Subcellular localisation of newly synthesized viral RNA in coronavirus infection by EM autoradiography

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1 Works for me dx.doi.org/10.17504/protocols.io.bfrtjm6n

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ABSTRACT

This protocol allowed the subcellular localisation of viral RNA synthesis in coronavirus-infected cells by EM autoradiography, though it is largely applicable to other types of EM autoradiography samples. Newly synthesized viral RNA is metabolically labelled by providing the infected cells with tritiated uridine while arresting cellular transcription. Subsequently, the samples are processed for EM, sectioned and incubated with a photographic emulsion. Radioactive disintegrations from the labelled viral RNA generate signal around the radioactive source that, after development, becomes apparent as electron-dense grains that can be detected by transmission electron microscopy. The advantages of this approach are its high sensitivity and its compatibility with high-contrast EM sample preparation protocols. The protocol presented here is an adaptation of different methods and tips to EM autoradiography discussed in detail by Williams, in Glauert, 1977, Practical Methods in Electron Microscopy, Volume 6, Chapter 4.

EXTERNAL LINK

https://www.kosterlab.nl/virus-replication-18

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Snijder EJ, Limpens RWAL, Wilde AHd, Jong AWMd, Zevenhoven-Dobbe JC, Maier HJ, Faas FFGA, Koster AJ, Bárcena M (2020) A unifying structural and functional model of the coronavirus replication organelle: Tracking down RNA synthesis. PLoS Biol 18(6): e3000715. doi: 10.1371/journal.pbio.3000715

ATTACHMENTS

Subcellular localisation of newly synthesized viral RNA in coronavirus infection by EM autoradiography.pdf

MATERIALS

NAME	CATALOG #	VENDOR
Ilford L4 nuclear emulsion	AGP9282	agar scientific
Gold(III) chloride trihydrate	520918	Sigma Aldrich
Metol	69750	Sigma Aldrich
L-Ascorbic acid	A2218	Sigma Aldrich
Borax Anhydrous	71997	Sigma Aldrich
Potassium thiocyanate	P2713	Sigma Aldrich
Potassium bromide	P9881	Sigma Aldrich
Sodium Thiosulfate Pentahydrate	15661350	Fisher Scientific

Metabolic labelling of newly-synthesized viral RNA and EM sample preparation

1 Infect cells with virus, preferably at a high multiplicity of infection (MOI ≥5)

Control samples:

It is important to include several control samples in the experiment. The first control sample does not receive radioactive label (skip step 3) and will be used to estimate the background levels. A second control consists of mock-infected cells (replace virus inoculum by mock-inoculum in step 1), and will serve to assess the inhibition of cellular transcription by actinomycin D.

2 1 hour before applying the radioactive label, replace the normal culture medium with culture medium containing 10 µg/ml actinomycin D, to block cellular transcription.

3 Remove the medium, and quickly add the radioactive label ([5-^{3}H]-uridine, 500 µCi/ml) in medium containing 10 µg/ml actinomycin D.

4 After the desired labelling time (20-60 min in our case), remove the label and chemically fix the cells to stop the incorporation of label. In our case, after fixing with 1.5% glutaraldehyde in 1 M cacodylate buffer (pH 7.4) for 30 minutes at room temperature, we keep the samples in the fixative overnight at 4°C (BSL3 biosafety requirements).

5 After this fixation step, wash extensively with 0.1 M cacodylate buffer to remove as much as possible of the unincorporated radioactive label.

6 From here on, samples can be processed for EM either by chemical fixation or high-pressure freezing and freeze-substitution, using any protocol for preparing and embedding the samples in epoxy resin.

Sectioning and post-staining

7 It is important to have a sufficient amount of grids per sample to follow the progression of the experiment. This is particularly important the first time, as the required incubation time will be hard to estimate, but also recommended in subsequent experiments because variations in the incubation times per experiment are not uncommon. It is advisable to have thinner sections than for most standard EM samples, because this will enhance the spatial resolution of the autoradiography signal.

Cut 50 nm sections of your sample and place them on EM grids. Prepare 10 grids of each sample.

8 If additional contrast is desired, post stain the grids with 7% uranyl acetate and Reynold's lead citrate.

Preparation of glass slides with grids

9 Clean microscope glass slides with ethanol and let them dry.

10 Apply a strip of double sided sticky tape along the long side of the glass slide.

11 Attach the grids to the glass slide by gently pressing the grid edge against the sticky tape.

Each glass slide should include a grid for every condition tested (e.g. different labelling times or times post infection) within a given experiment plus the appropriate controls, to be later developed simultaneously.

