Chapter 4

Dengue Virus Purification and Sample Preparation for Cryo-Electron Microscopy

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Abstract

Cryo-electron microscopy (cryo-EM) is a valuable tool used to study the structures of icosahedral viruses without having to resort to crystallization. During the last few decades, significant progress has been made where virus structures previously resolved only to low resolution have now breached the sub-nanometer threshold. Critical to such excellent results are the acquisition of highly purified virus samples and well-frozen samples in vitreous ice. With the virus particles locked in their native conformations, cryo-EM together with single-particle analysis can then be deployed to study the structures of the viruses in their fully hydrated states.

Key words Cryo-EM, Dengue virus, Virus purification, Potassium tartrate gradient centrifugation, Vitrobot

1 Introduction

Cryo-electron microscopy is utilized to study virus structures. The shape of the virus and the arrangement of its capsomeres can be visualized at low resolution (20–40 Å), while α-helices and β-sheets become apparent at sub-nanometer resolutions (<10 Å). C-α backbones as well as some side chains also start to become more evident when viruses are resolved to near-atomic resolution (<4.5 Å) [1]. The quality of the images is, however, dependent on the purity and quality of an adequately concentrated sample.

Structures of the immature and mature forms of dengue virus have been studied extensively. The immature dengue virus contains three structural proteins: capsid (C), precursor membrane (prM), and envelope (E) proteins [2]. The capsid protein packages the viral genomic RNA to form the nucleocapsid, while the prM and E proteins form heterodimers that organize into 60 trimeric spikes [3, 4]. These spikes project outwards from the lipid envelope of the virion, and in these trimeric structures, the pr portion of the prM proteins cap the E protein fusion loops. During the maturation

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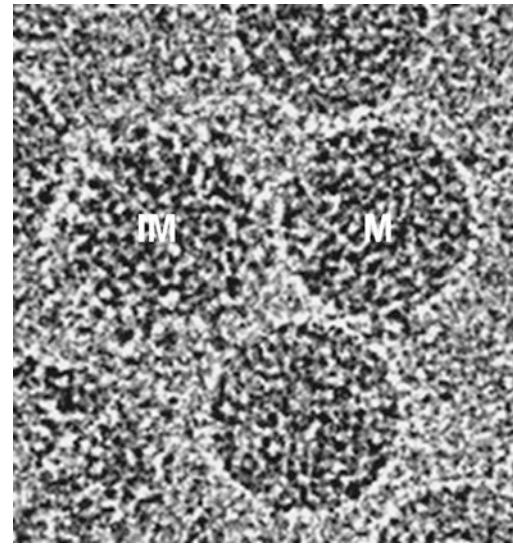

Fig. 1 Cryo-electron micrograph of a dengue 1 virus preparation showing an immature (IM) and a mature (M) virion particle

process, the immature virus particle passes through the trans-Golgi network (TGN) from the endoplasmic reticulum. The acidic environment in the TGN initiates the rearrangement of the spiky prM/E heterodimers into 90 E dimers that cover the virus surface in a herringbone formation [5]. At this stage, a host protease, furin, cleaves the pr peptide from the M protein [6]. However, the pr peptide still remains bound to the fusion loop of the E protein until the virus is released into the extracellular environment, forming the infectious mature dengue virus particle [7]. The function of the pr molecule is thought to prevent the newly synthesized virus particles from fusing back into the cell while moving through the acidic TGN compartments.

The preparations of mature and immature virus vary slightly. Growth of immature virus involves the addition of ammonium chloride (NH_4Cl) to the growth media during cell culture. This increases the pH of the TGN, preventing the rearrangement of the trimers and cleavage of viral prM molecule by furin protease. The immature virus particle thus remains spiky, while the mature virion appears smooth (Fig. 1).

Potassium tartrate gradients have been successfully used to purify dengue for cryo-EM as well as atomic force microscopy [8, 9]. This gradient, of low cost and toxicity, serves to separate the virus from cytoplasmic debris.

To freeze the virus on cryo-EM grids, a thin layer of freshly purified sample is applied to the grid and then plunge-frozen into liquid ethane. This procedure minimizes the formation of ice crystals that may potentially damage the structure of the particles and

allows the retention of the sample close to its original conformational state [10, 11]. Furthermore, since electron beams penetrate thin ice layers more easily, better contrast cryo-EM images can be obtained [1].

This chapter describes in detail the large-scale preparation of dengue viruses, its purification, and also the procedure to freeze samples on cryo-EM grids.

