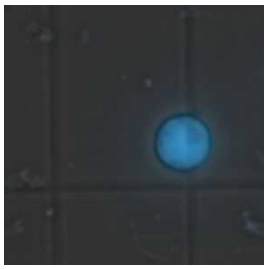
Aug 21, 2024

Intact and clean nuclei isolation from wheat meristems



DOI
dx.doi.org/10.17504/protocols.io.yxmvm3renl3p/v1

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Protocol Citation: Isabel Faci, Maximillian RW Jones, Cristobal Uauy 2024. Intact and clean nuclei isolation from wheat meristems. **protocols.io https://dx.doi.org/10.17504/protocols.io.yxmvm3renl3p/v1**

Protocol status: Working
We use this protocol and it's working

Created: February 14, 2024

Last Modified: August 21, 2024

Protocol Integer ID: 95212

Keywords: Nuclei, Wheat, Meristems

Funders Acknowledgement:
UK Biotechnology and Biological Sciences Research Council (BBSRC)
Grant ID: BB/X011003/1
UK Biotechnology and Biological Sciences Research Council (BBSRC)
Grant ID: BB/X01102X/1
European Research Council
Grant ID: 866328

Disclaimer

We highly recommend that you test the protocol with samples that are meristematic and easy to collect to get used to the protocol and familiarise yourselves with how the nuclei look. It is a fast-pace protocol and nuclei don't last very long intact. For example, we recommend samples from a single spike at Terminal Spikelet stage which should yield sufficient nuclei to test protocol. Avoid using precious samples before optimising protocol in your local lab conditions.

Abstract

This protocol describes how to perform nuclei isolation from wheat meristem tissues (spikes and carpels/ovaries), specifically from flash-frozen tissues. With this protocol, we obtain intact and clean nuclei for downstream applications.

Materials

To collect tissue:

- Ophthalmic surgery knife (see https://www.protocols.io/view/wheat-spike-meristem-micro-dissection-3byl49r2zgo5/v1)
- Stereo microscope (e.g. Leica S9)
- Gloves
- Scissors
- Metallic beads, previously cleaned with 0.5 M HCl and 70% ethanol respectively
- Dry ice
- Liquid nitrogen
- 2mL Eppendorf tubes (x 1 per rep/sample)
- 70% ethanol and/or RNase BLITZ

To grind and extract nuclei (recommended to run x 2 reps/samples at a time):

- Tissue grinder: QIAGEN TissueLyser II, with the Adapter Set 2 x 24. Alternatively: GenoGrinder
- pluriStrainer Mini (pluriSelect) filters sizes 100 µm, 70 µm, 40 µm **and** 20 µm (1 of each size per rep/sample)
- 2 mL Eppendorf tubes (x 4 per rep/sample)
- 5 mL Eppendorf tubes (x 1 per rep/sample)
- Dry ice
- Liquid nitrogen
- Ice
- Centrifuge (for 5 mL Eppendorf tubes)
- MOPS
- NaCl
- KCl
- EDTA
- EGTA
- KOH
- Tris-HCl (pH 7.5)
- Tris-HCl (pH 8)
- Spermine powder: 2.904 g dissolved in 10 mL of water, and store 2 M aliquots (1 mL) in the freezer.
- Spermidine powder: 0.405 g dissolved in 10 mL of water, and store 200 mM aliquots (1 mL) in the freezer.
- Triton X-100
- β-mercaptoethanol (Toxic)
- MgCl2
- Sucrose

Incomplete Nuclei Purification Buffer (**NPBi**):

	A	B
	MOPS	20 mM
	NaCl	40 mM

	A	B
	KCl	90 mM
	EDTA	2 mM
	EGTA	0.5 mM
	KOH (2 M)	Use to adjust solution to pH 7

Incomplete Nuclei Purification Buffer (NPBi).

