Identification and validation of maize enhancers
A cartography of the maize regulatory genome
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Chapter 5

3C in Maize and Arabidopsis

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Abstract

Chromosome Conformation Capture (3C) allows studying the relative frequency of interaction of one chromosomal fragment with another. The technique is especially suited for unravelling the 3D organization of specific loci when focusing on aspects such as enhancer-promoter interactions or other topological conformations of the genome. 3C has been extensively used in animal systems, among others providing insight into gene regulation by distant cis-regulatory elements. In recent years, the 3C technique has been applied in plant research. However, the complexity of plant tissues prevents direct application of existing protocols from animals. Here we describe an adapted protocol suitable for plant tissues, especially Arabidopsis thaliana and Zea mays.
1. Introduction

Since the first microscopic observation of nuclei, it has become clear that chromatin is not randomly organized [1]. A specific 3D architecture of the genome is established in each and every cell’s nucleus to ensure proper regulation of gene expression [2, 3]. Such architecture includes large-scale chromatin domains as well as specific enhancer-promoter interactions that together shape the cell’s transcriptome and its fate [4, 5]. Chromosome Conformation Capture (3C) allows to study chromatin organization and helps to understand how the spatiotemporal organization of chromatin influences gene expression [6]. 3C technology was first implemented in yeast [7] and rapidly adapted to other organisms including mice, human and Drosophila [8–10]. The 3C technique provides the attractive opportunity to study chromosomal interactions at a resolution that was previously difficult to achieve with cytological methods [6]. The method relies on the proximity ligation concept (Figure 1). In this method, chromatin from fixed nuclei is first subjected to digestion with a methylation insensitive restriction enzyme, followed by ligation in conditions favoring intramolecular ligations. Hereby, regions that are in close contact in 3D have a higher chance of being ligated together. Subsequently, interaction frequencies of specific ligation events are quantified, providing insight into the 3D organization of chromatin at the genomic locus of interest. Frequencies of interaction are measured using quantitative PCR (qPCR) in combination with primers specific for each interaction one desires to examine. The use of primers recognizing specific fragments makes 3C an hypothesis-driven approach.

Since the first publication in 2002, variants of the 3C method have been developed (e.g. 4C, 5C and Hi-C, see Chapter 29) that allow a more systematic analysis of chromosomal interactions [11–14]. These methods identify many more interactions at the same time (one to all, many to many, all to all) by including deep sequencing techniques. Hence they are associated with higher costs, and given the complexity and amount of data generated, also time consuming data analysis pipelines. Therefore, 3C remains a method of choice when special focus is given to one specific locus. It allows faster results and often offers better resolution.

The specific nature of plant cells hampers the direct application of published 3C protocols from other species. Therefore, 3C on plant tissues requires plant specific steps. In literature, 3C protocols have been described for Arabidopsis and Maize [15, 16]. In this article, we provide a step-by-step bench protocol, starting from the design and setup of a 3C experiment, up to the analysis and interpretation of the 3C data. In addition, we provide critical notes on different aspects that need to be adapted when applying this method to other plants or tissues of interest.
Fig. 1 Graphical overview of the 3C technique. (A) Schematic representation of a potential locus of interest. Primers (black arrows) are designed on one and the same strand for all fragments monitored. The bait is indicated in blue. (B) Schematic representation of an hypothetical chromosome conformation at the potential locus of interest. (C) Fixed chromatin is digested, ligated in a large volume and then de-crosslinked. The products of these processes are shown. (D) Schematic representation of a 3C template. Black vertical bar indicates the ligated restriction site (RS). Primers (black arrows) anneal on each side of the RS and now form a primer pair. A TaqMan probe can be used for more specific quantification by qPCR (see Note 15) and should be designed on the bait fragment, on the strand complementary to the strand on which the bait primer is designed. (E) Graphical representation of a 3C plot. The relative frequency of interaction of each fragment with the bait is plotted on the y-axis (black and red circles). The distances to the middle of the bait fragment are reported on the x-axis. Vertical boxes are used to indicate the different fragments monitored.

2. Materials

Prepare all solutions using autoclaved milliQ water and analytical grade reagents. Sterilization by autoclaving is performed at 121 °C for 10 min unless indicated otherwise. Diligently follow all waste disposal regulations when disposing of waste materials.
2.1 Tissue Fixation and nuclei isolation

1. 1 X Phosphate Buffered Saline (PBS): add 800 mL of milliQ water into a graduated cylinder together with a magnetic stirrer. Weigh 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na₂HPO₄, 0.24 g of KH₂PO₄ and add it to the measuring column. Adjust the pH to 7.4 with HCl and add milliQ water up to 1 L. Autoclave the solution and store at room temperature (RT).

2. 4% Paraformaldehyde (PFA) in PBS: Prepare this solution in the fume hood. Pour 100 mL of autoclaved 1 X PBS in a 250 mL glass bottle. Weigh 4 g of powdered paraformaldehyde and transfer it to the PBS-filled bottle. Adjust the pH to 9 with KOH, close the bottle and transfer it to a 65 °C water bath. Shake the bottle from time to time until the PFA is completely dissolved. Transfer it back to the fume hood and allow the bottle to cool down before adjusting the pH back to 7-7.5 with HCl. Prepare aliquots of 10 mL to be stored at -80 °C.

3. 2 M Glycine: Add 80 mL of milliQ water to a glass graduated cylinder or beaker, together with a magnetic stirrer. Weigh 15 g of Glycine and add it into the graduated cylinder or beaker. Dissolution can be enhanced by raising the temperature. After complete homogenization, adjust the volume to 100 mL with milliQ water and transfer the solution to a 100 mL glass bottle. Autoclave and store at 4 °C.

