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Correlative Light and Electron Microscopy of Influenza Virus Entry and Budding

Lorna Hodgson[1], Paul Verkade[1,2], Yohei Yamauchi[3*]

[1] School of Biochemistry, [2] Wolfson Bioimaging Facility, [3] School of Cellular and Molecular Medicine, University of Bristol, Bristol, U.K.

* Corresponding author:
School of Cellular and Molecular Medicine, Faculty of Biomedical Sciences, University of Bristol, BS8 1TD, Clifton, Bristol, United Kingdom

Abstract

Influenza A virus (IAV) entry is a stepwise process regulated by viral and cellular cues, and facilitating cellular functions. Virus entry begins by attachment of hemagglutinin to cell surface sialic acids, followed by endocytic uptake, vesicular transport along microtubules, low pH-mediated viral membrane fusion with the late endosomal membrane, capsid uncoating, viral ribonucleoprotein (vRNP) release, and nuclear import of vRNPs. Here we show a basic methodology to visualise incoming and egressing IAV particles by correlative light and electron microscopy (CLEM). We combine fluorescence microscopy of virus infected human lung carcinoma A549 cells with high pressure freezing (HPF) and in-resin fluorescence CLEM and the Tokuyasu CLEM method. This approach forms a basis to study the virus lifecycle and virus-host interactions at the ultrastructural level.

Running Head: CLEM of Influenza virus entry and budding

Key words: Influenza virus entry, Fluorescent confocal microscopy, Electron microscopy, CLEM, Tokuyasu, High pressure freezing, Endosome

1 Introduction

Virus-host interactions tightly regulate virus entry [2]. Influenza A virus (IAV) entry is a multistep process that is separated into single steps i.e. attachment, endocytosis, trafficking in endosomes, viral fusion at low pH (at late endosomes), capsid uncoating, genome penetration, and genome import into the nucleus [3,4]. Genome import triggers transcription of early genes such as nucleoprotein (NP). Although we have a basic understanding of virus entry and its major host players, the molecular details of how, for example, capsid uncoating is regulated is incomplete. During virus entry, receptor, chemical, and enzymatic cues trigger changes in the virus particle and propel the virus further downstream the entry pathway [5]. Cellular processes including endosome maturation, aggresome processing (a cellular mechanism for disposal of misfolded protein aggregates) and nuclear import promote IAV entry. Overall, viruses, as obligatory parasites, must communicate intimately with the host for successful entry and establishment of infection [5,6].

The fluorescent visualisation of IAV entry into cells can be done by labelling the viral envelope with lipophilic dyes such as R18, DiOC, DiD [7,8], or by primary amine labelling of the viral glycoprotein using NHS (N-hydroxysuccinimide) esters conjugated to a fluorescent probe. In the former approach, the lipophilic dye is primarily incorporated into the viral membrane at self-quenching concentrations. When the virus fuses with the endosomal membrane, the dye de-quenches and the fluorescence intensity increases. Alternatively, two lipophilic dyes (R18, red and DiOC, green) can be used simultaneously. In the labelled virus, the green fluorescence of DiOC is suppressed by both self-quenching and by fluorescent resonance energy transfer (FRET) from DiOC to R18, whereas R18 is partly self-quenched. The labelling approach enables virus imaging up to the step of fusion.

For large DNA viruses such as herpes simplex virus type 1 (HSV-1) and vaccinia virus (VACV), the genetic incorporation of a fluorescent protein-encoding gene into the viral core is feasible and supports the viral life cycle (for examples see [9,10]). The IAV genome can be modified using reverse

genetics [11,12] but the viral capsid cannot be genetically altered to fuse a fluorescent protein without permanent deleterious effects on virus viability. Of the IAV structural elements, the polymerase subunits PA and PB2 have been successfully tagged and used for fluorescence imaging. Examples are WSN PB2-GFP11 and WSN PA-GFP, A/WSN/33 (H1N1) strains in which PB2 is fused to the C-terminal 16 amino acids (residues 215 to 230) of GFP, and PA is fused to the full-length GFP, respectively [13,14]. To visualise the GFP fluorescence in WSN PB2-GFP11-infected cells, the split-green fluorescent protein (split-GFP) system is employed and the complementary GFP fragment (GFP1-10) is supplied *in trans* [15]. On one hand, the low abundance of IAV polymerase complexes per particle renders robust fluorescent imaging during entry a challenge. On the other hand, these fluorescent IAVs can be used to study post-entry, viral replication by live fluorescent imaging.

Fluorescence microscopy can be combined with a variety of different imaging techniques such as atomic force microscopy (AFM) [16], soft X-ray [17] and electron microscopy (EM) [18] to study viral structure and trafficking within host cells. The combination of light and electron microscopy is perhaps the most commonly used correlative microscopy technique. Correlative light and electron microscopy (CLEM) is a powerful tool for studying viruses as it provides both functional and structural information. The light microscopy stage enables visualisation of fluorescently tagged viral particles and allows study of dynamic or rare intracellular trafficking events, whilst the electron microscopy reveals the ultrastructural composition of the virus and/or localises it within its cellular context.

A variety of different CLEM workflows exist [19] utilising a wide range of imaging modalities from super resolution fluorescence microscopy [20] to 3D electron tomography [21]. The majority of electron microscopy and CLEM studies of viruses have involved using cryo electron microscopy to study viral structures in their near native state at high resolution [22,18,23]. This chapter describes two alternative CLEM techniques, in-resin fluorescence [20,16] and Tokuyasu CLEM [24,25]. In-resin fluorescence combines high pressure freezing with freeze substitution and embedding in a

hydrophobic resin, whilst Tokuasyu involves chemical fixation of cells, embedding in gelatin and cryosectioning (see Fig. 1). Both techniques preserve the fluorescence of the sample throughout processing and therefore enable imaging of the same region of a section by both modalities. In addition, both methods can be combined with immunolabelling to precisely localise viral proteins. Following processing, the sections are imaged using a standard epifluorescent microscope to identify regions of interest before transferring to the electron microscope where the sample is imaged at high resolution placing the fluorescence in its structural context within the cell. There are minimal processing and grid handling steps in-between the two imaging stages therefore enabling an accurate and direct correlation of the acquired LM and TEM images. Furthermore, the correlation is aided by the addition of fluorescent beads that are visible in both modalities and it is generated using open source software that helps facilitate registration of the two images [16,26].

In this chapter, we apply CLEM to visualise both IAV entry and budding using two different approaches (Fig. 5 and 6). For entry studies, it is difficult to validate whether a structure is an IAV particle or not only by its morphology. Therefore, we used Alexa Fluor-labelling to confirm that the structures are indeed virus particles. The advantage of Alexa Fluor is its photostability and brightness. The fluorophore is retained on the endosomal surface following viral fusion (Fig. 6F). Therefore, the application of CLEM allows us to determine the ultrastructural nature and cellular context of the fluorescence and distinguish between viral particles trafficking through the endocytic network and those that have fused and escaped the endosome. As a proof of principle, virus budding was imaged by CLEM using the WSN PA-GFP strain. This technique can be combined with multi-colour fluorescent tagging of cellular markers (e.g. endosomes, Rabs, cytoskeleton, essential host factors), antibody labelling, and allow ultrastructural examination of virus-host interactions during virus entry and replication.