2 Materials

2.1 Virus Culture

1. RPMI 1640 supplemented with 10 % fetal bovine serum (FBS), 25 mM HEPES, and 2.05 mM L-glutamine, pH 7.0.
2. Minimal essential medium (MEM) without HEPES, pH 7.0, supplemented with 10 % (v/v) FBS.
3. T175 tissue culture flasks.
4. Two- and ten-cell stacks (Thermo Scientific).

2.2 Virus Purification and Concentration

1. NTE buffer (1,000 mL): 120 mM NaCl, 12 mM Tris–Cl, 1 mM EDTA, pH 8.0.
2. 40 % (w/v) PEG 8000 (500 mL): 200 g PEG 8000, topped up with NTE buffer.
3. 24 % (w/v) sucrose (20 mL): 4.8 g sucrose topped up with NTE buffer.
4. 40 % (w/v) potassium tartrate-30 % glycerol (100 mL): 40 g potassium sodium tartrate, 30 mL glycerol, topped up with NTE buffer.
5. Light torch.
6. 18 G needle.
7. 5 mL syringe.
8. Gradient maker (Hoefer).
9. 100 kDa MWCO centrifugal filter (Millipore).
10. Centrifuges and rotors: benchtop microfuge and centrifuge, low speed centrifuge with a ≥1 L handling capacity, ultra-centrifuge.

2.3 Virus Quantitation

1. BSA standards: 0.25, 0.5, 1, and 2 mg/mL concentrations.
2. SDS-PAGE gel (4–15 %) (BioRad).
3. Bio-Safe Coomassie Blue stain (BioRad).

2.4 Cryo-EM Sample Preparation and Grid Freezing

1. Plasma glow discharger (Quorum Technologies).
2. Vitrobot Mark IV (FEI).
3. Vitrobot cup (FEI).

4. Ethane cup (FEI).
5. Metal spindle (thermal conductor) (FEI).
6. Vitrobot tweezers (FEI).
7. Long tweezers.
8. Liquid cryogens: nitrogen and ethane gas cylinders with attached flow regulators.
9. Lacey carbon grids (Ted Pella Inc).
10. Cryo-transfer dewar (SPI Supplies).
11. Filter paper (Ted Pella Inc).
12. Kimwipes.
13. Hairdryer.
14. Yellow tips.

3 Methods

The entire process of virus purification spans several days. To prevent the degradation of the virus, samples should be frozen on cryo-EM grids within 72 h after purification. The following methods have been adapted from previously published procedures [5, 7].

3.1 Virus Culture

Two methods are listed below for the large-scale production of immature and mature virus particles. Incubations are carried out at 29 °C with 5 % carbon dioxide. All centrifugation steps are carried out at 4 °C.

1. For mature dengue virus production, C6/36 *Aedes albopictus* cells were cultured in RPMI media supplemented with 10 % (v/v) FBS in two- and ten-cell stacks to around 90 % confluence (*see* **Note 1**). The total volume of media should be 1.5 L. For immature dengue virus production, C6/36 cells were cultured in MEM (without HEPES) with 10 % (v/v) FBS to approximately 90 % confluence [7] (*see* **Note 2**).
2. Ensure that the media in the cell stacks are level (*see* **Note 3**). Incubate the stacks for 2 days or until the cells have reached 70–90 % confluence.
3. Defrost the virus stock (*see* **Note 4**) and dilute it to 300 mL with either RPMI or MEM medium supplemented with 2 % (v/v) FBS. Discard the media from the cell stacks before infection. Aliquot proportional amounts of the virus stock into the cell stacks and incubate them for 1.5 h. Rock the stacks from side to side every 15 min.
4. The inoculum is removed from both cell stacks and replaced with a total of 1.5 L of fresh media containing 2 % (v/v) FBS.

For immature virus production, replace the medium with 1.5 L of MEM containing 2 % (v/v) FBS and 20 mM filter-sterilized NH_4Cl (*see* **Note 5**).

5. Harvest at 48–60 h or 4 days post-infection for immature and mature viruses, respectively. Centrifuge at 9,000 × *g* for 45 min.