4. 20 % Triton X-100: pipette twice 1 mL of Triton X-100 with a cut pipette tip and add to a 15 mL tube containing 8 mL of autoclaved milliQ water. Allow all Triton to get out of the tip by pipetting up and down. Avoid foaming as much as possible. Shield the tube from light with opaque tape or aluminium foil. Place the tube in a rotating wheel at RT overnight to allow complete homogenization. 20 % Triton X-100 can be stored shielded from light at RT for a month.

5. 100 mM Phenylmethylsulfonyl fluoride (PMSF): weigh 174 mg of PMSF and dissolve it in 10 mL of isopropanol. Prepare aliquots of 100 µL and store at -20 °C.

6. Complete Protease inhibitor (Roche): dissolve 1 complete protease inhibitor tablet in 2 mL of autoclaved milliQ water by vortexing vigorously. Dissolved tablets can be stored for 1-2 weeks at 4 °C, or up to 6 weeks at -20 °C.

7. Nuclei Extraction Buffer (100 mL, prepare fresh): 2 mL 1M Hepes pH 8, 25 mL 1M Sucrose, 0.1 mL 1M MgCl₂, 0.5 mL 1M KCl, 46 mL 87% Glycerol, 1.25 mL 20% Triton X-100, 26 mL autoclaved milliQ, 100 µl 100 mM PMSF, 100 µl Beta-Mercaptoethanol.

8. 50 mL tubes.
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9. Miracloth.
10. Sefar Nitek nylon filter 50 µm pore size.
11. Mortar and pestle.
12. Liquid Nitrogen.
13. Cooling centrifuge for 50 mL tubes.

2.2 Digestion and ligation of 3C and control samples

1. BAC-clone or other large plasmid that contains the region of interest (see step 3.2.1)
2. Suitable restriction enzyme (see Table 1 and section 3.1) with 10x restriction buffer.
3. Phenol:Chloroform:Isoamyl alcohol (25:24:1 v/v): under the fume hood, pipette 25 mL of Phenol equilibrated with 10 mM Tris-HCl, 1mM EDTA, pH 8 and transfer it to a 50 mL glass bottle. Add 24 mL of Chloroform and 1 mL of Isoamyl alcohol. Close the lid and mix. Allow the phases to separate, protect the bottle from light and store at 4 °C.
4. Chloroform:Isoamyl alcohol (24:1 v/v): under the fume hood, pipette 24 mL of Chloroform and transfer it to a 50 mL glass bottle. Add 1 mL of Isoamyl alcohol. Close the lid and mix. Protect the bottle from light and store at 4 °C.
5. 2 M Sodium Acetate (NaOAc) pH 5.6: Add 80 mL of milliQ water to a graduated cylinder. Weigh 16.4 g of Anhydrous Sodium Acetate and dissolve it in the 80 mL of milliQ water. Adjust pH to 5.6 with HCl and bring volume up to 100 mL with milliQ water. Autoclave and store at RT.
6. Glycogen 20 mg/mL
7. 96% and 70% Ethanol
8. 10 mM Tris pH 7.5
9. 1 M Tris-HCl pH 7.8: add 800 mL of milliQ water into a graduated cylinder, together with a magnetic stirrer. Weight 157.6 g of Tris and gradually add it to the column while stirring. Set the pH to 7.8 with HCl and adjust the volume to 1 L with milliQ water. Autoclave solution and store at RT.
10. 1 M DL-Dithiothreitol (DTT): dissolve 1.53 g of DTT powder into 10 mL of autoclaved milliQ water. Prepare aliquots of 100 µL and store at -20 °C.
11. 10x Ligase buffer for 3C samples (1 mL, prepare freshly): 300 µL 1M Tris-HCl pH 7.8, 100 µL 1M MgCl₂, 100 µL 1M DTT, 6 mg ATP (final concentration 10 mM), 500 µL autoclaved milliQ water.
12. Highly concentrated T4 DNA ligase
13. 20 % Sodium dodecyl sulfate (SDS): open SDS container under the fume hood and weigh 10 g of SDS. Still under the fume hood, transfer SDS to a 50 mL glass bottle filled with 35 mL of autoclaved milliQ water. Ensure complete homogenization and bring volume to 50 mL with autoclaved milliQ water. Store bottle at RT.
14. Gel running device and UV transilluminator.
15. Water bath
16. Heat block
17. Cooling microcentrifuge
18. Cooling centrifuge for 50 mL tubes.
19. 50 mL tubes

2.3 De-crosslinking, DNA purification and qPCR analysis
1. 10 mg/mL Proteinase K: weight 100 mg of proteinase K and dissolve in 10 mL of autoclaved milliQ water. Prepare aliquots of 100 µL and store at -20 °C.
2. 10 mg/mL RNase A: for a final volume of 1 mL, add 900 µL of 10mM NaOAc pH5.6 to a 1.5 mL eppendorf. Add 10 mg of RNase A. Mix until complete dissolved and place the tube into boiling water for 15 min. Allow solution to cool down at RT and add 100 µL of Tris-HCl pH 7.4. Store at -20°C.
4. Chloroform:Isoamyl alcohol (24:1 v/v), see step 5 of Subheading 2.2
5. Agarose.
6. Gel running device and UV transilluminator.
7. 10 µM primers for the ligation products to be tested (see step 3 of Subheading 3.1).
8. 10 mM dNTPs.
10. Reagents for real-time PCR analysis (e.g. SYBR Green Mastermix)

3. Methods

3.1 Study design
1. Define your region of interest: 3C can be performed at any specific locus or gene of interest for which the DNA sequence is known. In general, one should select a restriction enzyme (RE) that will generate a restriction pattern compatible with intramolecular
ligation (fragments smaller than 300 bp are more difficult to ligate) and allows the verification of hypothesized interactions (regions of interest should be located in fragments >300 bp). Define a “bait” or “viewpoint” (see Figure 1) by selecting the fragment for which you will quantify the frequency of interactions with other fragments.

