as I could see? And SMTs were harvested into buffer as well as embryo.
Response: We moist the grain to prevent its drying. We have now provided this information in a clearer way in the initial NOTE for the chapter 3. Dissection of seed tissues.

14. Line 276: "should be clean of any rests". This is not clear.
Response: We have specified this information as follows: "…should be clean of any rests of embryo tissues (Figure 2C)."

15. Line 398: "likely as both tissues are separated by endosperm". This is not true. Most of the embryo, at all stages of development, is surrounded by maternal tissue. It only borders endosperm on the apical face.
Response: We have modified the text to make it correct.

16. As such, the whole aspect of using ploidy to analyse contamination seems overly complex. It only tells you if endosperm contamination is present in embryo or SMT samples and vice versa. A much simpler test for endosperm contamination is simply to test for the presence of starch.
Response: Starch granules are not present in young endosperm. In addition, at early development of seed maternal tissues, there are parts which contains starch (see Sreenivasulu N et al. *Int Rev Cell Mol Biol*. 2010;281:49-89). Therefore, we still consider the proposed method based on cell nucleus ploidy as more reliable (especially for the young seed tissues).

17. Lines 409-410: If you have done RNAseq experiments, a much better test for tissue purity would be to look for the expression of tissue-specific marker genes in your tissues. How did they compare with your ploidy results?
Response: We believe that one of the strengths of our protocol is that the ploidy analysis allows to assess sample purity before going into an expensive library preparation and high throughput sequencing. To demonstrate the sample quality produced with this protocol, we have now included RNA-sequencing results of several tissue-specific marker genes (Figure 5).

Minor Concerns:
1. Line 164: stigma? At 4-8DAP? Did you mean seed?
Response: Corrected

2. Line 83: transfer cells
Response: Corrected

3. Details of protocol lines 1.1 and 1.2 are not necessary - the seeds can be sown directly into soil and grown as described in 1.3 without jeopardising grain development
Response: We have added alternative step including germination in soil.

4. Heading 2: Determination of anthesis and days after pollination
Response: Corrected

5. Line 250: DOP should be DAP
Response: Corrected

Reviewer #3:
Manuscript summary:
The authors describe a protocol to dissect and isolate the different tissues within the developing seed of barley. The protocol will be useful for the research community interested in working with

developing seeds from barley and probably for other cereals with a little optimization. I expect that the visualized narrative, when produced, will be extremely helpful to those wishing to reproduce the protocol. The figures the authors supply are well drawn.
The introduction describes well the process of seed development, but it would be helpful if the authors relate this to the time points they analyse. For example, they mentioned the development stages, I, II and III (line 64-69) in the introduction, but how do these relate to day of pollination (DOP) for barley as used in their protocol.
The protocol description uses clear language and is easy to follow. The authors described how to grow the plants, determine the day of pollination, dissect the different tissues, do a quality control of the dissection and isolate RNA from the collected tissues.
I found the representative results section contains information that I would have appreciated to read in the introduction, for example the definition of DOP (line 253) paragraph.
The manuscript requires proof-reading to correct some spelling mistakes and typos.

Response: We would like to thank reviewer #3 for supportive comments and for suggestions. We have now included in introduction on the correspondence between different DAPs and seed developmental scales.

Major Concerns:
None.

Minor Concerns:
I have below listed a few considerations that would benefit a researcher who is trying to use the method for the first time, as well as other minor details I encountered.

It would be useful for the reader to have an idea of RNA concentrations obtained from the isolated tissues and a comment on the minimal amount of RNA that gives a robust q-RT-PCR or RNA-seq results.
Response: We have added information on the typical amounts of total RNA obtained from isolated tissues. As to the requirements for different methods, this greatly varies on the used protocol. In general, the obtained amounts of total RNA are fully sufficient for standard methods.

Line 42-34, it states "this protocol provides information how to dissect pure…". I would suggest them to rephrase, for example: This protocol describes practically how to dissect pure…
Response: We have implemented the suggested modification.

Line 101, it states "Moisturize it with distilled water…". Please provide details of volume used to keep the moisture for 3 days.
Response: Final volume depends on many parameters, such as diameter of petri dish and number of seeds. In general, there the amount of water should not be too high, so there is no excess water. We have added this information to the text.

Line 120, authors mentioned the term anthesis in the title and do not describe nor mention it again.
Response: Right. We have now simplified the title to “Determination of pollination”. We also added information why this information is needed.

Line 122-123, it states "we monitored the day of self-pollination." But there is no mention around which day users should check. I suggest the authors provide a time frame to help novel users.

Response: We have now added more information in the manuscript: “To define day of pollination (DOP), we monitored the day of self-pollination. This is cultivar specific, but a good indicator is starting protrusion of the awns from the leaf sheath.”

Line 127, citation 15. The publication cited has no definition of DOP. Please double check it.
Response: We have modified the sentence as follows: Spikelets with yellow anthers and “fluffy” stigma will pollinate within few hours[15] and are considered as DOP.

Line 134, it states "Put the information…" I suggest the authors use a more precise term for example write down, transfer, annotate…
Response: Corrected

Line 135. What is "the proper day after pollination"? What do the authors refer to? Please be more specific.
Response: We have now corrected unclear formulation. It means the day after pollination (DAP) when tissue isolation will take place. We also added new images into the Figure 1 to clarify day of pollination.

Line 149. Should be "…moisten the samples with 1x PBS", not "…moist the samples by 1x PBS".
Response: Corrected

Line 164, it states "Extraction from …". What do the authors mean with extraction? Please correct onwards.
Response: Corrected.

Line 167-168, it states "removes excessive buffer with pipetting". Is the material wet when frozen in liquid nitrogen?
Response: We are afraid that this is misunderstanding from the reviewer #3. The protocol states that the removal of buffer is performed before freezing. Subsequently, the dried sample is frozen.