3.2 Virus Purification and Concentration

1. The supernatant is transferred to a large flask and swirled very gently with an adequate amount of 40 % (w/v) PEG 8000 to achieve a final concentration of 8 % (w/v) PEG (*see* **Note 6**). Leave the flask at 4 °C overnight.
2. Centrifuge the suspension at 14,000 × *g* for 1 h.
3. The pellet is gently resuspended in 10 mL NTE buffer using a pipette. Rinse the centrifuge bottle with a further 5 mL of NTE buffer after the initial 10 mL has been moved to another tube (*see* **Note 7**).
4. Purify the virus suspension through a sucrose cushion by transferring it into an ultracentrifuge tube. Gently add 5 mL of 24 % (w/v) sucrose to the bottom of the suspension using a Pasteur pipette. Do not mix the two solutions. Centrifuge at 105,000 × *g* for 1.5 h.
5. Invert the tube to remove the sucrose and wick off the excess solution. Add 500 μL NTE buffer to cover the pellet. This is kept overnight at 4 °C to allow the pellet to soften (*see* **Note 8**).
6. Gently resuspend the virus and transfer the supernatant into a new 1.5 mL Eppendorf tube. Centrifuge on a benchtop centrifuge at 16,000 × *g* for 2 min. Transfer the supernatant into a new Eppendorf tube and repeat the centrifugation process.
7. Prepare a potassium tartrate-glycerol gradient ranging from 10 to 30 % in an ultra-clear tube suitable for the specific ultracentrifuge rotor. If the Beckman Ti70 rotor is used, prepare 10 mL of the gradient in a 12 mL tube. The stopcocks of the gradient maker should be opened completely (*see* **Note 9**).
8. Carefully layer the virus suspension over the top of the potassium tartrate-glycerol gradient. Ultracentrifuge the sample at 175,000 × *g* for 2 h. The deceleration of the rotor should be set to zero.
9. To visualize the virus band, the tube is clamped such that the bottom of the tube is suspended above an inverted torch discharging a narrow beam (Fig. 2) (*see* **Note 10**).
10. The virus band is harvested using an 18 G needle connected to a 5 mL syringe. With the bevel facing up, pierce the tube about 1–2 mm below the virus band (*see* **Note 11**). Recover the entire band containing the virus fraction while pulling slowly on the plunger.

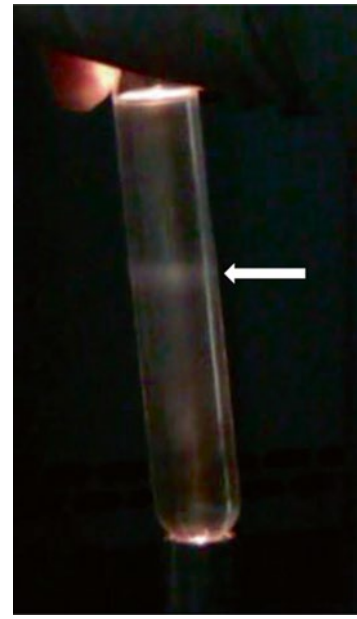

Fig. 2 The virus band (*arrowed*) can be visualized by shining a torch through the bottom of the tube, in a darkened room

11. Transfer the virus fraction to a 100 kDa MWCO centrifugal filter and conduct a buffer exchange with NTE buffer to remove the potassium tartrate. The virus is concentrated to 100–150 µL by centrifuging at 16,000 × *g* at 4 °C (*see* **Note 12**). The final concentration of the potassium tartrate should be below 0.01 %. Use enough NTE buffer to bring the potassium tartrate down to this concentration.

3.3 Virus Quantitation

1. Run 5 µL of the reduced purified virus sample on an SDS-PAGE gel alongside twofold dilutions of BSA starting from 2 mg/mL (until 0.25 mg/mL) and a molecular weight ladder (*see* **Note 13**). The gel is stained with Coomassie Blue for 1 h at room temperature and then destained with water (Fig. 3). A 2.5 µL sample from a virus concentration of around 0.75–1 mg/mL should produce an adequately populated grid.

3.4 Cryo-EM Sample Preparation and Grid Freezing

1. Glow discharge the required number of grids (with the carbon films facing upwards) in the plasma glow discharger at 5 mA for 50 s.
2. Assemble the Vitrobot cup by fitting the ethane cup into the inner receptacle and the grid holder into the outer ring followed by the metal spindle. The metal spindle acts as a thermal connector to conduct heat away from the ethane cup (Fig. 4).
3. Up to four grid boxes can be placed into the grid box holders (*see* **Note 14**).