2. **Selection of the Restriction Enzyme:** REs used in the 3C procedure need to efficiently digest crosslinked chromatin, which is challenging. Different REs or combinations of REs that allow digestion of fixed chromatin have been reported in published 3C protocols and studies. Using such REs (see Table 1) is a safe choice. However, if the restriction patterns generated by these enzymes are not compatible with one’s hypothesis at the locus of interest, newly selected enzymes should fulfill a number of requirements. First, the selected RE should be methylation insensitive, since methylation sensitive enzymes may result in partial digestion and thus introduce a bias. Second, the selected RE should ideally display optimal efficiency for digesting fixed chromatin at 37 °C, and preferably maintain its activity over a long period of time (e.g. overnight). In case of a short survival time, aliquots of restriction enzymes can be added sequentially during the digestion time. Thirdly, high ligation efficiency is crucial for a successful 3C experiment. Thus, favor enzymes generating sticky ends (the larger the overhang, the better), and ensure that the re-ligation efficiency is high without the need of adding any macromolecular crowding agent such as polyethylene glycol (PEG).

Once the digestion is complete, to stop digestion the RE needs to be inactivated by elevated temperatures. In addition, for some enzymes detergents need to be added for inactivation (see Table 1). Note that the addition of SDS is associated with negative effects on ligation efficiency.

**Table 1: List of REs regularly used in 3C experiments.**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Heat Inactivation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Hind</em>III</td>
<td>Yes</td>
<td>[17, 18]</td>
</tr>
<tr>
<td><em>Eco</em>RI</td>
<td>Yes</td>
<td>[7, 18]</td>
</tr>
<tr>
<td><em>Bgl</em>II</td>
<td>No</td>
<td>[18–20]</td>
</tr>
<tr>
<td><em>Bam</em>HI</td>
<td>No</td>
<td>[18, 20, 21]</td>
</tr>
<tr>
<td><em>Dpn</em>II</td>
<td>Yes</td>
<td>[22, 23]</td>
</tr>
<tr>
<td><em>Mfe</em>I</td>
<td>No</td>
<td>[24]</td>
</tr>
<tr>
<td><strong>NlaIII</strong></td>
<td>Yes</td>
<td>[25]</td>
</tr>
<tr>
<td>------------</td>
<td>-----</td>
<td>------</td>
</tr>
<tr>
<td><strong>XhoI</strong></td>
<td>Yes</td>
<td>[18]</td>
</tr>
</tbody>
</table>

Column “Heat Inactivation” indicates if an enzyme can be heat-inactivated without addition of SDS.

3. **Type of tissue and number of nuclei:** chromosome conformations are mostly cell type-dependent [8, 19, 26]. Therefore, ideally, for 3C one uses fresh (see Note 1), homogeneous and synchronized cell populations. Plant tissues have an heterogeneous cell type composition, and their cells are not synchronized. The heterogeneity of plant tissues does not preclude the use of 3C technology, however, one should keep in mind that the results obtained will reflect an average of the chromosomal interactions occurring in different and unsynchronized cell types. Combining the 3C procedure with methods that allow the isolation of specific cell types (e.g. FACS or INTACT) could, although technically very challenging, allow studying cell type-specific interactions [27–29].

The type of plant tissues used for 3C analysis is dependent on the research question. Theoretically, most tissues are compatible with 3C analysis, however, tissues that are highly lignified or have a high starch content pose difficulties at grinding and/or downstream process. In addition, the amount of starting material needs to be sufficient to allow isolating 1 to 10 million nuclei (to assess the number of nuclei see Note 2). Tissues that are difficult to harvest in bulk, such as meristems, are therefore challenging.

4. **Primer Design:** Specific primers need to be designed for all DNA fragments one wishes to study. 3C primers need to be orientated uni-directionally, meaning that all primers are designed complementary to the same strand. This way, only ligation events between two different fragments will result in amplicon formation (see Fig. 1). Primers are usually designed 40-150 bp away from the restriction sites. Furthermore, they are preferably 18-27 bp long with a GC content of about 50 %, a Tm between 57-63 °C and no more than 2 °C difference in Tm between them. Primers need to be very specific, which should be determined using BLAST (High Throughput Genome Sequence database). Select primers only if one perfect match is found; homology to sequences elsewhere in the genome should be less than 75 % of the primer length and exclude the 3’ end of the primer. Primers should be tested experimentally on control template (Random Ligation Library; see section 3.2.1) and genomic DNA to ensure that only the correct amplicon size is amplified when using the Random Ligation Library.
3.2 Controls in the 3C procedure

The correct interpretation of 3C results demands the use of a number of controls. For instance, controls are used to ensure an optimal digestion efficiency (a non-digested 3C sample and digested gDNA), correct for primer efficiency (Random Ligation Library), and also account for technical and biological variation between 3C libraries (endogenous locus).

3.2.1 Random Ligation Library (RL-Library)

To determine the relative frequency of interaction of one fragment with another, one needs to ensure that no quantification bias arises due to primer pairs with different amplification efficiencies. Therefore, to normalize for the primer efficiency, a random ligation library (RL-Library) is prepared that consists of all possible ligation products that need to be analyzed by qPCR (see 3.7). The RL-Library can be used as a template to first test primer efficiency and specificity. An RL-Library can be generated in different ways. One option is by digesting a bacterial artificial chromosome (BAC) or other large plasmid that contains the locus of interest, followed by re-igation. The digestion of the BAC is performed with the same RE selected to generate the 3C library but in a smaller volume compared to the ligation step for the 3C library. This allows all fragments to randomly ligate with one another. Alternative to using a BAC-derived RL-Library in the qPCR experiments, one can PCR amplify all potential ligation products from a 3C library, and mix those in equimolar amounts. Finally, note that a different RL-Library has to be prepared for every locus one examines (locus of interest and the endogenous locus).