Nuclei Purification Buffer (**NPB**):

	A	B
	NPBi	3 mL
	Spermine 0.2 M	3 uL
	Protease inhibitor	1X

Nuclei Purification Buffer needed for 2 samples (2 mL + error)

Lysis buffer:

	A	B
	Tris-HCl pH 7.5	15 mM
	NaCl	20 mM
	KCl	80 mM
	Spermine	0.5 mM
	β-mercaptoethanol	5 mM
	Triton x-100	0.2% (v/v)

Lysis buffer (toxic), final concentrations needed. Use sterilized water.

Sucrose buffer:

	A	B
	EDTA	2 mM
	MgCl2	20 mM
	Sucrose	1 mM
	Tris-HCl pH 8.0	2 mM
	β-mercaptoethanol	15 mM

Sucrose buffer (toxic), final concentrations needed. Use sterilized water.

To visualise and quantify nuclei (optional, but encouraged)**:**

- DAPI (4',6-diamidino-2-phenylindole)
- Bürker haemocytometer and coverslips
- Tin foil
- Fluorescence microscope (e.g. Zeiss AXIO Imager Z2)
- Kimtech wipes (or equivalent)
- 70% ethanol

Safety warnings

β-mercaptoethanol: can be toxic if ingested, and fatal if inhaled or absorbed through the skin. Vapours can irritate the eyes, mucous membranes, and respiratory tract. Symptoms of inhalation exposure may include coughing, sore throat, and/or shortness of breath). Work

Before start

NPBi can be prepared in advance and stored for up to 3 months in the fridge.
It is highly recommended to read the full protocol before doing it.

Tissue collection

1 First, gather all the materials and prepare the necessary buffers described in the Materials section.

2 Next, for each sample, label 2 mL Eppendorf tubes for collection and place two nitrogen-cooled metallic steel beads (previously cleaned with 0.5 M HCl and 70% ethanol, diameter 3 mm) in each tube. For each sample, we will pool a number of meristematic spikes/ovaries/spikelets. Depending on the stage/tissue, we will pool a different amount per sample (Table 1). Note that this is an indicative number for cultivar Chinese Spring. It is important not to use more tissue than indicated, as the filters may become blocked later in the protocol.

	A	B	C
	Waddington scale	Tissue to harvest	How much tissue (number of spikes, ovaries or spikelets) is needed?
	1	Spike	63
	2	Spike	25
	2.5	Spike	10
	3	Spike	6
	3.25	Spike	4
	3.5	Spike	1.5
	4	Spike	1
	5	Spikelets (4 per plant; middle)	4.5
	6	Ovaries or carpels (12 per plant; middle)	25
	7	Ovaries or carpels	20
	7.5	Ovaries or carpels	15
	8	Ovaries or carpels	10
	8.5	Ovaries or carpels	7
	9	Ovaries or carpels	5
	9.5	Ovaries or carpels	4
	10	Ovaries or carpels	4

Table 1. Amount of tissue (approximately) needed for a single nuclei isolation reaction, per sample/rep.

* Waddington scale stages: Waddington-scale.tif 1.7MB

3 Once you have the number of plants that you need ready, prepare a stereo microscope to dissect and harvest the meristematic tissue. Make sure the area and the ophthalmic knives are clean (sterilise with 70% ethanol or RNase blitz, depending on what the nuclei isolation is for).

4 Place the labelled 2 mL Eppendorf tubes with metallic beads on dry ice.

5 Dissect the spikes/spikelets/ovaries/carpels under the microscope and place them in the pre-cooled collection tubes. For help with microdissections, take a look at this protocol: (**dx.doi.org/10.17504/protocols.io.3byl49r2zgo5/v1**).

We usually follow this steps (considering all tubes being in dry ice, labelled, with metallic beads and lids opened):

Clean surfaces and knives -> Dissect spike 1 -> Place spike 1 in sample A --> Clean surfaces and knives -> Dissect spike 2 -> Place spike 2 in sample B -> (...)

We follow these steps during up to 30 minutes, or up to when the required amount based on Table 1 is reached. Then we close all the tubes and put them in liquid nitrogen. Then, if the tubes aren't completed yet, we put them back in dry ice, and repeat the same steps again, until the required amount based on Table 1 is reached.