Line 178. Should be "…separated part in half", not "…separated part by half".
Response: Corrected

Line 191, it states "Collect endosperms…. with 1x PBS". I would mention that you are collecting liquid endosperms. How much volume of PBS do you use here?
Response: We have added “liquid”. As to the volume, we have now modified the protocol and provide information that the sample should be frozen with a buffer used in the next downstream application. The application will also determine the volume.

Line 203-205, the calibration method with beads is poorly explained.
Response: The information was added.

Line 227, it states "Estimate the nuclear DNA amounts…", I would suggest the authors use "… DNA content". Please modify accordingly onwards.
Response: Corrected

Line 232-235. RNA extraction isolation description is not specific enough. I would suggest adding citations e.g. TRIzol and starchy endosperms protocols.

Response: Corrected

Line 279, it states "purity of separated tissues", I would suggest using "…isolated tissues" instead.
Response: Corrected

Line 280, it states "young barley leaves", how old were they?
Response: We have now modified the text to “fresh barley leave”. The leaves can be also from older plants, but should bear no signs of infection or senescence (browning, drying etc.).

Line 281, it states "contained > 95 % nuclei with 2C and 4C DNA amounts". I suggest rephrasing to, for example, "contained > 95% of 2C and 4C nuclei".
Response: To be consistent we use “DNA content” in the whole protocol now.

Line 283. Please state the flow cytometry settings. These would be very hard for an inexperienced user to work out for themselves.
Response: Unfortunately, the settings are very much dependent on different types of flow cytometers. Providing the settings for the ploidy analysers used in our lab (Sysmex) may just mislead the users of other types of machines.

Line 287. Should be "mixing tissues" instead of "mixing more tissues".
Response: Corrected

Line 291, authors state "As an example of downstream use of the samples" but they are only isolating RNA and assessing the quality of the RNA in a Bioanalyzer. Could the authors provide any successful downstream analysis (PCR or RNA-seq results) to validate their protocol?
Response: Yes, we have done RNA sequencing. We have now added new Figure 5 showing example expression data for some tissue-specific marker genes.

Line 294, it states "older endosperm samples", what do the author mean with older? Age or previously isolated?
Response: Corrected

Line 310. Figure 1B. The images of the barley seed look very green and it is difficult to distinguish clearly the tissues. I would also suggest the authors to provide more detail on the seed structures, since it will help understanding when they mention them in other sections of the protocol (e.g. stigma in line 164).
Response: We have now corrected colours of barley seed and added section in Figure 1 showing morphology of stigma and anthers before, at and after DOP. We have also labelled next important part of the seed – nucellar projection.

I would also suggest adding a figure 1, a picture of the barley spike with structure names to help users with the identification of DOP and DAP.
Response: We have now added photo of parts of barley spikelet into Figure 1 and described structures important for correct identification of DOP.

Line 320. "DNA amounts" correct accordingly onwards.
Response: Based on the previous comment, we modified “DNA amounts” to “DNA content”.

Line 379, it states "…presence of cell walls in plant tissues prevents separation of intact cells". What about preparing protoplasts?
Response: Protoplasting has a profound effect on gene transcription and would introduce massive transcriptional changes. Therefore, we would like to avoid this approach.

Line 382, it states "protocol presented here provides relatively large amounts of material". What is the concentration range obtained from the different tissues? Could you provide numbers?
Response: We have now added information about typical amount of total RNA isolated from different tissues into representative results.

Reviewer #4:
Manuscript Summary:
OK
The authors described and visualized a protocol for manual separation of different tissues of the developing barley grains. As such, the protocol is not new and has being used in many laboratories dealing with cereal grains. However, all these techniques and tricks were not summarized so far. as additional advantage is the usage of the measurement of ploidy levels to check the tissue purity. Its limitations are also mentioned. The advantage of the proposed protocol in its systematic and precise description making it useful for both beginner and experts, as well as for students.

Line 65. It is not fully correct that the first phase of barley grain development is characterized by minimal weight increase. This phase is rather characterized by proliferation and the intensive tissue growth which is also reflected in weight increase but storage product synthesis does not still begin.
Response: The literature is not consistent in description of this stage. We have now modified this to consensual “typified by cell proliferation and not yet initiated starch synthesis”.

Lines 83-86. Besides pericarp and seed coats, the maternal seed part encompasses such tissues as the nucellus (disintegrating during early development) and the vascular bundle. The nucellar projection, noted in the manuscript, is developed from the nucellus that adopted a new transport-related function. It may be of importance to mention that the nucellar projection is embedded into the endosperm making difficult the careful separation of these tissues by manual tissue dissection.
Response: Corrected

Line 395: please correct "conained" to "contained"
Response: Corrected

Figure 1. Images in B may be much bigger to visualize the structure in greater details. There is a plenty of empty black space around them.
Response: We have now used the space to highlight some of other critical stages, which should help the users.

Figure 3. The description of the contamination peaks may be labeled by red letters for better visualization.
Response: Changed as suggested

Major Concerns:
no

Minor Concerns:
listed above

Reviewer #5:
The protocol provided is of value and illustrations are OK. I have a few suggestions:

- Include a Figure (photograph?) to show the nucellar projection mentioned in Lines 269-272.
Response: We have now added label of the nucellar projection which is visible in Figure 1 section B, Transverse sections.

- I could not find in the text an estimation of the total number of seeds that should be processed to perform RNA and flow cytometry analyses for the different tissues. Also, could the Authors inform the number of plants that should be grown to provide enough material?
Response: We have now included NOTE in section 1. which provides basic information on the numbers of seeds/spikes that can be used per typical plant.