1. Prepare a 1.5 mL eppendorf tube with 10 µg of template (e.g. BAC) in a final volume of 100 µL with a two-fold excess of restriction enzyme (e.g. 1µl of 20,000 units/mL HindIII) and its recommended restriction buffer at a final concentration of 1x.
2. Incubate for 5 hours at 37 °C.
3. Check the digestion efficiency by running 10 µL of the digestion mixture on a 0.8 % agarose gel. Satellite bands should be visible.
4. Add 210 µL of sterile milliQ to bring the volume up to 300 µL.
6. Centrifuge at full speed for 10 min at RT.
7. Transfer the top aqueous phase to a new 1.5 mL eppendorf tube and add 1 volume of Chloroform:Isoamyl Alcohol (24:1). Mix thoroughly.
8. Repeat the centrifugation step at full speed for 10 min at RT.
9. Transfer the top (aqueous) phase to a new 1.5 mL eppendorf tube and precipitate the DNA with 1/10 volume of 2 M NaOAc (pH 5.6), 2 volumes of 96 % Ethanol and 10 µL of Glycogen (20 mg/ml).

10. Store the tube at -80 °C for 2 hours to overnight.
11. Centrifuge at full speed for 30 min at 4 °C.
12. Gently pour off the supernatant and wash the pellet with 1 mL of 70 % ethanol.
13. Centrifuge at full speed for 10 min at 4 °C.
14. Gently pour off the supernatant and use a pipette tip to remove the remaining droplets.
15. Let the pellet air dry for 2-5 min and resuspend the DNA in 22 µL of 10 mM Tris pH 7.5.

16. Transfer 20 µL of the digested DNA to a new eppendorf tube. Add 5 µL of fresh 10x ligation buffer, sterile milliQ water and 20 Units of T4 DNA ligase up to a final volume of 50 µL (see Note 3). Store the remaining 2 µL of digested BAC DNA at -20 °C.

17. Incubate the ligation reaction overnight at 16 °C.
18. Bring the volume to 300 µL by addition of 250 µL of sterile milliQ water.
20. Centrifuge at full speed for 10 min at RT.
21. Transfer the top phase to a new 1.5 mL eppendorf tube and add 1 volume of Chloroform:Isoamyl Alcohol (24:1). Mix thoroughly.
22. Centrifuge at full speed for 10 min at RT.

23. Transfer the top phase to a new 1.5 mL eppendorf tube and precipitate the DNA with 1/10 volume of 2 M NaOAc (pH 5.6), 2 volumes of 96 % Ethanol and 10 µL of Glycogen (20 mg/ml).

24. Store the tube at -80 °C for 2 hours to overnight.
25. Centrifuge at full speed for 30 min at 4 °C.
26. Gently pour off the supernatant and wash the pellet with 1 mL of 70 % ethanol.
27. Centrifuge at full speed for 10 min at 4 °C.
28. Gently pour off the supernatant and use a pipette tip to remove the remaining droplets.
29. Let the pellet air dry for 2-5 min and resuspend the DNA in 22 µL of 10 mM Tris pH 7.5.

30. Prepare a 0.8 % Agarose gel and mix both the 2 µL of the RL-Library, and the 2 µL of digested 3C sample (3.6.1 step 16) with 7 µL of sterile milliQ and 1 µL of 10x loading buffer.
31. Store the RL-Library at -20 °C.
32. Run the digested and ligated samples on the prepared 0.8 % agarose gel to check the ligation efficiency.
33. Prepare 1/10, 1/100, 1/1000 and 1/10,000 dilutions of the RL-Library in sterile milliQ, and add non-digested gDNA to each dilution (final concentration of 50 ng gDNA/µL). Addition of non-digested gDNA mimics the PCR conditions with the 3C library as a template. Measure the Ct values for the different primer pairs of interest using qPCR. For each 20 µL PCR reaction prepare a qPCR mix according to your own setup. Use 1 µL of template (concentration depends on the dilution) and 1 µL of each primer (10 µM). When analyzing the 3C library for the first time with qPCR, use the different dilution series of your RL-Library complemented with gDNA. Subsequently, for normalization use the dilution showing Ct values in the range of the Ct values obtained for the 3C library. In later qPCR analyses, one can use only the relevant dilution(s) of the RL-Library.

3.2.2 Endogenous Control

Another crucial 3C control, an endogenous control locus, accounts for technical and biological variation between samples. Technical variation hereby refers to differences in quantity and quality of the sample, biological variation refers to differences in interaction frequencies at the locus of interest between different tissues. To this end, an endogenous locus is chosen that shows similar RNA expression levels across the different tissues examined by 3C. A similar RNA expression level indicates a similar chromatin conformation. Typically, genic loci referred to as housekeeping genes are known to be similarly expressed and can be assumed to show similar chromatin conformation in different tissues. The frequency of interactions at such loci can therefore be used to normalize the data between biological samples.

1. Identify a proper endogenous control locus (e.g. SAM (GRMZM2G154397) in Z. mays or TIP41 (AT4G34270.1) in A. thaliana). Do this by checking if RNA transcript levels are similar in the tissues of interest.
2. Design primers complementary to multiple restriction fragments at the endogenous control locus and its flanking sequences.
3. Using qPCR and your 3C library as a template, measure the frequencies of interaction between the selected bait fragment and the other fragments of the endogenous locus. Usually the bait fragment consists of the fragment that contains the TSS of the selected
endogenous locus. Select a primer pair displaying a frequency of interaction comparable to the mean of your frequency of interactions at the studied locus and take this primer pair along at subsequent 3C experiments for normalization.