Note: Alternating which tube you deposit successive spikes/carpels/spikelets into will help to reduce variation between samples. Variation can arise due to the total collection time for a tube, the person dissecting, the time of day (many genes are regulated by the circadian clock), and many other factors!

6 You can continue the protocol on the same day or you can store the samples in -70 °C to -80 °C.

Tissue grinding

7 Next, grind the samples under **cold** conditions. We use the QIAGEN TissueLyser II, with the Adapter Set 2 x 24. Each adapter consists of a top plate, a bottom plate and a tube holder for holding 24 x 2 mL tubes. We pre-chill the 2 x top, 2 x bottom plates with dry ice for ~ 1 minute. We also fill the 2 x tube holders with dry ice, leaving free the spaces you will use for your 2 mL tubes. Once the adapter sets are cold, we quickly remove the dry ice from the 2 x bottom and 2 x top plates (but not from the tube holders), take the tubes from the liquid nitrogen and place them in the tube holders. Close them with the plates, and place them in the TissueLyser II, as

quickly as possible. Grind them for 30 seconds at a frequency of 27.5 Hz (s^{-1}). Put the tubes back in liquid nitrogen once finished.

You can also grind with GenoGrinder, pre-cooling the tube holders with liquid nitrogen.

Note: You can grind the samples in advance and keep them in -70 °C to -80 °C.

Nuclei isolation (we recommended to do two samples at a time with two people)

8 You can prepare in advance (up to 3 months earlier) the "incomplete Nuclei Purification Buffer" (**NPBi**) Stock (1 L) (Bajic et al., 2018).

Once all the reagents are added and well dissolved, and the pH adjusted to 7, filter sterilise with a syringe filter of 0.45 µm, 25 mm diameter. Then, degas under vacuum for 10 min.
NPBi can be stored at 4 °C for up to 3 months.

9 On the day, before starting the protocol, make sure you **pre-chill the centrifuge to 4 °C**.

10 On the day, prepare the buffers (lysis, sucrose and complete Nuclei Purification Buffer (NPB)):

Prepare **lysis buffer** (contains β-mercaptoethanol, toxic: can be toxic if ingested, and fatal if inhaled or absorbed through the skin. Vapours can irritate the eyes, mucous membranes, and respiratory tract. Symptoms of inhalation exposure may include coughing, sore throat, and/or shortness of breath.) Prepare under the hood:

For 2 reps/samples, we make 5 mL (4 mL needed). Make lysis buffer and **keep on ice**.

11 Prepare **sucrose buffer** contains B-mercaptoethanol (toxic), so should be prepared under the hood:

For 2 reps/samples, we make 5 mL (4 mL needed). Make lysis buffer and keep on ice.

Label 2 x 5 mL tubes and place 2 mL of sucrose buffer in each ("sucrose cushion" tubes). **Keep them on ice.**

12 Prepare Nuclei purification buffer (**NPB**) (Final volume needed for 2 reps/samples (1 mL per rep/sample) + error ~ 3 mL).

Make buffer and **keep on ice**.

ALL THE BUFFERS NEED TO BE PRE-CHILLED.

13 Prepare 2 mL Eppendorf tubes with 100 µm filter and 70 µm filter (Figure 1).

Figure 1. 2 mL Eppendorf tubes with 100 µm filter (brown, left) and 70 µm filter (white, right).

14 Prepare cut-wide tips (1 per rep/sample): cut 0.5 cm of 1,000 µL tips.

15 Make sure you have all the components ready to start:

- Ground tissue reps/samples in liquid nitrogen
- Centrifuge pre-chilled to 4 °C, with the right tube holders: 5 mL tubes
- Buffers (lysis, NPB) pre-chilled on ice
- "Sucrose cushion" tubes (5 mL size) pre-chilled on ice
- 100 µm filters in 2 mL tubes on ice
- 70 µm filters in 2 mL tubes on ice
- Cut-wide 1,000 µL tips
- 5 mL pipettes
- Timer (20 secs)

Bear in mind, the next steps need to be done very quickly, but also gently. Stick to the timing, otherwise you will get fewer intact nuclei and more debris. Too much time or too much tissue results in lower quality. We recommend you memorise the steps and practise them beforehand. We also recommend that you do this between two people as each can do one sample at the same time. If only one person, we would recommend processing only one sample, and using a dummy sample to balance the centrifuge.