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12 Evaporate a thin layer of carbon (around 10 nm) on each glass slide, to prevent direct contact between the stained section and the photographic emulsion, that could lead to undesired chemographic effects.

Application of the photographic emulsion

13 Applying the photographic emulsion to the glass slide is performed in a dark room. Make sure to have the Ilford 902 or 904 safelight filter installed.

14 Add **10 ml** of Ilford L4 nuclear emulsion to **25 ml** of milliQ water in a glass cylinder.

15 Heat the emulsion to **42 °C** while stirring in a water bath for **00:30:00** .

16 Cool down the emulsion to roughly **20 °C** At this temperature the emulsion will form a nice thin and clear membrane in a wire loop, which can then be applied on top of the glass slide. If the emulsion is still too warm, the emulsion will be visibly fluid and the membrane will break easily. In case the emulsion cools down too much, the emulsion layer will be too thick and you will see irregularities in its opacity. In this case, put it at **42 °C** again for a while.

17 When the emulsion forms an even layer in the loop, it can be applied to the glass slides. Take a glass slide and press the loop parallel onto the samples, so the emulsion layer will attach to the grids.

18 Lay the glass slides on a flat surface for **00:10:00** to let the emulsion dry.

19 Place the slides in a plastic box for microscopy slides, containing silica gel, and seal the box with dark tape to make it light-tight. Do not use cardboard or wooden slide boxes, since these can create chemographic background.

20 The samples are stored at **4 °C** until further processing.

Development & imaging

21 The progress in the exposure of the nuclear emulsion to radioactive disintegrations is evaluated regularly by EM until the number of autoradiography grains is sufficient for analysis. This can take from weeks to several months, depending on your sample.
The development method here described is based on Ginsel et al. 1979, Histochemistry; 61(3):343-6. (PMID: 478993).
For the development, it is important that the temperature is at all times around 20°C, because the indicated time for the developing step is based on this temperature.

22 Clean all the glassware with 30% acetic acid for 10 minutes to make it phosphate free, rinse with milliQ water several times and dry the glassware.

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23 Prepare the solutions for development of the samples. The gold solution and the fixative can be stored (at **4 °C** and **Room temperature**, respectively) for successive development sessions. Solutions A & B need to be freshly prepared every time. Development starts 1h after the last chemical of solution B has dissolved. It is convenient to prepare solutions A & B in T175 cell culture flasks, because these are clean and phosphate free.

Gold-solution:
2% $HAuCl_4$ in autoclaved milliQ water

Gold latensification (Solution A):
1 ml 2% H-$AuCl_4$ in **100 ml** autoclaved milliQ water

Add **250 mg** potassium thiocyanate + **300 mg** KBr

Add autoclaved milliQ water to **500 ml**

Elon-Ascorbic acid (Solution B):
225 mg elon (metol) and wait until it dissolves

Add **1500 mg** ascorbic acid and wait until it dissolves

Add **1300 mg** **anhydrous** borax (or 2500 mg borax decahydrate) and wait until it dissolves

Add **500 mg** KBr and wait until it dissolves

Add autoclaved milliQ water up to **500 ml**

Fixative (Solution C):
24% sodium thiosulphate in milliQ water

24 Transfer the glassware, solutions and the box containing the samples to the darkroom.

25 Inside the darkroom, fill Coplin jars with solutions A, B and C, and have some extra containers for milliQ water.

26 Take one slide with samples out of the box, and close the box again with tape.

27 Place the glass slide for **00:05:00** in solution A.

28 Rinse briefly in milliQ water.

29 Develop for **00:07:30** in solution B.

30 Rinse briefly in 2% acetic acid.

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31 Fix for **00:02:00** in solution C.

32 Rinse 3x **00:01:00** in milliQ water.

33 Remove the grids from the glass slide immediately after the last rinse, and let the grids dry on filter paper.

34 When the grids are dry, store them in a gridbox and transfer them outside the darkroom.

35 Have a look at the samples in the electron microscope to assess if there is enough autoradiography signal for analysis. If there is not enough signal, then wait longer (usually 1-3 weeks, depending on the progress) before developing another slide. If there is enough signal, then develop more slides to have extra grids for analysis.

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