3.2.3 Positive Digestion Control
The positive digestion control is used as a reference to determine the efficiency of digestion. The positive digestion control consists of fully digested gDNA. In this sample, the pattern of digestion (size range of the smear and satellite bands) should be clearly visible.

1. Prepare a 1.5 mL eppendorf tube with 10 µg of gDNA template in a final volume of 100 µL with a two-fold excess of restriction enzyme and its recommended buffer at a final concentration of 1x.
2. Incubate overnight at 37 °C.
3. Check the digestion efficiency by running 10 µL of the digestion mixture on a 0.8 % agarose gel. Satellite bands should be visible. If the pattern of digestion is not clearly visible, extend the incubation time.
4. Add 200 µL of sterile milliQ to bring the volume up to 300 µL.
6. Centrifuge at full speed for 10 min at RT.
7. Transfer the top aqueous phase to a new 1.5 mL eppendorf tube and add 1 volume of Chloroform:Isoamyl Alcohol (24:1). Mix thoroughly.
8. Repeat the centrifugation step at full speed for 10 min at RT.
9. Transfer the top (aqueous) phase to a new 1.5 mL eppendorf tube and precipitate the DNA with 1/10 volume of 2 M NaOAc (pH 5.6), 2 volumes of 96 % Ethanol and 10 µL of Glycogen (20 mg/ml).
10. Store the tube at -80 °C for 2 hours to overnight.
11. Centrifuge at full speed for 30 min at 4 °C.
12. Gently pour off the supernatant and wash the pellet with 1 mL of 70 % ethanol.
13. Centrifuge at full speed for 10 min at 4 °C.
14. Gently pour off the supernatant and use a pipette tip to remove the remaining droplets.
15. Let the pellet air dry for 2-5 min and resuspend the DNA in 50 µL of 10 mM Tris pH 7.5.
16. Store the tube at -20 °C.
17. Load 5 µL of the positive digestion control on a 0.8% agarose gel when checking the digestion efficiency of the 3C library (see 3.6.16).

### 3.3 Plant tissue fixation and nuclei isolation

The following procedure describes the handling of one biological sample. Multiple samples can be handled at the same time. In our hands, working with more than four samples at once is cumbersome and might result in suboptimal 3C library quality.

1. Prepare Nuclei Extraction Buffer (NEB) and place on ice. Also, pre-cool the centrifuge for 50 ml tubes (swing-out) and eppendorf centrifuge to 4 °C.
2. Fill a 50 mL centrifuge tube (preferably PPCO tubes – Poly Propylene COpolymer) and fill it with 10 mL of 1x PBS. Place the tube on ice.

![Fig. 2 Preparation of a “tea bag” from Miracloth. One black square is 4 by 4 cm. (A) Cut a 12 by 12 cm piece of Miracloth. (B) Place the plant tissue at the center of the piece of Miracloth. (C) Fold by joining all corners and staple them together.](image)

3. Harvest tissue of interest (1 to 3 g; see Note 2) and place it in a large petri-dish on ice. If necessary, cut tissue into pieces with a sharp scalpel to improve penetration of the fixative (see Note 4). Place the tissue on top of a 12x12 cm piece of Miracloth (see Note 5). Enclose the tissue into the Miracloth by folding and stapling the corners, generating a “tea bag” (see Figure 2). Completely submerge the bag into the PBS solution. [Figure 2 here]

4. Place the tube under the fume hood. Add 10 mL of 4 % PFA.
5. Vacuum infiltrate the tissue at RT (to determine the time of fixation, see Note 4). During fixation turn vacuum OFF and ON again 3 times to ensure good penetration of the fixative.
6. Stop fixation by adding 1.25 mL of ice-cold 2 M Glycine (final concentration is 0.125 M) and vacuum infiltrate for 5 min.

7. Place the tube back on ice under the fume hood and discard PFA solution. Add autoclaved milliQ water to the tube, close the lid and shake vigorously to wash the tissue. Repeat this step twice. Discard PFA solution and milliQ water used for the washes according to your waste disposal regulations for fixative.

8. Place the tea bag in between two stacks of paper towels and press to dry the tissue. Repeat this process with new paper towels until the tissue is sufficiently dry (see Note 6).

9. Open the tea bag and place the tissue into a pre-chilled mortar containing liquid nitrogen. Grind the tissue into a fine powder. Avoid thawing the ground material.

10. Add sufficient NEB to submerge all the ground material (usually 10 to 20 mL). The NEB may freeze upon addition into the frozen mortar. Wait for it to thaw and mix from time to time. Avoid the suspension to warm up higher than 4 °C.

11. Place a new 50 mL tube on ice with a funnel on top.

12. Prepare a 12 by 12 cm piece of Sefar Nitek nylon filter (50 µm pore size) and of Miracloth. First place the Sefar Nitek filter in the funnel and then cover it with the piece of Miracloth, resulting in a two-layer filter.

13. Pipette the tissue suspension (from step 10) onto the two-layer filter and allow it to flow through by gravity. Rinse the mortar with an additional 5 to 10 mL of NEB and pipette it on top of the filter. Do not compress the filter! Upon squeezing you also obtain undesirable debris. Let gravity do its work. The filtrate contains your nuclei.

14. Centrifuge filtered nuclei at 1900 g for 15 min at 4 °C.

15. Promptly place the tube back on ice and gently pour off the supernatant. Resuspend the pellet in 1 mL of NEB and transfer the nuclei suspension into a 1.5 mL eppendorf tube. At this step the number and quality of extracted nuclei can be determined (see Note 2).

16. Centrifuge at 1900 g for 5 min at 4 °C.

17. Promptly place the eppendorf tube back on ice and gently pipette off the supernatant. Resuspend the pellet in 1 mL of NEB and repeat the centrifugation step (1900 g for 5 min at 4 °C).