16 1) Take samples out of liquid nitrogen. Place them in a rack and open lids. Wait ~ 20 secs to add the lysis buffer (if you add it too fast, it will instantly freeze. Also, if there is too much tissue, it will more likely freeze). Try to add the **lysis buffer** at the exact same time in both samples (this is why having two people helps). Once added, put timer on for 20 s.

2) In those **20 s**, gently invert the tube a couple of times, carefully. When the timer is reaching 0, hold the Eppendorf tube with the **100 µm** filter with your other hand. You will pour the liquid onto the filter, however the volume is 2 mL, so it is important that you hold the filter slightly higher than it would naturally do. Once the 20 s are over (and no later!), pour the sample onto the filter. **If the filter blocks, don't force it.** If you force it, you will get broken nuclei and debris. If you don't force it, you might lose yield but the nuclei will more likely be intact. (Most times the filter blocks due to having too much tissue, you can try with less if it blocks too much).

3) Right after filtering with the 100 µm filter, remove the 100 um filter and do the exact same with the **70 µm** filter (which should be in another 2 mL Eppendorf tube). Again, if the filter blocks, don't force it.

4) Remove the filter and pipette the 2 mL carefully with the cut-wide tip on top of the 2 mL **sucrose cushions**. Pipette it very gently, on the wall of the tube, so it falls into the cushion.

5) Centrifuge at 4 °C (2,200 g) for 20 minutes. 2200 x g, 4°C

17 While the centrifuge runs, we prepare:
- the foil-covered DAPI tubes (to observe the nuclei under a fluorescence microscope): 0.5 µL 1 X DAPI per sample. Cover them in foil (DAPI is light-sensitive)
- Fluorescence microscope: Prepare ZEISS image software to take pictures under bright field and DAPI channels combined.
- Haemocytometer, cover slips
- Kimtech wipes and 70% ethanol

- Prepare 2 mL Eppendorf tubes with 40 µm filter and 20 µm filter, keep on ice (Figure 2)

Figure 2. 2 mL Eppendorf tubes with 40 µm filter (blue, left) and 20 µm filter (green, right).

18 Once the centrifuge finishes, pick up the tubes carefully. Remove supernatant (the nuclei pellet is not visible, so just remove the supernatant slowly without touching the bottom of the tube).

19 Resuspend pellet in 1 mL of pre-chilled NPB by very gentle pipetting.

20 Pipette carefully and very slowly the 1 mL NPB with re-suspended nuclei into the filter walls of the 40 µm filter that is in a 2 mL Eppendorf tube (kept on ice). The filter will most likely block. Wait for 1 minute to see if it goes down by itself. If it doesn't, hit the filter ONCE very gently to help it go through. If it doesn't, wait 1 minute again. If after that 1 minute it is still blocked, don't do anything, just keep going with the volume that has gone trough.

If you force it, you will get broken nuclei and debris. If you don't force it, you might lose yield but the nuclei will be more likely intact. (Most times the filter blocks due to having too much tissue, you can try with less if it blocks too much).

21 Repeat the exact same as step **22** with the 20 µm filter.

The final volume will normally be ~ 0.9 - 1 mL, but it can also be lower if you have lost some sample because of filters blocking. If you want to calculate the amount of nuclei, you should record now how much approximate volume you have.

If you want to calculate the total number of nuclei, which is optional: Only 9.5 µL of each rep/sample will be used to quantify/visualise, keep the rest of the sample on **ice** as intact nuclei are more stable at cold temperatures.

