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Standard Operating Procedure for karyotyping non-model organisms

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Abstract

The SOP we developed for karyotyping non-model organism is a combination of several protocols, with modifications (see references in document). Metaphases obtained from cell cultures have a higher quality compared to the ones from tissues, since cell division can be observed and controlled, and the chromosomes are more distended. It requires, however, development of a cell culture, which is not trivial for many taxa. It also needs adjustments in relation to the time of action of colchicine in different taxa and modifications of buffers to maintain osmolarity when dealing with marine/freshwater organisms. Overall, karyotyping methods from cell cultures are well established and have been done routinely at our labs for mammals, birds, reptiles, and fishes. For the latter two groups, it is more time consuming since the cells must be exposed to colchicine for longer periods (minimum 3h until overnight). Karyotyping tissues can prove more challenging due to variation in penetration of colchicine, timing of the incubation in the different reagents, which also needs to be adjusted for every target group, among other things. In addition, in some cases, it requires the injection of colchicine *in vivo*, which makes the process more bureaucratic since it requires obtaining local permits, approval from local ethics committees, and in some cases the presence of a veterinarian. Furthermore, it is difficult to control cell division and consequently the metaphase index is lower compared to karyotypes from cells. Even though cell culture is the best source of karyotyping (with the advantage of being permanently stored and reused), tissue karyotyping has proven to be a very useful alternative in cases where cells are difficult to obtain (some fishes, amphibians, snakes and invertebrates). Furthermore, cell culture karyotypes are more subject to mutation if many passages occur.

SOP: Karyotyping non-model organisms

The present SOP is a compilation of several protocols (see below in each case), with modifications. This protocol was developed by:

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1.1. Obtention of metaphases from cell cultures

(based on Freshney 2015 and Houck et al., 2017 – with modifications)

- Check if the cells are in log phase of growth. For this, you can count your cells at the beginning of the replication, and then at several points since then (usually 24, 48, and 72 h). When you find the biggest increase in the number of cells with respect with the previous time, your culture has entered the log phase.
- Add 0.1 ml of colchicine (50 µg/ml) per ml of cell culture medium to the proliferating cell culture in the flask. You can also use colcemid (Roche) at 10 µl/ml of culture. Incubate for 1 hour up to 1 day. Note that time depends on the species, longer times are usually applied to cells from invertebrates, but this has to be established by a test. Time should be optimized since longer incubations in colchicine tend to produce more condensed and less quality chromosomes.
- Subsequently, trypsinize the culture (to digest proteins attached to chromosomes and allow Giemsa to stain later) by adding 1 ml of trypsin and centrifuge the cells (500xg during 10 min). Make sure the trypsin solution covers your culture.
- Add 7 mL 0.075 M potassium chloride solution (KCL) previously pre-warmed at 37°C. Gently homogenise and leave for 20 min at 37°C (water bath). For marine invertebrates, place your cells in a hypotonic solution composed of colchicine (0.04%) in artificial seawater (Sigma – S9148) for 45 minutes in the dark.
- Pre-fixation: add 6 drops of Carnoy's fixative (3 methanol: 1 acetic acid ratio). Carnoy's solution must be prepared fresh and left in the freezer. Incubate 5 minutes at room temperature. Add 3 mL more of Carnoy's

fixative (3 methanol: 1 acetic acid ratio) and gently pipette. Incubate for 10 minutes at room temperature.

- Centrifuge for 10 minutes (500xg). Discard supernatant.
- Add 6 mL Carnoy's fixative (3 methanol: 1 acetic acid ratio), pipette and centrifuge for 10 minutes at 500g. Discard supernatant.
- Repeat the previous step one or two more times, depending on the amount of the pellet.
- Drop the cell suspension (dropwise) from at least one foot above the surface of the slide (2 drops/slide). Cells suspension can be stored at -20°C but it is better to prepare several slides on the same day.
- Stain slides with Giemsa (1:30 dilution) (Giemsa Modified Stain – Sigma GS-500) for at least 15 minutes (adapt according to species). Alternative for cells of marine organisms: Stain with 7 ml of Giemsa solution in 100 ml of Sorensen-phosphate buffer (pH 6.8, WVR 100496-398).
- Wash slides with distilled water. Analyse under microscope and photograph chromosome spreads to count.

1.2. Obtention of metaphases from embryos

(based on Imsiecke et al. 1995; Ishijima et al. 2008; Guo et al. 2018 – with modifications)

Because embryos can be extremely fragile during cleavage steps, solutions should be added slowly dropwise and enough solution should be left in the petri dish to keep the embryos fully submerged to avoid blastomeres being burst as a result of the surface tension exerted by the solutions. Do not puncture the embryos.

Treat your embryos with either:

For freshwater species: Add colcemid (1mg/ml) or colchicine (2ug/ml) to a final concentration of 0.02 – 0.04 mg/ml. (Stock of 1–2 mg/ml in water) and incubate for 6 hours up to 1 day, depending on the species. Sometimes for flimsy embryos 45 minutes to 1 hour is enough.