18. Promptly place the tube back on ice.
3.4 Digestion

1. Gently pipette off the supernatant and resuspend the nuclei into 400 µL of 1.2x restriction buffer (refer to the manufacturer’s instructions for the optimal restriction buffer).
2. Centrifuge at 1900 g for 5 min at 4 °C.
3. Promptly place the tube back on ice and gently pipette off the supernatant. Resuspend the nuclei into 500 µL of 1.2x restriction buffer.
4. Add 7.5 µL of 20 % SDS (final concentration 0.3 %) to permeabilize the nuclei and inactivate endogenous nucleases. Incubate at 65 °C for 40 min in a shaker at 900 rpm (see Note 7).
5. Place the tube at 37 °C for 20 min, still shaking at 900 rpm.
6. Add 50 µL 20 % Triton X-100 (final concentration is 2 %). Incubate at 37 °C for 1 hour while shaking at 900 rpm. The Triton X-100 will sequester the SDS, preventing a negative impact on the digestion efficiency. The susceptibility to SDS varies from one restriction enzyme to another. In case of poor digestion efficiency, the final concentration of SDS might have to be adjusted (see Note 8).
7. For a non-digested control sample: transfer 28 µL of the nuclei suspension to a new 1.5 mL eppendorf tube containing 272 µL of milliQ water. Store the tube at -20 °C until all samples will be decrosslinked (see step 7 of Subheading 3.5.).
8. Add 400 Units of Restriction Enzyme and incubate overnight at 37 °C while shaking at 900 rpm (see Note 9).

3.5 Intra-molecular Ligation and de-crosslinking

The ligation of fragments crosslinked together needs to be favored. Therefore, the volume of ligation needs to be sufficiently large to favor intramolecular ligations. At the same time, a too large volume will result in low DNA recovery. Thus, genome size-specific adaptations are required. For small-genome organisms such as *A. thaliana* the volume of ligation needs to be decreased compared to the volume used for large-genome organisms such as *Z. mays*. The following part of the protocol describes volumes based on the *Z. mays* genome size. To determine in which volume intramolecular ligation should be performed for other organisms see Note 10 and Table 2.
Table 2: Reported volumes of ligation reactions in different 3C protocols and organisms with their respective haploid genome size

<table>
<thead>
<tr>
<th>Organism</th>
<th>S. cerevisiae</th>
<th>A. thaliana</th>
<th>Z. mays</th>
<th>M. musculus</th>
<th>H. sapiens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genome size (Mbp)</td>
<td>~12.5</td>
<td>~135</td>
<td>~2100</td>
<td>~2800</td>
<td>~3300</td>
</tr>
<tr>
<td>Volume ligation reaction (mL)</td>
<td>0.8</td>
<td>2</td>
<td>7</td>
<td>7.5</td>
<td>7.5</td>
</tr>
<tr>
<td>Reference</td>
<td>[7]</td>
<td>[16]</td>
<td>[15, 19]</td>
<td>[25]</td>
<td>[18]</td>
</tr>
</tbody>
</table>

1. Prepare 10x ligation Buffer and store at RT (see Note 11).
2. Inactivate the restriction enzyme either by heat inactivation (refer to the manufacturer’s instructions, shake at 900 rpm) or by addition of 40 µL of 20% SDS (final concentration is 1.6%) followed by incubation for 25 min at 65 °C, 900 rpm.
3. Transfer the digested sample to a 50 mL tube and add 7 mL of 1x ligation Buffer (700 mL of 10x Ligation buffer plus 6.3 mL of sterile milliQ water).
4. Sequester the SDS by addition of 375 µL of 20% Triton X-100 (final concentration is 1%) and incubation for 1 hour at 37 °C.
5. For a digested control sample, pipette 300 µL of the digested sample into a 1.5 mL eppendorf tube and store the tube at -20 °C until the de-crosslinking step (see step 7).
6. Add 100 units of highly concentrated T4 DNA ligase to the ligation mix and incubate for 5 hours at 16 °C, followed by 45 min at RT (see Note 3).
7. Add 30 µL of 10 mg/mL proteinase K to the ligation mix, and 5 µL to the non-digested and digested samples previously stored at -20 °C (see step 7 of Subheading 3.4 and step 5 of Subheading 3.5).
8. Incubate all tubes overnight in a 65 °C water bath.