Visualise and quantify nuclei (optional)

22 Gently invert each sample to make sure that nuclei are well distributed within the sample. Then, add 9.5 µL of each rep/sample to the already prepared foil-covered tubes with 0.5 µL 1X DAPI. Mix by gentle pipetting.

23 Place the slide coverslip on top of both chambers of the Bürker hemocytometer. Load 10 µl of your DAPI stained sample into one of the counting chambers with a 10 µl pipette. Capillary effect ensures even distribution of the suspension within the chamber. Place the haemocytometer under the microscope. The haemocytometer has 9 x 1 mm² squares. We often count DAPI stained (blue) intact (more or less round, with no membrane ruptures) nuclei in 3 random squares (out of 9), and calculate the total.

Example:

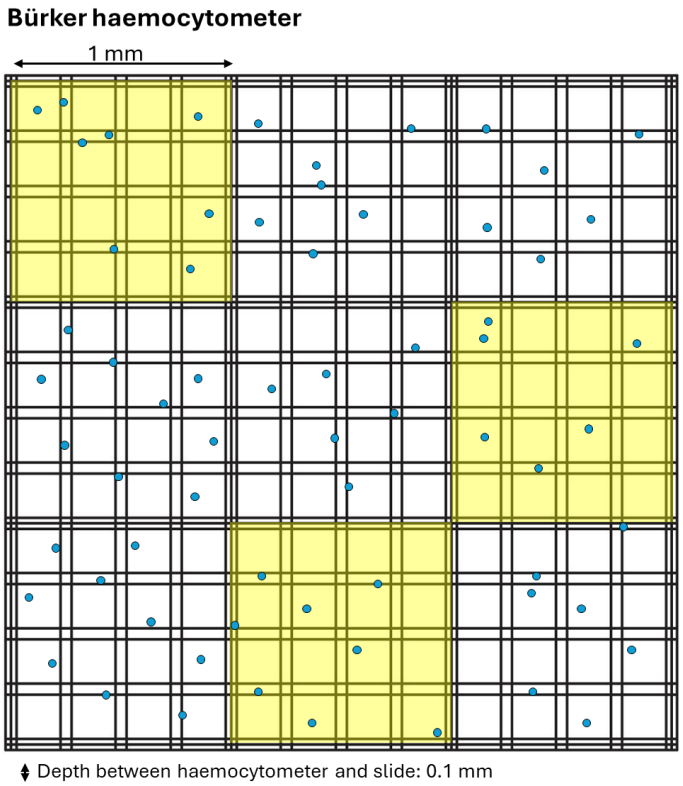


Figure 3. Bürker hemocytometer representation. The yellow squares represent three random squares chosen to count nuclei out of the nine. The blue circles represent the intact DAPI stained nuclei.

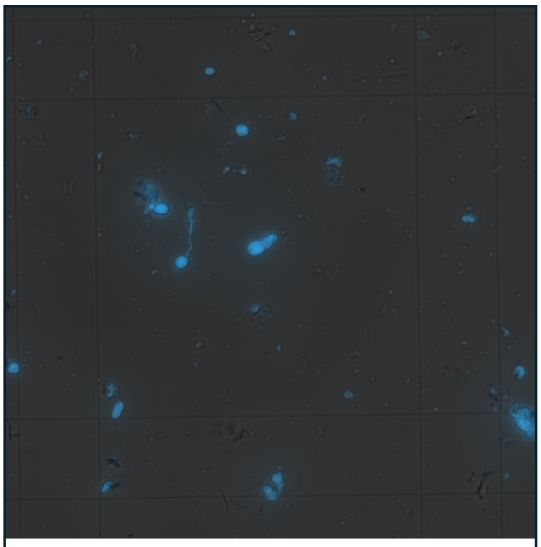
This is how we would then calculate the nuclei in this example:

1. Considering the volume of whole haemocyometer square: (9 mm2 x 0.1 mm depth = 0.9 mm3 = 0.0009 mL)
2. Volume of our sample is 0.9 mL. (Number here depends on the volume you observed in step 21)
3. Count (intact DAPI stained) nuclei in 3 yellow squares: 8 + 6 + 8 = 22 nuclei
4. Calculate nuclei in whole haemocytometer square. We counted 3/9 squares, so need to multiply by 3 to get an estimate for the whole haemocytometer chamber: 22 x 3 = 66 nuclei
5. Calculate total nuclei in 0.9 mL of sample:

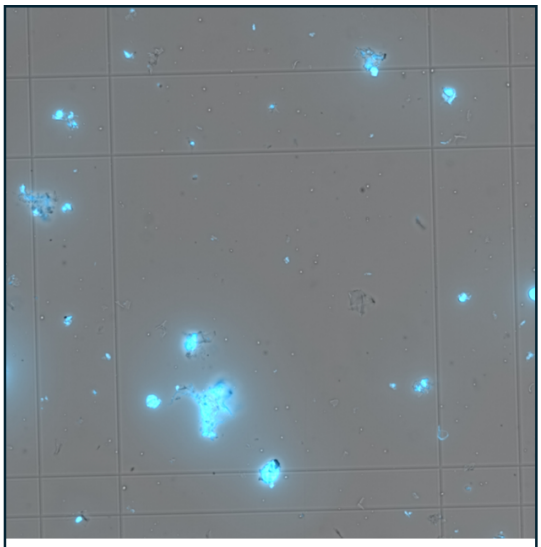
0.9 mL total / 0.0009 mL haemocytometer square = 1,000
66 nuclei in haemocytometer x 1,000 = **66,000 nuclei in total**

Note: To count nuclei, we often look closely into each of the yellow squares: Each of these contain 16 mini-squares. We take pictures in bright field and DAPI combined of each of the 16 mini-squares, to make sure we identify well the intact nuclei. The ***shape is well captured with bright field*** *(rounded and intact), and the* ***DAPI in the nuclei will be stronger than in other tissues****. This is why is important to adjust well the microscope beforehand.*

Examples of real samples that can be improved (Figure 4):



X Broken nuclei
X Debris
X Brightfield channel too low: hard to see edges of haemocytometer



X Debris
X DAPI channel too high in visualization: does not help identify nuclei

Troubleshooting:
Try using less tissue to avoid filters blocking, and be gentler (don't force filters) throughout protocol

Figure 4. DAPI stained nuclei visualised under fluorescence microscope.

This is how a better sample (clean with intact nuclei) might look (Figure 5):

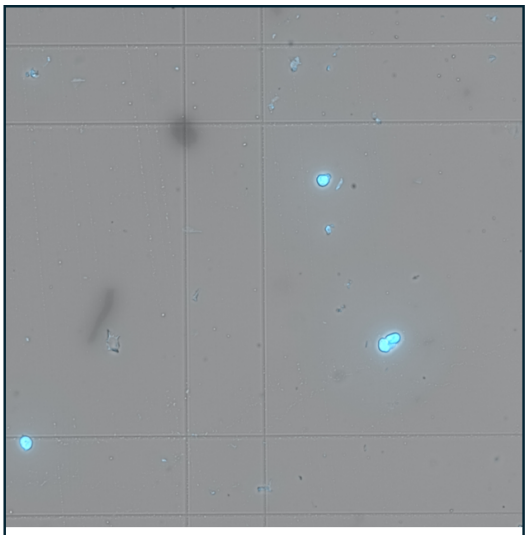


Figure 5. DAPI stained nuclei visualised under fluorescence microscope.

To visualise the next sample, clean with Kimtech wipes and ethanol, and load your next stained sample.

Note: The nuclei will start bursting after roughly 15 minutes, especially if not kept on ice and if physical impact applied. We recommend you use them immediately after this protocol. This is also why we recommend doing few samples at a time, to be able to do all the steps faster and more gently.

Protocol references

Bajic, M., Maher, K. A., & Deal, R. B. (2018). Identification of open chromatin regions in plant genomes using ATAC-Seq. *Plant chromatin dynamics: Methods and protocols*, 183-201.