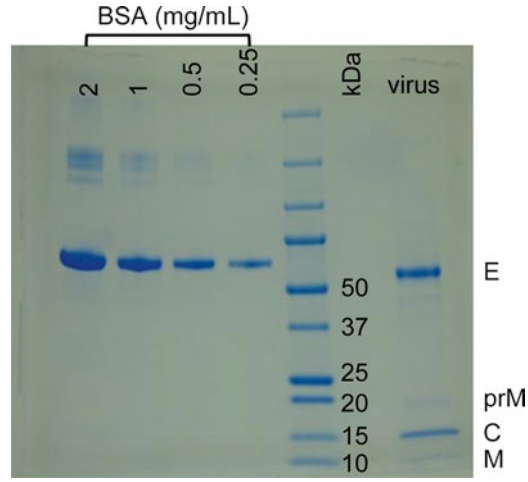

Fig. 3 Coomassie Blue-stained SDS-PAGE gel. 5 μL of the virus sample was run under reducing conditions against a BSA standard. The separated viral proteins are indicated: envelope (E), pre-membrane (prM), core (C), and membrane (M)

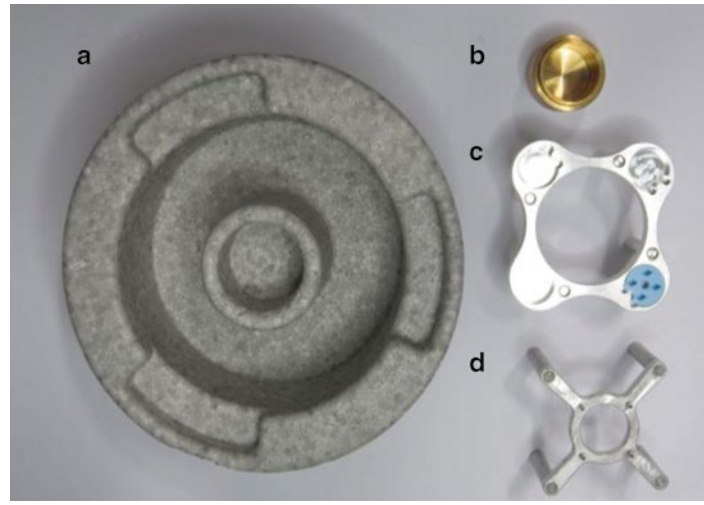

Fig. 4 Components of the Vitrobot cup (**a**). The ethane cup (**b**) should sit in the inner receptacle of the Vitrobot cup. The grid holder (**c**), holding one grid box (*lower right*), is placed over the ethane cup followed by the metal spindle (**d**)

4. Fill the humidifier reservoir in the Vitrobot with distilled water (Fig. 5).
5. Turn on the Vitrobot and program the conditions for the Vitrobot control chamber using the touch screen. At the "Console" page, set the temperature to the required value and the humidity to 100 %. At the "Options" page, select "Use foot pedal" and "Skip grid transfer." The former option allows for a hands-free control of the raising and lowering of the stage

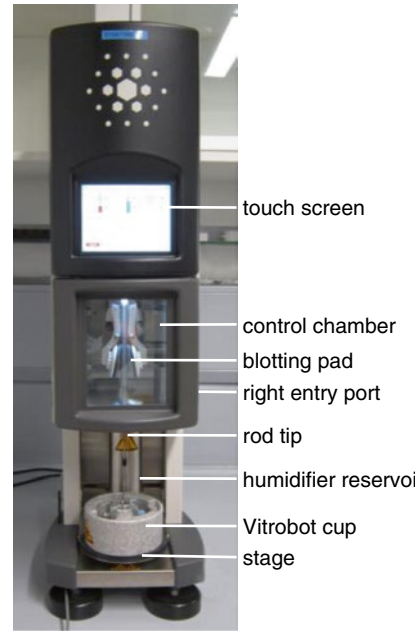

Fig. 5 The Vitrobot Mark IV machine and its component parts

and blotting and plunge-freezing of the grid, while the latter option keeps the plunger holding the grid immersed in ethane while the stage is being lowered. We use the following parameters in our grid preparations: Blot force = 1; Blot total = 1; Blot time is usually between 1 and 3 s (the optimization of blotting time is required as it varies between samples) (*see* **Note 15**).