3.6 DNA purification

1. Place the Phenol:Chloroform:Isoamyl Alcohol and Chloroform:Isoamyl Alcohol solutions under the fume hood at RT at least 2 hours before starting (see Note 12).
2. Add 30 µL of 10 mg/mL RNase A to the ligation sample, and 5 µL to the non-digested and digested samples.
3. Incubate all tubes at 37 °C for 30 to 45 min.
4. Place all tubes under the fume hood and add 10 mL of Phenol:Chloroform:Isoamyl Alcohol to the ligated sample and 300 µL to the non-digested and digested samples. Close all lids tightly and shake vigorously.
5. Spin all tubes at 4500 g for 10 min at RT.
6. Transfer the aqueous phase to a new 50 or 1,5 mL tube.
7. Precipitate the DNA by first doubling the volume with milliQ, followed by addition of 1/10 volume of 2M NaOac (pH 5.6) and 2 volumes of 96 % Ethanol. To promote high precipitation efficiency the addition of Glycogen is strongly recommended. Typically, 20-40 µL (20 mg/ml) is added to the ligated sample and 5 µL to the non-digested and digested samples.
8. Incubate all tubes at -80 °C for at least 2 hours.
9. Cool the centrifuge for 50 ml tubes (swing-out) and eppendorf centrifuge to 4 °C.
10. Centrifuge all tubes at 4500 g for 60 min at 4 °C.
11. Pour off the supernatant and wash the pellet of the ligated sample with 10 mL and the non-digested and digested samples with 1 mL of 70 % Ethanol.
12. Centrifuge all tubes at 4500 g for 20 min at 4 °C.
13. Promptly place the tubes back on ice and gently pour off the supernatant. With a pipette tip, remove the residual droplet of ethanol and let the pellet air-dry for 2-5 min. Alternatively, when dealing with multiple samples one can use a pump to remove the supernatant and then dry the pellet. Using a pump is certainly faster, but be cautious as loose pellets might get lost.
14. Resuspend the ligated sample in 150 µL, and the non-digested and digested samples in 15 µL Tris-HCl pH 7.5.
15. Incubate all tubes at 4 °C overnight to optimally resuspend the DNA pellet.
16. To assay the digestion and ligation efficiency, mix 2 µL of the generated 3C library with 1.5 µL of 10x loading buffer and 11.5 µL of milliQ water. Load the sample, together with the entire 15 µL of the non-digested and digested samples, and a positive control for digestion consisting of digested gDNA (see step 3 of Subheading 3.2), all with 10x loading buffer, on a 0.8 % Agarose gel. For the expected results, see Fig. 3 and Note 13.
17. The 3C library concentration can be estimated using a Qubit fluorometer. Alternatively, the concentration can be estimated on gel by comparison to a dilution series of a gDNA sample of known concentration. For more accuracy, load several dilutions of the 3C library on gel (1:1, 1:2, 1:4, 1:8). Note that nanodrop measurement is not reliable for quantification of complex DNA samples such as 3C libraries.

Fig. 3 Non-digested (ND), digested (D) and Ligated 3C library (L) samples on a 0.8% agarose gel. The ND and L samples show one high molecular weight band, indicating intact DNA (ND sample) and efficiently ligated DNA (L sample). The presence of low molecular weight products in the ND and L samples would indicate DNA degradation and inefficient ligation, respectively. The digestion (D) sample has to show a similar banding pattern, including signs of satellite bands, as the positive digestion control (+), which consists of digested genomic DNA. M indicates the size marker lane.

3.7 Quantification of interaction frequencies and Data analysis

In 3C experiments the Relative Frequency of Interaction (RFI) is measured between a fixed fragment, the bait or viewpoint, and another fragment at the locus of interest. To estimate the RFI of a given fragment (e.g. the red fragment in Fig. 1) with the bait fragment (blue fragment in Fig. 1), one needs to compare it to the RFI of other fragments (black fragments in Fig. 1)
with the bait. Below, we provide step-by-step qPCR and RFI quantification methods. The qPCR protocol is based on using SYBR green. The use of TaqMan probes is an alternative to SYBR green technology. For this, please see Note 14.

1. Prepare the qPCR mix, excluding primers, according to your own setup. The final volume for each reaction should be 20 µL.
2. Use 50 ng of 3C library as a template per reaction. Prepare similar reactions for the locus of interest and the endogenous control locus.
3. For the RL-Library controls, use the previously prepared dilution(s) complemented with gDNA (see step 33 of Subheading 3.2.1). Prepare similar reactions for the RL-Library dilution of the locus of interest and the endogenous control locus.
4. Add 2 µL of primer (1 µL bait primer + 1 µL fragment primer from 10 nM stock) to each well.
5. For each primer pair, perform a triplicate qPCR reaction on the 3C library and RL-Libraries.
6. For each primer pair, the RFI is calculated as follows:
\[
RFI = \frac{2^{-(C_{t_{3C}} - C_{t_{RL}})}}{2^{-(C_{t_{3CE}} - C_{t_{RLe}})}}
\]
where:
\[
C_{t_{3C}} = \text{mean value from the 3C triplicate for a given primer pair } i.
\]
\[
C_{t_{RL}} = \text{mean value from the RL-Library triplicate for primer pair } i.
\]
\[
C_{t_{3CE}} = \text{mean value from the 3C triplicate for the primer pair of the endogenous locus.}
\]
\[
C_{t_{RLe}} = \text{mean value from the RL-library triplicate for the endogenous locus.}
\]
7. Normalize all RFIs to the highest RFI value and plot the normalized RFIs as a function of their distance to the bait (Fig. 1E).

4. Notes
1. Use always fresh plant material if possible. Fresh material results in the most optimal digestion of fixed chromatin. In case material collection and the 3C protocol cannot be performed simultaneously, we advise to store fixed, dried material rather than fixed, ground material. This appeared more effective in our hands.
2. The amount of tissue to be processed per sample ranges between 1 to 3 grams of fresh plant material. This range should not be exceeded as too much tissue will affect the efficiency of fixation. At the same time, the amount of tissue used in each experiment should yield a sufficient number of nuclei (1 to 10 x 10⁶). To estimate the number of nuclei isolated, take a 2 µL aliquot after the first resuspension of nuclei in 1 mL of NEB
(step 15 of Subheading 3.3). The nuclei have the tendency to sink to the bottom of the tube. Therefore, invert the tube with the resuspended nuclei gently three times before pipetting to avoid underestimating the actual yield. Add 2 µL of DAPI stain to the sample (final DAPI concentration of 2 µg/mL; Dilution Factor of 2) and count the nuclei on a hemocytometer using fluorescence microscopy (10-20x magnification, use DAPI filter). The total number of nuclei is estimated with the following formula: Total number of nuclei = (total number of counted nuclei x Dilution Factor x 10^4)/(number of chambers counted). In case of a low number of nuclei per gram of tissue, one could consider generating multiple tissue samples and subsequently pool the nuclei together when resuspending the nuclear pellets (step 15 of Subheading 3.3).

3. Crowding reagents such as PEG cannot be used to increase the efficiency of the 3C ligation as it compromises the intramolecular nature of the ligation. Note that the addition of PEG 4000 (10 % final concentration) can be used to increase the ligation efficiency of the RL-library.