2 Materials

2.1 IAV labelling with NHS-Ester Alexa Fluor 488

1. Purified Influenza A virus X31 strain (an H3N2 reassorted strain derived from the A/Puerto Rico/8/34 (PR8) and A/Hong Kong/1/68 strains) (Virapur Inc., CA, USA) in formulation buffer (40 % sucrose, 0.02 % BSA, 20 mM HEPES pH 7.4, 100 mM NaCl, 2 mM $MgCl_2$). The protein concentration is approximately 1.5 mg/mL.
2. 0.2 M sodium bicarbonate in distilled H_2O (pH 8.3).
3. Alexa Fluor™ 488 NHS Ester (succinimidyl ester) (Thermo Fisher), 10 mg/mL (15.5 mM) in DMSO.
4. MNT buffer: 20 mM MES buffer, 150 mM NaCl, 30 mM Tris-HCl pH 7.5, sterile filtered through a 0.22 μm filter.
5. Ultrapure 30 % and 60 % sucrose in MNT buffer.
6. Ultracentrifuge buckets (JS-24.15, Beckman, or equivalent).
7. Ultracentrifuge.
8. Ultracentrifuge tubes (15 mL).
9. 5 mL Syringe.
10. 22 Gauge needle.
11. Rotating mixer.
12. Phosphate buffered saline (PBS).
13. 10 kDa cut-off Amicon centrifugal filter.
14. 1.5 mL tubes.

2.2 Plaque assay

1. Madin-Darby canine kidney (MDCK) II cells (ATCC, #CRL-2936).
15. MDCK growth medium: Dulbecco's Modified Eagle Medium (DMEM), 10 % (v/v) fetal calf serum (FCS), 100 U/mL penicillin G, 100 μg/mL streptomycin.
16. PBS.

17. 7.5 % (w/v) sodium bicarbonate solution.
18. 10 % (w/v) Endotoxin-Free Bovine Serum Albumin (BSA).
19. 100× Mg^{2+}/Ca^{2+}: 0.2 M magnesium and 0.1 M calcium chloride in distilled H_2O, filtered through a 0.2 μm membrane filter.
20. Infection PBS (PBSi): 500 mL PBS supplemented with 15 mL 10 % BSA, 100 U/mL penicillin G, 100 μg/mL streptomycin, and 5 mL of 100× Mg^{2+}/Ca^{2+}.
21. 2× Minimum Essential Medium (MEM): To make 500 mL, combine 100 mL of 10× MEM, 10 mL of 200 mM L-glutamine, 10 mL of Pen-Strep (10,000 U/mL penicillin G, 10,000 μg/mL streptomycin), 20 mL of 7.5 % sodium bicarbonate, 10 mL of 1 M HEPES, and 6 mL of 35 % BSA. Add 350 mL of ddH_2O and filter through a 0.2 μm membrane filter and store at 4 °C.
22. 1 mg/mL TPCK-trypsin in distilled H_2O.
23. 2.4 % (w/v) Avicel in distilled H_2O, autoclaved.
24. 4 % (w/v) formaldehyde solution in PBS.
25. 1 M MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) in distilled H_2O, store at - 20 °C in 0.5 mL aliquots.

2.3 IAV infection assay

1. A549 (human lung adenocarcinoma) cells (ATCC, #CCL-185).
2. A549 growth medium: DMEM, 10 % (v/v) FCS.
3. Trypsin-EDTA.
4. IAV X31-Alexa Fluor 488 (from section 2.1).
5. IAV WSN PA-GFP [14].
6. Infection medium: DMEM, 50 mM HEPES (pH 6.8), 0.2 % BSA.
7. 60-mm petri dish.
8. Crushed ice.
9. Ice bucket.

10. 2x metal plates: roughly 8 cm (W) x 10 cm (L) x 5 -10 mm (D) (*see* **Note 1**).

2.4 Chemical fixation and embedding for Tokuyasu CLEM

1. Cell scraper.
2. Fixative: 4 % (w/v) paraformaldehyde, 0.1M phosphate buffer.
3. Benchtop microcentrifuge.
4. 0.15 % glycine.
5. 12 % gelatin.
6. Ice.
7. Razor blades (single and double edged).
8. Cryoprotectant: 2.3 M sucrose, 0.1 M PB.
9. Rotator.
10. Tweezers.
11. Aluminum pins for cryomicrotomy.
12. Liquid nitrogen.
13. Whatman 1 filter paper.

2.5 Cryosectioning of gelatin embedded samples

1. Diamond knives for trimming and ultrathin cryo-sectioning (e.g. Cryo trim 45 ° and Cryo immuno 35 °, Diatome).
2. Pick up solution: 1 % methylcellulose, 1.15 M sucrose.
3. Carbon and pioloform coated H6 copper finder grids.
4. Cryoultramicrotome (e.g. Leica EM UC7 with FC7 cryo attachment).
5. Pick up the loop.

2.6 High pressure freezing and freeze substitution for in-resin CLEM

1. High Pressure Freezer (e.g. Leica EM PACT2 with EM RTS).
2. Leica 1.5 x 0.1 mm membrane carriers.
3. Long, narrow tweezers for loading carriers into RTS (#72919-SS, EMS).
4. Stereo microscope.
5. Cell scrapers.
6. Cryoprotectant: 15 % BSA, phenol red free DMEM containing 10 % FBS (*see* **Note 2)**.
7. Benchtop microcentrifuge.
8. Liquid nitrogen.
9. Freeze substitution and low temperature embedding system (Leica EM AFS2).
10. AFS2 consumables (reagent baths with flow through rings, reagent containers, 10 mL syringes and dispensing tips).
11. Freeze substitution mixture: 0.2 % uranyl acetate, 0.01 % tannic acid, 5 % H_2O, acetone) (*see* **Note 3**).
12. MonoStep Lowicryl, HM20.
13. 100 % Ethanol dried over a molecular sieve.
14. Acetone (99.5 %).
15. Trypsin-EDTA.

2.7 Ultramicrotomy of Lowicryl embedded samples

1. Plyers.
2. Specimen carrier detaching tool (Leica).
3. Liquid nitrogen.
4. Razor blades (single and double edged).
5. Ultramicrotome (eg. Leica UC7).
6. Perfect loop.
7. Carbon coated Formvar films on copper F1 finder grids (Agar Scientific).
8. Diamond knife (e.g. Ultra 45 °, Diatome).