For marine species: Add colcemid or colchicine (2ug/ml) (to a final concentration of 0.04% colchicine in artificial seawater for 45 min with gentle rotation in the dark.

- Fix embryos in freshly made Carnoy's fixative (3:1 methanol: acetic acid) on ice.
- Prepare slides: Wash slides in Carnoy's fixative (3:1 methanol: acetic acid) and then soak them in ice cold water until ready to use (distilled water plus some ice). It is important for the slides to be both cold and wet when ready to go. You can also siliconize your coverslips. For this: Siliconized coverslips (22 × 22 mm) are submerged in Sigmacote for 2–3 s in a fume hood. The coverslips are then propped up vertically for 10 min to dry, rinsed in DI (deionized water) H₂O for 2–3 s, and propped up vertically to dry.
- Trypsinize embryos (not always needed, this may require some trial/error) by adding 1-2 ml of trypsin to the plate: Wash your embryos in 0.25% trypsin/ CMFM for 5 s, and then resuspend in CMFM by gentle pipetting. Spin down dissociated cells at 1000 rpm for 3 minutes. Remove supernatant. CMFM is calcium and magnesium-free medium (see below).
- Place your embryos on the slide ensuring you have enough solution to keep them submerged. Remove the solution gently and add 1 ml 0.56% KCl dropwise. Incubate at RT for 6 minutes. (Prepare 3 or 4 slides for each sample).

- If you have not trypsinized your embryos (sometimes it is not necessary, as in cnidarian embryos), place your embryos on the slide ensuring you have enough solution to keep them submerged. Remove supernatant and add 60% acetic acid and incubate 5 mins RT.
- Place a siliconized coverslip on top of the embryos. The weight of the siliconized coverslip will be sufficient to flatten the cells, do not apply additional pressure. Be careful to keep the coverslip from shifting laterally during this procedure.
- After an overnight (ON) incubation at 4 °C, the slides are chilled on dry ice for 10–20 min.
- While the slides are still sitting on dry ice, remove quickly the coverslips using a blade. Warm slides to RT.
- Rinse slides 3 times with 1× phosphate-buffered saline (PBS).
- Stain slides with Giemsa (1:20 dilution) for at least 15 minutes (can go longer... even overnight). Wash slides with water. Alternatively stain with 7 ml of Giemsa solution in 100 ml of Sorensen-phosphate buffer (pH 6.8).
- Photograph chromosome spreads to count.

1.3. Obtention of metaphases from tissues

All the procedures to obtain tissues are performed on living animals. You should ensure that you work according with

your country's regulations for responsible handling of animals regarding animal welfare.

1.3.1 Invertebrates

(based on Imsiecke et al. 1995; Tosuji et al. 2004; Ishijima et al. 2008; Guo et al. 2018; Kenny et al. 2020 – with modifications)

- Treat your animals with:
Freshwater animals: 0.25% colchicine in freshwater and incubate for 1 hour up to 1 day.
Marine organisms: 0.25% colchicine in artificial seawater.
- Rinse the tissues with deionized water (DI H₂O).
- Puncture the tissues with a needle to increase permeability and incubate in DI H₂O for 20 minutes at RT.
- Fix tissues in freshly made Carnoy's fixative (3:1 methanol : acetic acid) on ice.
- Prepare slides: Wash slides in Carnoy's fixative (3:1 methanol: acetic acid) and then soak them in ice cold water until ready to use (distilled water plus some ice). It is important for the slides to be both cold and wet when ready to go. You can also siliconize your coverslips. For this: Siliconized coverslips (22 × 22 mm) are submerged in Sigmacote for 2–3 s in a fume hood. The coverslips were then propped up vertically for 10 min to dry, rinsed in DI H₂O for 2–3 s, and propped up vertically to dry.
- Trypsinize tissues with trypsin/EDTA in PBS or 0.25% trypsin/CMFM for 5 s to 2–3 minutes (not always needed, this may require trial/error).
- Stop the proteolytic reaction by removing supernatant or adding 0.5% KCl.
- Fix tissues in freshly made Carnoy's fixative (3:1 methanol:acetic acid) on ice.
- Place a small portion of animal/tissue onto a slide using a pair of forceps or a pipette.
- Soak your tissue in 60% acetic acid (10–20 µl) and incubate for 5 min.
- Place a siliconized coverslip on top of the tissue.
- Squeeze your tissue by applying constant pressure to the coverslip for 2–3 s to create a single layer of nuclei. Take care to keep the coverslip from shifting laterally during its application.
- After an overnight (ON) incubation at 4 °C, the slides are chilled on dry ice for 10–20 min.



- While the slides are still sitting on dry ice, remove quickly the coverslips using a blade. Warm slides to RT.
- Rinse slides 3 times with 1× phosphate-buffered saline (PBS)
- Stain slides with Giemsa (1:20 dilution) for at least 15 minutes (can go longer... even overnight). Wash slides with water.
Alternatively stain with 7 ml of Giemsa solution in 100 ml of Sorensen-phosphate buffer (pH 6.8).
- Photograph chromosome spreads to count.