4. Some tissue types can be used directly for fixation, other types need to be cut in smaller pieces. For instance, relatively permeable tissue like Arabidopsis rosettes can be used directly, while maize inner stem and husk tissue needs to be cut in ~1 cm² pieces before fixation. For each tissue type the optimal fixation time needs to be determined. Underfixation will negatively affect the ability to detect chromosomal interactions. Overfixation will negatively affect digestion and de-crosslinking efficiency, but also increase the background level of interactions. A good indication that tissue gets fixed is when it gets a translucent appearance. To define the optimal fixation time, one should perform a time series, fixing tissue samples for different time periods, and proceed with nuclei isolation and digestion (from step 6 of Subheading 3.3 to step 8 of Subheading 3.4), followed by de-crosslinking and DNA precipitation (from step 7 of Subheading 3.5 to step 14 of Subheading 3.6). Run the DNA samples on a 0.8 % agarose gel in parallel with an unfixod, de-crosslinked sample (no fixative added, no vacuum infiltration, positive control for DNA isolation) and a fixed, non-decrosslinked sample (negative control). At the optimal time of fixation, samples display efficient digestion and a relatively high DNA recovery after de-crosslinking and DNA isolation. Alternatively, one could test the effect of different formaldehyde concentrations while using a fixed incubation time.

5. For efficient fixation, wrap the tissue or pieces of tissue in a 12 by 12 cm piece of Miracloth, and close the Miracloth with a staple, generating a “tea bag” (see Fig. 2). The
tea bag ensures complete submersion of the tissue during fixation, prevents spilling of tissue, and allows easier handling of the sample during subsequent washes.

6. To efficiently dry the tissue, place the Miracloth “tea bag” containing the tissue between a stack of paper towels and use a bottle or something similar as a roller to remove water. Repeat this procedure till the paper towels do not get wet anymore. Then the tissue is considered dry. Remaining water negatively impacts the grinding efficiency and hence the yield of nuclei.

7. The incubation of the chromatin for 40 min at 65 °C prior to digestion is crucial for inactivation of endogenous nucleases that would otherwise become active during the digestion step at 37 °C.

8. Digestion efficiency is sensitive to the chemicals present in a solution, including the SDS used to permeabilize the nuclei (step 4 of Subheading 3.4). In case of poor digestion efficiency, we recommend testing the effect of different SDS concentrations (0.05 to 0.3 % final concentration) on the digestion efficiency of the restriction enzyme.

9. Efficient digestion is key to a successful 3C experiment. For enzymes with a low performance over a long incubation period, we recommend to add fractions of the total number of enzyme units (400) at different time points during the digestion procedure. This helps to maintain high digestion efficiency.

10. Good intramolecular ligation conditions are important to ensure that only fragments crosslinked with one another are being ligated. To favor intramolecular ligation events, the ligation reaction is carried out in a large volume. This volume needs to be adapted to the genome size, as a low DNA concentration hampers an efficient precipitation of the ligation products, while a too high DNA concentration also allows intermolecular ligation events. In Table 2 we report volumes of intramolecular ligation used in different published 3C protocols for different organisms. Note that the appropriate ligation volume (step 3 of Subheading 3.5) is influenced by the concentration of SDS present before the ligation step. If one wishes to lower the ligation volume, the volume of digestion should be adapted such that the SDS concentration will not hamper ligation efficiency. For instance, when working with Arabidopsis thaliana, in our hands a ligation volume of 2 mL (see Table 2) requires a digestion volume of 250 µL (step 3 of Subheading 3.4) followed by addition of SDS to a final concentration of 0.2 % (step 4 of Subheading 3.4).
11. We highly recommend preparing a fresh 10x ligation buffer each time to ensure efficient ligation. Addition of extra ATP (0.6 mg/mL) after 2 to 3 hours of incubation of the ligation reaction can further improve the ligation efficiency.

12. In case of low DNA recovery after the ligation step: make sure that the Phenol:Chloroform:Isoamyl-Alcohol and Chloroform:Isoamyl-Alcohol solutions are well equilibrated at RT before adding them to the samples. Cold Phenol:Chloroform:Isoamyl-Alcohol and Chloroform:Isoamyl-Alcohol are more prone to phase inversion. Increasing the amount of Glycogen added and incubation of the precipitation reaction overnight at -80 °C can help to increase precipitation efficiency.

13. Digestion efficiency can be evaluated on an agarose gel as indicated at step 16 of Subheading 3.6 (see Fig. 3). A more accurate evaluation of digestion efficiency can be achieved by designing a few primer pairs spanning restriction sites, followed by qPCR. The digestion efficiency of the 3C sample should be evaluated by comparison to a non-digested and digested gDNA template. Ideally the digestion efficiency is above 80%. To check for variation in the amount of each template, use a primer pair amplifying a region not cut by the restriction enzymes used.

14. Plant genomes can harbor a very high density of repetitive elements (see e.g. *Zea mays*) [30]. Specific amplification of the desired amplicons can therefore be challenging. When analyzing the qPCR results, always perform melting curve analyses for all primer pairs to check amplicon specificity. If obtaining specific primers appears to be very difficult, the design and use of a TaqMan probe (Fig. 1) can help to increase signal specificity. With a TaqMan probe one does not rely on the use of a non-sequence-specific fluorescent dye such as SYBR. A TaqMan probe should be designed for the bait fragment, on the opposite strand of the bait primer (see Fig. 1). In this way, the quencher from the probe can only be removed when a new strand is synthesized using the primer annealed at the ligated fragment. TaqMan probes are usually designed as an approximately 30 bp oligo with a Tm 7-10 °C above the Tm of the primers [25].

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6. References