2.8 Light microscopy imaging of CLEM sections

1. Glass slides.
2. High precision glass coverslips, No. 1.5 thickness.
3. 50 % glycerol in H_2O.
4. Deep red (633 / 660 nm) PS-Speck™ microspheres (Thermo Fisher).
5. Hoescht 33342 nucleic acid stain.
6. Fluorescent microscope with high NA oil immersion lens (e.g. Leica DMI4000B inverted epifluorescence microscope with a 63 x NA 1.4 objective, DFC365 FX CCD camera and ebx 75-04 Xenon lamp).

2.9 Immunolabelling of CLEM sections

1. Fine, angled (15 °) DUMONT tweezers (EMS).
2. Parafilm.
3. PBS.
4. Rabbit anti-GFP (Thermo Fisher).
5. Rabbit anti-HA (Pinda) polyclonal Ab (produced in-house).
6. Donkey anti rabbit IgG (H&L) 10 nm (Aurion).
7. 0.15 % glycine.
8. Blocking solution: 1 % Acetylated BSA (BSA-c, Aurion), PBS.

2.10 Poststaining of Tokuyasu sections

1. Ice.
2. Parafilm.
3. Distilled H_2O.
4. Pick up solution: 1.8 % methylcellulose, 0.3 % uranyl acetate.

5. Drying loop.
6. Whatman 1 filter paper.

2.11 Poststaining of Lowicryl embedded sections

1. Parafilm.
2. Distilled H_2O.
3. 3 % uranyl acetate.
4. Reynolds lead citrate solution: lead nitrate, sodium citrate, sodium hydroxide
5. Sodium hydroxide pellets.

2.12 TEM and correlation

1. Transmission electron microscope (e.g. 120 kV BioTwin TEM, FEI).
2. Icy imaging software with ec-CLEM plugin [26].

3 Methods

3.1 IAV labelling with NHS-Ester Alexa Fluor 488 (*see* Note 4)

1. Thaw one vial of 100 μL of IAV X31 and transfer to an Eppendorf tube.
2. Add 210 μL of fresh 0.2 M sodium bicarbonate buffer.
3. Add 1 μL of 15.5 mM Alexa Fluor NHS-Ester (final 50 μM) and immediately vortex briefly.
4. Rotate or rock the tube for 1 h at room temperature in the dark.
5. Take 3.5 mL of 60 % sucrose in MNT into an ultracentrifuge tube.
6. Carefully layer on top, 6 mL of 30 % sucrose in MNT.
7. Layer the virus solution from step 4 on top of the sucrose cushion.
8. Ultracentrifuge at 24,000 rpm (110,500 x g) for 90 min at 4 °C.

9. To harvest the virus, puncture the ultracentrifuge tube right beneath the interphase of the two sucrose layers, using a 22-gauge needle attached to a syringe, and draw out 1-1.5 mL of liquid containing virus.
10. Dilute the liquid into PBS to a total of 15 mL in an Amicon centrifugal filter. (*see* **Note 5**)
11. Centrifuge at 4,000 x g for 10-15 min at 4 °C (*see* **Note 6**).
12. Aliquot the concentrate (X31-Alexa Fluor 488) and store at -80 °C until use. Make a single tube of 5 μL for titration analysis.

3.2 IAV X31-AF488 titration

1. Seed MDCK II cells in 6-well plates so that they are confluent at the time of the assay.
2. Thaw one aliquot of 100 μL of X31-Alexa Fluor 488 and transfer to an Eppendorf tube.
3. Generate 10-fild dilution for the plaque assay in PBSi. In an Eppendorf tube, take 200 μL of PBSi and pipette 2 μL of labelled IAV and mix well. This is the 10^{-2} dilution.
4. Take 100 μL from step 3 and pipette into a fresh Eppendorf tube containing 900 μL of PBSi, and mix well. This is the 10^{-3} dilution.
5. Repeat this up to 10^{-8} dilution, using new tips for each dilution.
6. Wash the MDCK II cells with PBSi (2 mL/well).
7. Inoculate 200 μL of the virus dilutions (10^{-3} to 10^{-8}) into each well of the 6-well plate.
8. Incubate at 37 °C for 1 h. Swirl the plate every 15 min in order to spread the virus evenly on the cell surface.
9. Prepare a 1:1 mixture of 2x MEM and 2.4 % Avicel (overlay medium), mix well and warm to 37 °C.
10. Add TPCK-trypsin to a final concentration of 1 mg/mL.
11. Wash the cells with 2 mL of PBSi.
12. Overlay with 2 mL per well of overlay medium from step 9.
13. Incubate at 37 °C, 5% CO_2 for 3 days on a flat surface for plaques to form (*see* **Note 7**).
14. Remove the overlay, and wash the cells once in PBS.

15. Incubate in 2 mL/well of PBS, 0.5 mg/mL MTT for 30 min at 37 °C (*see* **Note 8**).
16. Wash once in PBS.
17. Count the plaques and calculate the plaque forming unit (PFU) per mL e.g. if 15 plaques exist in the 10^{-6} dilution inoculant, the viral titer is 15 x 10^{6} x (1 mL/0.2 mL inoculant) = 7.5 x 10^{7} PFU/mL.

3.3 IAV infection assays

13. Grow A549 cells on 60-mm petri dishes to ~80 % confluency.
14. Wash once in 5 mL of infection medium.
15. Add 1 mL of infection medium containing virus at a multiplicity of infection (MOI) of 10.
16. Bind on ice for 45 min with occasional tilting of the dish every 15 min to evenly distribute the inoculum.
17. Aspirate inoculum and wash once in 5 mL of infection medium.
18. Replace with 5 mL of warm (37 °C) infection medium.
19. Place on a pre-warmed metal plate in a 37 °C, 5 % CO_2 incubator.
20. Incubate for 30 min (X31-Alexa Fluor 488) or 16 h (WSN PA-GFP strain).
21. Proceed to sample processing for CLEM.

3.4 Sample processing for Tokuyasu CLEM

1. Grow A549 cells on 60-mm petri dishes to ~80-100 % confluency and infect with X31-Alexa Fluor 488 for 30 min, or WSN PA-GFP for 16 h, respectively.
2. Remove infection medium from dish and wash in 1mL warm PBS. Carefully add an equal volume of fixative, 4 % PFA in 0.1 M PB and fix at room temperature for 20 min (*see* **Note 9**).

3. Remove fixative and replace with fresh 4 % PFA in 0.1 M PB for 2 h at room temperature or overnight at 4 ºC.
4. Wash the culture dish in 2 x PBS for 5 min each.
5. Quench aldehydes in 0.15 % glycine in PBS for 10 min
6. Remove glycine and add ~250 μl of 1 % gelatin in 0.1 M PB. Mix by hand to cover all cells in gelatin and gently scrape the cells from the surface of the dish with a cell scraper. Transfer cells to a 1.5 mL Eppendorf tube and centrifuge at 13, 000 rpm for 1 min.
7. Remove the supernatant and resuspend in 0.5 mL of warm 12 % gelatin. Place at 37 °C for 10 min.
8. Centrifuge at 13, 000 rpm for 1 min and remove the supernatant leaving ~100 μl of supernatant. Place immediately on ice for 20 min.
9. Carefully cut off the tip of the Eppendorf just above the cell pellet using a single edged razor blade and cut the tip in half (see Figure 2B). Remove the gelatin embedded pellet from the tube using tweezers and place into a vial containing 2.3 M sucrose. Leave on ice for a further 20 min.
10. Cut the solidified gelatin pellet into small (~0.5 – 2 mm) cubes on ice using a double-edged razor blade and submerge into 2.3 M sucrose. Infiltrate with sucrose overnight at 4 ºC on a rotator.