1.3.2 Vertebrates

(based on Ford and Hamerton, 1956; Schmidt, 1978; and Ezaz et al., 2005 – with modifications)

1.3.2.1 Small mammals

- About 30 minutes before sacrifice, inject subcutaneously 0.1% colchicine (1mL for each 100g of animal weight).
- Remove the femurs, clean and cut the epiphyses. Then remove the spleen. Place them all in a Petri dish containing 3 to 5 mL of Hanks' balanced solution.
- Using a syringe with a needle, wash the bone canal and spleen successively until all the material is removed.
- Transfer the material to a 50 mL centrifuge tube and centrifuge for 10 minutes at 1200 rpms.
- Discard the supernatant, add 7mL of hypotonic solution (KCl 0.075 M) and gently resuspend. Incubate for approximately 20 minutes in a water bath at 37°C.
- Add 6 drops of Carnoy's fixative (3:1 methanol-acetic acid) and gently resuspend. Leave for 10 minutes at room temperature.
- Add 3 mL of Carnoy's fixative, mix slowly and leave for 10 minutes at room temperature. Centrifuge at 1200 rpm for 10 minutes.
- Discard the supernatant and add 7 mL of Carnoy's solution.
- Homogenize. Centrifuge again and discard the supernatant. Repeat two more washes, changing the Carnoy's fixative in each wash.
- Add 1 or 2 drops of the cell suspension onto clean histological slides.
- Stain slides with Giemsa (1:30 dilution) for 10 minutes. Wash slides with distilled water. Analyse under microscope and photograph chromosome spreads to count.

1.3.2.2 Amphibians and Reptiles

- Inject phytohemagglutinin 24 or 48 hours before preparation (0.1 mL each 10 g of the animal's weight).
- Colchicine: Inject 0.1% for each 100g of body weight for 4h30min.
- Remove the intestine, clean excess residue, and place it directly (without opening) in 5 ml of distilled water (Hypotonic) for 30 minutes in amphibians and lizards and 60 minutes in snakes.
- Remove the distilled water and add freshly prepared ethanol-acetic acid fixative (3:1).
- Remove the ethanol-acetic acid fixative (3:1) and add 50% acetic acid for 10 minutes at room temperature.
- Discard the 50% acetic acid, open the intestine to its extent, add methanol/acetic acid fixative (3:1) (Carnoy's fixative) and scrape the internal epithelium.

- Transfer the solution to a 50 ml centrifuge tube, complete to 7 ml of Carnoy's fixative and pipette repeatedly. Centrifuge at 1.200 rpm for 10 minutes and discard the supernatant.
- Repeat two more washes, changing the Carnoy's fixative in each one.
- Add 1 or 2 drops of the cell suspension onto clean histological slides.
- Stain slides with Giemsa (1:30 dilution) (Giemsa Modified Stain – Sigma GS-500) for 10 minutes.
- Wash slides with distilled water. Analyse under microscope and photograph chromosome spreads to count.

Lab equipment

Centrifuge
 Culture flasks
 Desk lamp
 Forceps
 Glass slides (acid-washed, grease-free)
 Glass tube (2 mL, conical)
 Falcon tubes or centrifuge tubes (50 ml)
 Green filter for microscope
 Kimwipes or any coarse, grease-free tissues
 Microscope with 100X oil immersion objective and 40X phase-contrast objective
 Pasteur pipettes (~0.2-mL capacity, drawn to deliver 10-µL drops)
 Water bath preset to 60°C-65°C

Lab reagents

Colchicine: Colchicine – Sigma C9754

Colcemid: Demecolcine – Sigma D-7385

Artificial seawater (Sigma; S9148)

Carnoy's fixative:

Recipe:

Methanol, three parts

Acetic acid (glacial), one part

Prepare fresh for each use.

Trypsin Trypsin (0.025% [w/v]) in 0.85% (w/v) NaCl

The age of the trypsin powder is not important, but the solution should be made ~30 min before use.

Trypsin/EDTA (0.25%) – Sigma T4049

Sigmacote: Sigma Aldrich – SL2-25ML

Calcium magnesium-free medium (CMFM)

Reagent	Amount to add	Final concentration



	5M NaCl	17.6 ml	88 mM
	1M KCl	1 ml	1 mM
	1M NaHCO ₃	2.4 ml	2.4 mM
	1M Tris-Cl (ph7.6-7.8)	7.5 ml	7.5 mM
	H ₂ O	971.5 ml	

Recipe for CMFM

Giemsa Modified Stain: Sigma – GS-500. Dilute 1:20 with H₂O.

Growth medium and serum

Acetic acid

NaCl (0.85% [w/v])

PBS: Phosphate buffer (pH 6.8) (BDH)

Potassium chloride (KCl) (0.56% [w/v])

Sorensen-phosphate buffer

Recipe:

0.133 M Na₂HPO₄

0.133 M KH₂PO₄

Mix 71.5 mL of Na₂HPO₄ and 28.5 mL of KH₂PO₄ to obtain pH 7.2.

Phytohemagglutinin (Sigma L1668)

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