6. Secure the filter papers onto the blotting pads with the clip rings and close the door to the Vitrobot.
7. Fill both the ethane cup and the outer nitrogen ring of the Vitrobot cup with liquid nitrogen to hasten the cooling process (*see* **Note 16**).
8. After the liquid nitrogen has completely evaporated from the central ethane cup and the ethane cup is cooled down to liquid nitrogen temperature, blow ethane gas at a moderate speed into the ethane cup using a yellow tip held against the side of the cup. The gas will liquefy. Fill the cup to the brim and wait for the liquefied ethane to appear cloudy indicating that the temperature has reached −170 °C.
9. Remove the metal spindle carefully (*see* **Note 17**) to prevent further cooling.
10. Holding the Vitrobot tweezers such that the spring faces the operator, pick up a grid such that the carbon side faces the right. Slide the black clamp ring on the tweezers down so that the grid is gripped tightly (*see* **Note 18**).

11. Step on the foot pedal to lower the rod tip from the control chamber so that the Vitrobot tweezers with the grid can be attached to the rod tip. Retract the tweezers into the control chamber by stepping on the foot pedal again and place the Vitrobot cup onto the stage (*see* **Note 19**).
12. Step on the foot pedal to raise the stage with Vitrobot cup. Apply 2.5 μL of sample to the entire surface of the carbon side of the grid through the right entry port found on the side of the Vitrobot.
13. Step on the foot pedal to activate the blotting of excess liquid on the grid using the blot force and time set previously. The blotted grid will then be plunge-frozen into liquid ethane.
14. Slide the tweezers out of the rod tip while keeping the grid immersed under liquid ethane. Very swiftly, transfer the grid into the liquid nitrogen in the outer ring. While applying pressure on the tweezers, raise the clamp ring and insert the grid into the preferred position in the grid holder.
15. The grids can then be used for imaging or stored in liquid nitrogen (*see* **Note 20**).

4 Notes

1. We find that growing the cells in 13–14 T175 flasks is sufficient for seeding both cell stacks. Confluency is reached approximately 2 days later but this is dependent on the rate of growth of the cells. A two-cell stack is needed as this can be placed under a light microscope to check the cell confluency. This will give an indication of how the cells are growing in the ten-cell stack.
2. A different medium, MEM, was used for the production of immature virus as we did not have good success producing immature dengue virus in RPMI supplemented with HEPES. Note that the C6/36 cells need to be adapted to MEM before use if they have been previously cultured in RPMI.
3. The growing cells will tend to cluster at the sides of the chambers if the stacks are incubated on an uneven surface. It may be easier to place the cell stacks on a level bench surface for 20–30 min prior to placing them into the incubator to let most of the cells settle rather than attempting to balance it while it is on a shelf in the incubator.
4. The virus stock for infecting both cell stacks can be prepared in advance and frozen at −80 °C. Stocks for mature virus preparations are harvested from three T175 flasks (4 days post-infection), each containing 25 mL of media. The clarified

supernatant is mixed with enough FBS to give a final concentration of approximately 25 % (v/v) FBS. Stocks for producing immature virus preparations are harvested from 10 T175 flasks 4 days post-infection. More viruses are needed to infect cells to generate enough immature viruses as less replication cycles occur during the shortened incubation period. Each stock is sufficient to infect two- and ten-cell stacks.

5. The presence of NH_4Cl elevates the pH in the Golgi and prevents furin from cleaving prM. This keeps the virion particles produced in the immature state.
6. PEG 8000 does not readily dissolve in NTE. Heating the mixture up to around 85 °C on a magnetic stirrer will help the PEG dissolve more quickly.
7. The pellet should be visible at the bottom of the centrifuge bottle. If you are using a fixed angle rotor, some of the PEG-precipitated virus will be present down the side of the centrifuge bottle. This may not be very visible, but do remember to rinse that portion of the bottle as well to maximize the virus yield.
8. Occasionally, the pellet may be very translucent and hard to visualize. In any case, the sucrose must still be removed as soon as possible.
9. The stir bar in the gradient maker should be adjusted to spin at a moderate pace at a medium setting so that will not cause the formation of air bubbles that may compromise the linearity of the gradient.
10. It will be easier to visualize the virus band if all the lights in the room have been turned off and the torch held against the bottom of the centrifuge tube.
11. The virus will be visible as a thin, concentrated band sandwiched between two layers of slightly cloudy cellular material. To control the rate of flow of the virus into the syringe, the syringe must first be connected to the needle with the plunger pushed all the way down. Otherwise, when the centrifuge tube is pierced, the liquid within the tube will start to flow into the syringe immediately.
12. Centrifuge the sample for an initial period of 5–10 min. Use this time as a gauge to set the amount of time needed for complete buffer exchange. Depending on the viscosity of the sample, the time needed for buffer exchange will vary depending on the quality of the harvested virus.
13. The BSA standard is relatively similar in size to E protein and provides a rough estimate of the amount of E protein that is present in the virus preparation. Load equivalent volumes of BSA and virus. If the concentration of E protein is below 0.25 mg/mL, it should preferably be concentrated further