11. Working on ice and under a stereo microscope, remove the bocks from sucrose using precooled tweezers and place onto aluminium pins. Orientate the specimen blocks as required on the tip of the pin, ensuring that the cells are facing upwards (Figure 2C). Trim the block if too large to fit on the centre of the pin and remove excess gelatin with a double-edged razor blade. Remove excess sucrose from around the base of the block using filter paper, but do not let the block dry out (*see* **Note 10**).
12. Freeze pins by rapidly plunging in liquid nitrogen and store pins in liquid nitrogen until needed.

13. Cool the cryoultramicrotome chamber to -100 °C and place in trimming (Cryo trim 45 °) and cutting (Cryo immuno 35 °) Diamond knives to cool. Mount the frozen specimen into the pin holder.
14. Trim the surface of the block at 100 mm/s with a feed of 200 nm using the trimming knife until the surface of the block is flat and the cells have been reached. (*see* **Note 11**). Trim the block face with the ioniser set high to remove trimmed material from the knife edge.
15. Trim 50μm from all four sides of the block to create a block face of approximately 250 μm x 375 μm (*see* **Note 12**).
16. Rotate the trimmed block to ensure that the longest side is orientated vertically, replace the knife with the cryo immuno knife and cool the chamber to -120 °C.
17. Cut ultrathin sections (70-100 nm) with a cutting speed of ~0.8 – 2 mm/s
18. Pick up ribbons of cryosections in a droplet of 1 % methylcellulose, 1.15 M sucrose and mount onto Pioloform and carbon coated copper finder grids.
19. Store sections at 4 °C for up to 2 months.

3.5 Sample processing for in-resin CLEM

1. Grow A549 cells on 60-mm petri dishes to ~80 % confluency and infect with IAV X31-Alexa Fluor 488 for 30 min, or WSN PA-GFP for 16 h, respectively.
2. Wash cells in 1 mL PBS and dissociate from the culture dish by digestion with 0.5 mL of warm trypsin for 5 min at 37 °C (*see* **Note 13**). Add an equal volume of warm cryoprotectant to the dish of cells and resuspend by pipetting. Transfer to a 1.5 mL Eppendorf tube and centrifuge at 3200 rpm for 2 min (*see* **Note 14**).
3. Remove the supernatant, being careful not to disturb the cell pellet and load 1 μl of cells into a 0.1-mm membrane carrier, pre-loaded into the Rapid Transfer System (RTS). Ensure that the inner well of the carrier is slightly overfilled before transferring the RTS to the EMPACT2 for high pressure freezing (Figure 3C). Care must now be taken to keep membrane carriers under liquid nitrogen to prevent ice crystal contamination through

warming of the samples. Therefore, all tweezers and tubes that will come into contact with the frozen samples, must be pre-cooled.

4. Fill the AFS2 dewar with liquid nitrogen and precool to -130 °C. Fill the reagent bath / flow through ring with freeze substitution (FS) mixture, 0.2 % uranyl acetate (diluted from 5 % UA in methanol), 0.01 % tannic acid (from 10 % stock in acetone), 5 % H_2O in acetone). Place the carriers into the transfer box under liquid nitrogen and transfer into the AFS chamber (Figure 3D). Load carriers into the flow through rings so that the cells are in contact with the FS mixture (*see* **Note 15**).
5. Attach the FSP and begin automated FS as described by Johnson et al [20]. Briefly, hold temperature at -130 ºC for 1 h, warm to -90 ºC at 20 ºC/h, hold at -90 ºC for 4 h, increase temperature to -45 ºC with a slope of 5 ºC/h and finally hold at 45 ºC for 2 h.
6. Pause the FS program and reorientate the samples so that the carrier is sitting flat in the flow through ring, with the cells facing upwards. Wash the samples in pure acetone for 30 min and 2 x 30 min in 100 % ethanol at -45 ºC.
7. Infiltrate with increasing concentrations (25 %, 50 %, 75 %) of Lowicryl HM20 in pure ethanol for 2 h each before incubating overnight in 100 % resin followed by a further 3 washes in 100 % Lowicryl for 2 h each. UV polymerise for 24 h at -45 ºC, before raising the temperature to 0 ºC for 12 h and holding at 0 ºC for a further 12 h with UV light on throughout. Finally, warm samples to room temperature and store protected from light.
8. Remove polymerised blocks from the flow through rings using plyers to cut away plastic reagent bath and excess resin.
9. Trim resin from the surface and sides of the carrier using a single edged razor blade. Carefully remove the carrier from the block either by hand using a single edged razor blade or by dipping the tip of the block in liquid nitrogen for ~30 s and breaking the carrier off with the specimen detachment tool warmed to 40 ºC (Figure 3F).

10. Cut 300 nm thick sections from the surface of the untrimmed-block using a glass knife and UC7 ultramicrotome (Leica Microsystems). Mount thick sections onto glass slides and screen fluorescence using a light microscope (*see* **Note 16**).
11. Trim the surface of the block using a razor blade to a small pyramid to include the fluorescent region of interest visualised previously.
12. Cut 70-150 nm sections using a Diamond knife and pick up onto a Formvar and carbon coated copper finder grids using a perfect loop (*see* **Notes 17, 18, 19**). Store grids in grid boxes in the dark and proceed to LM imaging immediately.

3.6 Light microscopy imaging of CLEM sections

1. Remove methylcellulose and gelatin form the Tokuyasu sections by incubating the grids, section facing down on drops of PBS for 2 x 10 min at 37 °C and 1 x 10 min at room temperature. Lowicryl sections do not need washing before imaging, therefore proceed straight to step 2.

2. Incubate grids on 10 μl of PS-Speck™ microspheres diluted in PBS (~1:10 – 1:50) for 10 min (*see* **Note 20**).
3. Blot the grids with filter paper and wash in distilled water, 3 x 1 min.
4. Incubate grids on a drop of Hoescht nuclear dye diluted in water (1:10,000) for 5 min before washing the grids in 3 drops of water.
5. Mount the grids for LM imaging using 50 % glycerol in water (*see* **Note 21**). Apply a 20 μl drop of 50 % glycerol to the centre of a square coverslip and place the grid onto the drop, with the sections facing down. Gently lower the glass slide onto the coverslip to avoid generation of air bubbles (Figure 4A).

6. Place the glass slide onto the fluorescence microscope so that the section side of the grid is facing the objective. Take an overview of the grid at low magnification (10 or 20 x lens) to locate the sections on the grid and identify areas of interest. Switch to a higher magnification oil immersion lens (63 x or 100 x) and image the areas of interest using

both fluorescence and transmitted light. Take care to record the precise position of the region of interest on the finder grid and capture multiple z positions of each grid square (*see* **Note 22**).

7. Following LM imaging, detach the coverslip from the glass slide by pipetting distilled H_2O around edges of the coverslip (Figure 4D). After a few seconds, the coverslip will begin to float. Pick up the coverslip using tweezers and invert so that the grid is on top and float the grid from the coverslip using a further drop of water.
8. Rinse any remaining glycerol from the grid by 3 x 1 min washes in water and carefully dry the backside of the grid with filter paper.

3.7 Immunolabelling of CLEM sections

All immunolabelling incubations are performed on 100-200 μl drops, unless otherwise stated, on parafilm and grids are floated on the incubation solutions, section-side down (Figure 4E).

1. Incubate grids on 2 x 5 min drops of 0.15 % glycine in PBS to quench aldehydes.
2. Block non-specific binding sites in 1 % BSA-c in PBS for 2 x 5 min
3. Incubate grids in 10 μl primary antibody diluted in 1 % BSA for 1 h at room temperature (*see* **Note 23**).
4. Wash 4 x 2 min in 0.1 % BSA in PBS
5. Incubate grids in 10 μl of donkey anti rabbit IgG conjugated to 10nm gold diluted in 1 % BSA for 1 h (*see* **Note 24**).
6. Wash 2 x 2 min in 0.1 % BSA in PBS
7. Wash in 2 x 2 min in PBS

3.8 Poststaining of in-resin CLEM sections

1. Poststain the grids, section facing down on a drop of 3 % uranyl acetate for 10 min in the dark.

2. Wash 2 x 5 min in distilled H_2O.
3. Incubate grids on a droplet of Reynolds lead citrate solution [27] for 10 min in the presence of NaOH pellets.
4. Wash 2 x 5 min in distilled H_2O and dry the grids with filter paper.

3.9 Poststaining of Tokuyasu CLEM sections

1. Rinse the grids 8 x 1 min in distilled H_2O. Dry the tweezers with filter paper between each grid transfer.
2. Counterstain on a 200 μl drop of 1.8 % methylcellulose, 0.3 % uranyl acetate on ice for 5 min (Fig. 2E).
3. Loop out the grid from the MC/UA drop and dry on filter paper by touching the loop to the paper and slowly moving the loop along the filter until the paper is dry (Figure 2F). This should leave a small film covering the grid with the thickness being determined by the speed at which the loop was moved along the filter paper. Leave the grids to dry for 20 min before placing into a grid box.

3.10 TEM and correlations

1. Image the grids in TEM using the finder grid references and fluorescent microsphere fiducials to relocate the regions that were imaged by LM. Record low magnification (~690 – 1400 x) images of the grid square and then increase the magnification (~2400 x) to record an image containing the cell of interest.
2. Correlate the low magnification EM images with the LM images using the ec-CLEM plugin within the ICY imaging platform (*see* **Note 25**) [26]. Mark the position of the microspheres in the TEM image and identify the corresponding fluorescent spot in the LM image. The LM image is then transformed to overlay the TEM image. Perform a further correlation using the high magnification image of the cell of interest and transform

the newly created correlated image onto this source image using either the fluorescent beads or easily identifiable structures, such as mitochondria or endosomes as correlation points.

3. Re-image the grid using the correlated images generated in ec-CLEM to identify points of interest and image at higher magnification to pinpoint the precise location of the fluorescent virus within the cell.

4 Notes

1. Metal plates facilitate temperature control and stability during the ice binding step and the warming step. Prior to warming, the plates should be left in the incubator to equilibrate to 37 °C (see Figure 3A).
2. The cryoprotectant (15 % BSA, 10 % FBS, phenol red free DMEM) should be freshly prepared prior to each experiment and kept at 37 °C until required. Pipette 850 μl of DMEM containing 10 % FBS into a 2 mL Eppendorf tube ensuring that all the medium is below the meniscus and not on the side of the tube [28]. Carefully add 0.15 g BSA to the top of the medium and centrifuge at 13,000 rpm for 20 s to dissolve the BSA. Phenol red free DMEM (#31053, Thermo Fisher) is used to reduce autofluorescence from the medium.
3. The composition of the FS mixture is crucial and must be optimised for each cell line and fluorescent protein or dye in order to achieve good membrane contrast whilst preserving fluorescence following embedding. Johnson et al demonstrate that low concentrations of tannic acid can improve intensity and photo-switching of fluorescent proteins in resin. We find that 0.01 % TA improves the in-resin fluorescence intensity of X31-Alexa Fluor 488 with minimal effects on the ultrastructural preservation. However, when working with bright, over expressed fluorescent proteins the TA can most likely be omitted from the FS mixture. Prepare the FS (0.2 % UA, 0.01 % TA, 5 % H_2O) mixture fresh for each experiment by first making up stocks of 10 % low molecular weight TA in acetone and 5

% UA in methanol. Mix 5 % ultrapure water with pure acetone and cool to -20 °C before adding the UA and TA. Upon addition of TA, the solution will turn a red/brown colour and a precipitate may be formed due to the interaction of the UA and TA, but this will not affect the quality of the freeze substitution.

4. Alexa Fluor dyes of different wavelengths such as 594, 647 can be used.
5. This step is performed to dilute the sucrose concentration.
6. After 10 min of centrifugation, the liquid will be concentrated to approximately 200 μL containing several percent of sucrose. For the complete removal of sucrose, skip steps 10-11 of section 3.1, and pellet the banded virus in MNT buffer by ultracentrifugation at 24,000 rpm (110,500 x g) for 90 min at 4 °C, and finally resuspend the pellet in MNT buffer at a desired volume.
7. X31 plaques are small and require 3 days for visualisation. WSN plaques can be visualised in 2 days.
8. MTT stains living cells in blue, allowing visualisation of plaques. Alternatively, plaques can be stained with crystal violet following fixation of cells
9. The composition of the fixation needs to be optimised for different CLEM samples in order to achieve good preservation of ultrastructure without loss of fluorescence or antigenicity. Low concentrations (0.05 – 0.1 %) of glutaraldehyde (GA) can be included in the fixation to improve the ultrastructure of the sample. However, this may lead to increased autofluorescence quenching of specific fluorescence signal and reduced antibody labelling efficiency. It is therefore important when testing a new sample or antibody to fix in the presence and absence of GA and compare the differences in ultrastructure and antibody labelling.
10. Mount the blocks onto the pins quickly to prevent the samples drying out and sucrose crystals forming. If possible, mount the samples under a stereomicroscope situated in a cold room.