prior to freezing onto grids. We generally find that concentrations of 0.75–1 mg/mL will give a good density of virus particles for cryo-EM. The Coomassie-stained gel will also allow you to check if the sample has been contaminated with immature virus. This will be evident by the presence of large amounts of prM (19–23 kDa) protein on the gel (Fig. 3). Some dengue strains may produce larger amounts of immature virions than others. The presence of large amounts of spiky immature virus particles will be detrimental if the downstream process involves complexing with antibodies since both populations of immature virus and mature virus complexed with antibody will look spiky under the microscope.

14. The grid holder is not numbered. To differentiate between the different grid boxes, mark the rim of the Vitrobot cup with a number so that each digit corresponds to the location of each grid box. This preempts a mix up if the grids are to be transferred for storage in liquid nitrogen.
15. The blot time can be varied between 1 and 4 s. A longer blot time is sometimes required for virus samples that contain a larger amount of cellular debris.
16. The level of the liquid nitrogen must be monitored constantly and replenished should the level fall. The outer receptacle should be at least ¾ filled with liquid nitrogen so that the grid boxes always remain completely submerged.
17. You may find it easier to remove the metal spindle if you press the flat blades of the long tweezers downwards against the horizontal arms of the metal spindle. Be careful not to sweep any frost found on the arms of the metal spindle into the ethane cup.
18. For operators who are left handed, position the grid with the carbon side facing the left-hand side. In this case, application of the virus sample will be made through the side port on the left of the machine.
19. You may want to conduct a dry run to ensure that the apparatus is functioning correctly. Attach the Vitrobot tweezers (without a grid) to the rod, place the Vitrobot cup on the stage, and perform a mock plunge-freeze into the ethane cup.
20. It is necessary to screen one grid from each sample before attempting to freeze the rest of the sample. Adjustments to the blot time can be made if the ice thickness is not optimal. A very concentrated virus sample may sometimes lead to uneven ice formation on the grid and may cause particles to overlap, so a slight dilution of the sample may help and cause the ice quality to improve. However, this can lead to a smaller concentration of particles on the grid. But generally, 3–4 grids for each sample should provide sufficient numbers of particles for reconstruction.

References

1. Chang J, Liu X, Rochat RH et al (2012) Reconstructing virus structures from nanometer to near-atomic resolutions with cryo-electron microscopy and tomography. Adv Exp Med Biol 726:49–90
2. Lindenbach BD, Rice CM (2001) Flaviviridae: the viruses and their replication. In: Knipe DM, Howley PM (eds) Fields virology, vol II, 4th edn. Lippincott Williams and Wilkins, Philadelphia, PA, pp 991–1042
3. Zhang Y, Corver J, Chipman PR et al (2003) Structures of immature flavivirus particles. EMBO J 22(11):2604–2613
4. Zhang Y, Kaufmann B, Chipman PR et al (2007) Structure of immature West Nile virus. J Virol 81(11):6141–6145
5. Kuhn RJ, Zhang W, Rossmann MG et al (2002) Structure of dengue virus: implications for flavivirus organization, maturation and fusion. Cell 108:717–725
6. Stadler K, Allison SL, Schalich J et al (1997) Proteolytic activation of tick-borne encephalitis virus by furin. J Virol 71(11): 8475–8481
7. Yu IM, Zhang W, Holdaway HA et al (2008) Structure of the immature dengue virus at low pH primes proteolytic maturation. Science 319(5871):1834–1837
8. Ferreira GP, Trindade GS, Vilela JMC et al (2008) Climbing the steps of viral atomic force microscopy: visualisation of dengue virus particles. J Microsc 231(Pt1): 180–185
9. Junjhon J, Edwards TJ, Utaipat U et al (2010) Influence of pr-M cleavage on the heterogeneity of extracellular dengue virus particles. J Virol 84(16):8353–8358
10. Iancu CV, Tivol WF, Schooler JB et al (2006) Electron cryotomography sample preparation using the Vitrobot. Nat Protoc 1(6): 2813–2891
11. Dobro MJ, Melanson LA, Jensen GJ et al (2010) Plunge freezing for electron cryomicroscopy. Methods Enzymol 481:63–82