11. A 200nm thick section can be picked up and stained with toluidine / methylene blue to determine whether the cells have been reached in the block. Place wire loop containing a droplet of methylcellulose / sucrose into the cryochamber above the sections. Touch the loop onto the sections just as it begins to freeze and withdraw the loop from the chamber. Once the droplet has thawed, touch the loop onto a glass slide and stain with methylene blue.
12. Cryosections undergo compression and stick to the surface of the knife during sectioning. The sides of the block perpendicular to the knife edge will be compressed by approximately 30 %. A rectangular block is therefore cut in order to compensate for this, with the longer sides being 1.5 times larger than the shorter side. A rectangular block that is orientated with the shorter side parallel to the knife edge will produce a square section when sectioned. Compression can be reduced by controlling the ioniser and altering the cutting speed but it cannot be avoided.
13. A cell scraper can also be used to detach cells from the surface of the culture dish when it is not possible to use trypsinisation. For example, if trypsin would break down fluorescent proteins or particles on the surface of cells. Gently scrape cells from the dish and pipette up and down to break apart cell clumps. Transfer to a 2 mL Eppendorf tube and centrifuge at 3000 rpm for 2 min. Remove the supernatant and add an equal volume of cryoprotectant. Resuspend the cells in cryoprotectant by pipetting up and down, transfer to a 0.5 mL Eppendorf tube and spin again at 3000 rpm for 2 min. This method also can be applied to cells grown in suspension or loosely adhered to the culture dish. However, the cells can clump together which leads to incomplete resin infiltration and therefore is only used when trypsinisation is not possible [20]. Alternatively, cells can be grown on sapphire discs and frozen by HPF. The sapphire discs are dipped in 20 % BSA, 10 % FCS in medium before loading into the carrier and freezing. However, using cells grown on sapphire discs is slightly more challenging when removing sapphires from the

polymerised block and in our hands, produces more variable results compared to cell pellets.

14. An alternative approach described by Kent McDonald and adapted by Christopher Peddie can be taken when cell numbers are low or when it's not possible to culture and infect a 60-mm dish [28,29]. Scrape or trypsinise cells, pellet in a centrifuge and resuspend in 100 μl or less of cryoprotectant. Pipette ~30 μl of cell suspension into a sealed, shortened 200 μl pipette tip with a small 'breather hole', place inside a 2 mL Eppendorf tube without a lid and spin at 5600 rpm for 15 s [29]. Attach the tip to a pipette and cut off the end of the pipette tip with a single-edged razor blade to remove the blockage and pipette ~1 μl of cells into the HPF carrier.
15. A quick freeze substitution can also be performed using standard laboratory equipment as described by McDonald and Webb and produces comparable results to samples freeze substituted in the AFS [30]. Samples can be freeze substituted in less than 90 minutes in a polystyrene box containing liquid nitrogen and then dry ice with agitation. The shorter incubations with solvents and agitation may result in improved preservation of fluorescence.
16. The sections can also be stained with methylene / toluidine blue and imaged using a standard inverted microscope with a 10 or 20 x lens to identify the position of cells within the block and determine the quality of the sample. Cut a 300-nm section and mount onto a drop of water before allowing the section to dry onto the slide. Add a drop of methylene blue stain to identify the position of cells in the block face. Once cells have been located, cut a further thick section and mount onto a glass slide, place a coverslip on top and image on a standard epifluorescence microscope.
17. The section thickness will depend on the intensity of the fluorescence of the sample before processing. For example, over expressed proteins will be brighter than endogenous proteins and therefore thinner sections can be cut and the fluorescence will still be easily visualised. 200 – 300-nm sections can be cut and tomography performed using a 200 kV

TEM. The fluorescence within the section reduces with time, therefore it is important to perform LM imaging immediately following cutting.

18. We choose to use Formvar / Pioloform coated grids despite their inherent auto fluorescence in GFP and RFP channels [16]. The plastic film acts as a support stabilising the section on the grid and preventing it from being lost during retrieval following LM imaging. The grid is further strengthened by a layer of evaporated carbon. The carbon coat makes the grid hydrophilic so resin sections and fluorescent beads will stick to the film more readily. Furthermore, the presence of the carbon coat decreases the autofluorescence of the plastic film and reduces charging in the electron microscope. Composite films of both carbon and a Formvar are particularly useful when doing immunolabelling following LM imaging as the grids have to undergo multiple washes and transfers with tweezers. 200 mesh grids are preferable as one grid square is roughly the same size as the field of view of the light microscope when at 100 x. In addition, the high number of grid bars provide more support, helping to keep the film and therefore section flatter.
19. Section pick up from the knife boat can be performed in a variety of different ways. We favour pick up with the perfect loop as this produces minimal section wrinkles and folds which can affect fluorescence imaging (Figure 3I). However, section pick-ups can also be performed without a perfect loop. Glow discharged coated grids can be immersed in the knife boat and slowly brought up underneath the section using an eyelash to keep the section in place. Alternatively, coated grids can be placed directly on top of the sections and floated on the water. Leave the sections to float for a few minutes to allow any bubbles to disperse and then pick up the grid using tweezers. Turn the grid 90 ° keeping the edge of the grid in contact with the water and slowly bring the grid out of the water. This will drain any water from the grid.
20. Fluorescent beads are used as fiducial markers to produce accurate alignments of LM and EM sections. A variety of fluorescent fiducials are commercially available, but we favour

the PS-Speck™ microspheres or the FluoSpheres™ available from Thermo Fisher. In general, a good fluorescent fiducial is one that is bright, monodispersed and easily visible in the TEM. The choice of fiducial colour will primarily be determined by the wavelength of the protein of interest unless using TetraSpeck microspheres which contain four fluorescent dyes (blue, green, orange and dark red). For instance, when using Alexa Fluor 488 – tagged virus, we opt to use either deep/far red or blue beads as there is no bleed through into the green channel. Ideally the fluorescent fiducial would be in the same channel as the protein of interest to remove any shift generated by switching between channels or chromatic aberrations. However, we found it difficult to distinguish between specific signal from the virus particles and the fiducials due to their similar size, shape and intensities. Both PS-Speck beads and FluoSpheres evenly distribute across grids when sonicated and diluted in PBS or treated with Tween-20 before use [16]. Despite this pre-treatment, beads tend to concentrate at the edges of sections or in folds within the section. Therefore, it is important to choose regions of interest outside of these areas and to ensure that sections are lying as flat as possible on the grid. Note that it is important to dry grids with filter paper before imaging to remove any unbound beads and adhere the beads onto the sections. Any unbound beads will exhibit Brownian motion when imaging by LM and will not be present in the EM section despite fluorescence on LM. Finally, we find that it is important to perform fiducial labelling prior to Hoescht incubations or immunolabelling with antibodies as this leads to the most efficient binding of the beads to the sections.

21. Glycerol is used as a mounting reagent to prevent sections sticking to the glass slide or cover slip. It is important to image mounted grids quickly or mount one grid at a time to prevent the medium drying out and making section recovery difficult. Alternatively, coverslips can be sealed with nail polish or vacuum grease to prevent them drying out.
22. Multiple z planes are taken as the section will not sit perfectly flat on the grid. An average intensity of these multiple z positions is generated and used for correlation.

23. Antibody dilutions will vary between applications and will need to be tested using appropriate controls. In general antibodies are used at a high concentration compared to those used in immunofluorescence, but this is not always the case.
24. Protein A gold can be substituted for IgG gold. It is smaller in size and is only capable of binding to one site on the primary antibody, so labelling is 1:1. However, it is only able to recognise certain IgG subclasses, so is not always appropriate to use. Grids can also be incubated with fluorescently tagged secondary antibodies to help identify endogenous proteins or particles that are weakly fluorescent. Incubate grids in secondary antibody for 30 min, wash and then proceed to gold labelling.
25. The low magnification TEM images are required to achieve an accurate correlation between the LM and EM. It is important to select regions and magnifications containing no less than 7-10 clearly visible fiducials in both LM and EM. The lower magnification TEM images are then correlated with the 63 – 100 x LM image. A further correlation is then performed using the generated low magnification CLEM composite as a source image. Fiducials or easily identifiable structures, such as mitochondria or endosomes within both the low magnification and high magnification TEM images are selected and the composite CLEM image is manipulated onto the high magnification TEM image. The correlation of high and low magnification TEM images can be improved by using smaller fiducials (~10-20nm) often used in tomography. These smaller fiducials can be applied to the grids following the LM imaging of the fluorescent fiducials aiding in correlation of the low and high magnification TEM images.

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Figure Legends

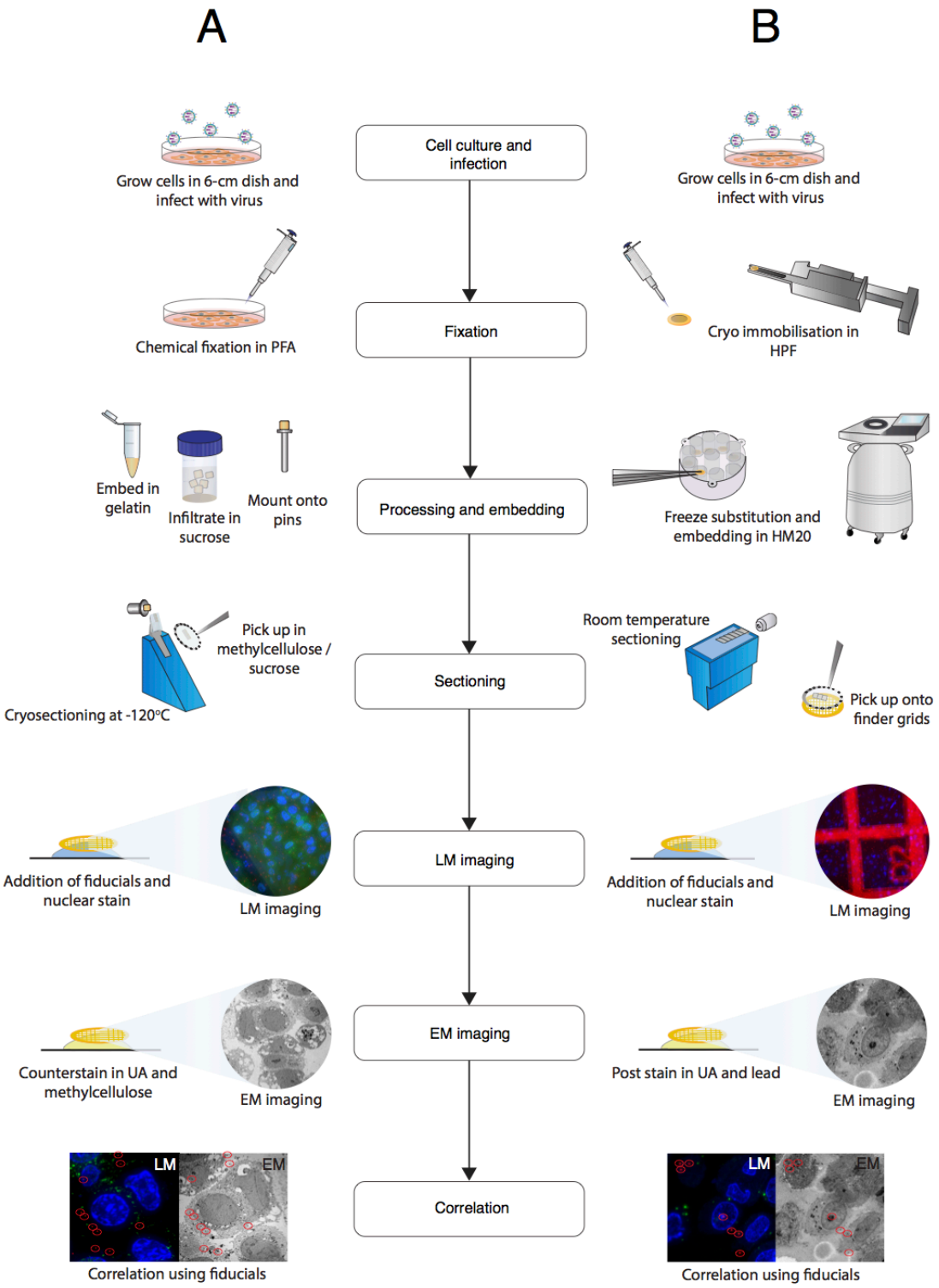

Figure 1: Schematic overview of CLEM workflows for investigating influenza virus entry and budding using Tokuyasu CLEM (A) and in-resin fluorescence CLEM (B). (A) Cells are grown on 60-mm dishes and infected with virus before fixing in PFA, pelleting by centrifugation in an Eppendorf tube and embedding in gelatin. Cell pellets are cut into smaller blocks, infiltrated in sucrose and

mounted onto pins. Pins are frozen in liquid nitrogen and cryosections cut from the blocks using a diamond knife. Sections are picked up in methylcellulose / sucrose and mounted onto coated finder grids. Fiducials and nuclear dye are applied to the grids and the grids imaged by light microscopy and subsequently electron microscopy following post staining in UA and methylcellulose. Correlations of LM and EM images are performed using ec-CLEM. (B) Cells are grown on 60-mm dishes, infected with virus and frozen by HPF. Samples are freeze substituted and embedded in resin. Resin blocks are subsequently trimmed and sectioned. Sections are mounted onto finder grids, fiducials and nuclear dyes are applied before imaging by LM and EM.

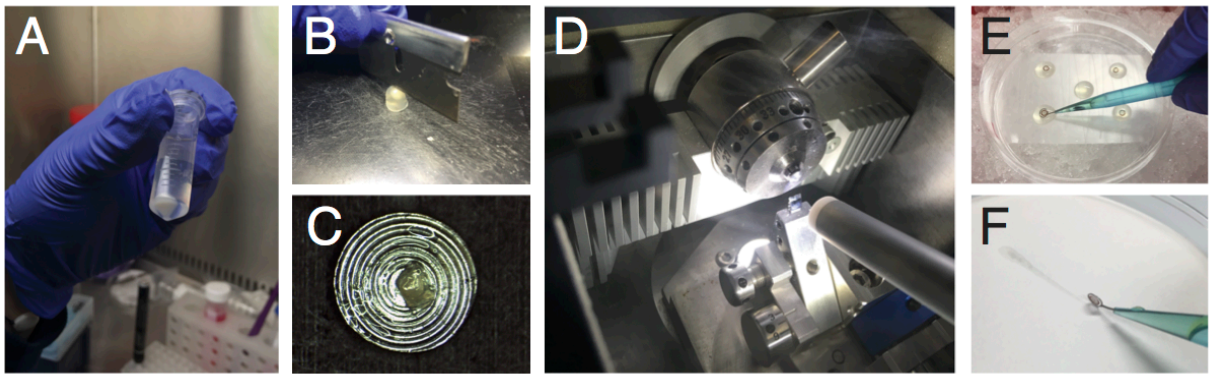

Figure 2: Key steps and essential equipment required for processing of samples for Tokuyasu CLEM. (A) Eppendof tube containing cell pellet (B). Embedded cells in gelatin which are cut into small blocks using a razor blade. (C) Mounted blocks on pins. (D) Cryomicrotome and diamond knives for trimming and cryosectioning gelatin blocks. (E) Post staining of grids in UA and methylcellulose on ice and (F) pick up in a loop and drying on filter paper.

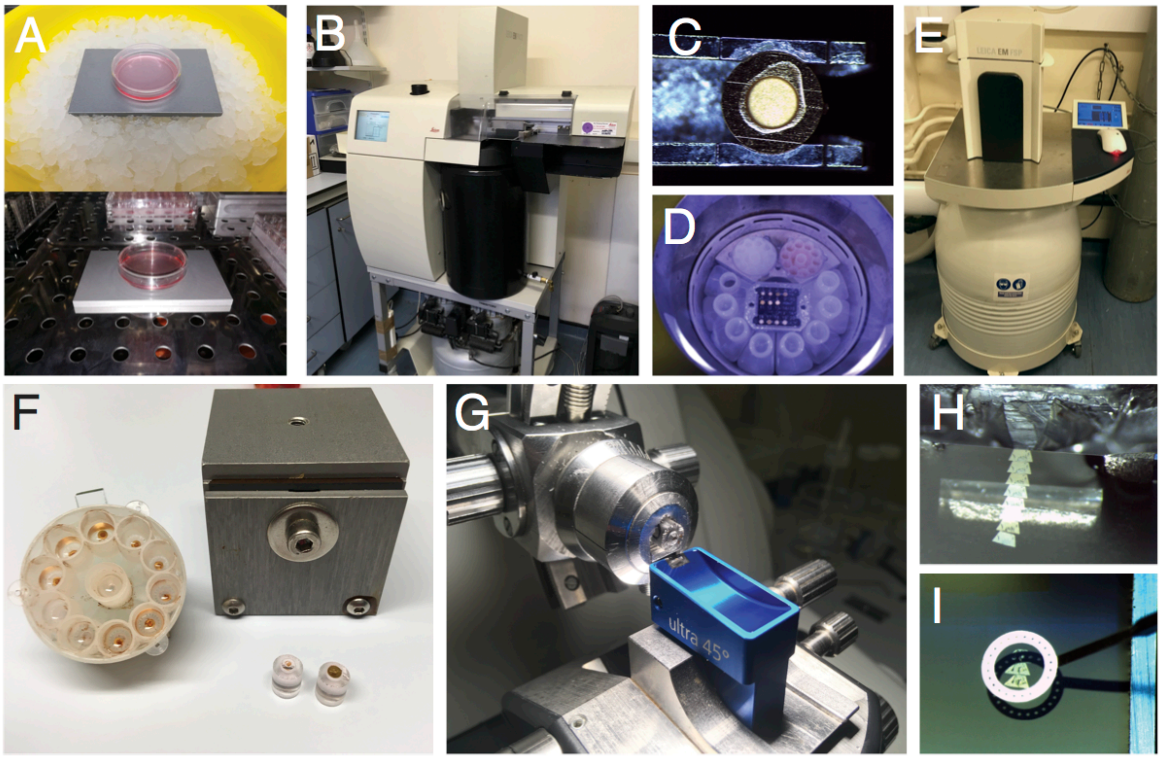

Figure 3: Key steps and essential equipment required for processing of samples for in-resin fluorescence CLEM. (A) Metal plate for infections on ice and at 37ºC. (B) HPF and (C) carrier containing cells before freezing. (D) Samples loaded into chamber of the (E) automatic freeze substitution machine. (F) Embedded samples in reagent baths with flow through rings, lowicryl embedded blocks and the carrier removal tool. (G) Microtome and diamond knife for trimming and sectioning of blocks following removal of the carrier. (H) section ribbons and (I) pick up of sections with loop.

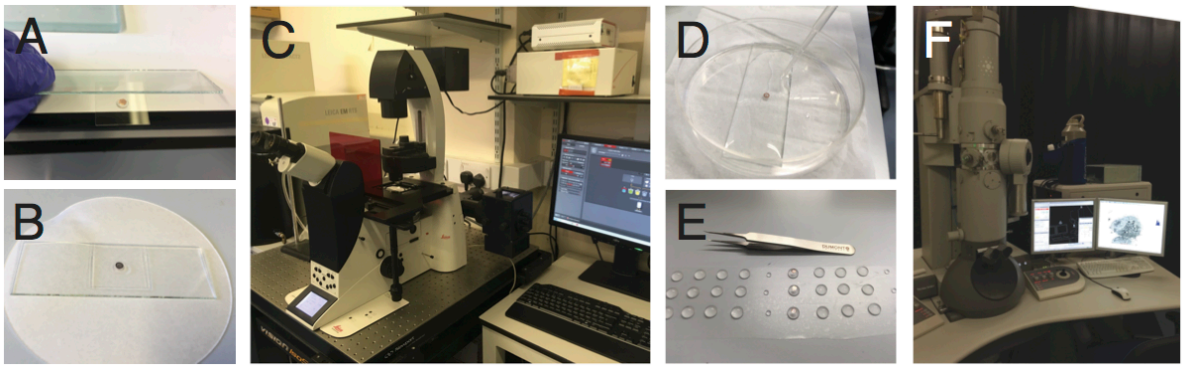

Figure 4: Key steps and essential equipment required for light microscopy and electron microscopy imaging of sections collected on finder grids. (A) Mounting of EM grids between glass slide and coverslip in a drop of glycerol to form a (B) sandwich. (C) Light fluorescence microscope set up for imaging of glass slides. (D) Removal of grid from the sandwich by washing and immersion in water. (E) Immunolabelling or washes of grids on parafilm. (F) TEM imaging set up.

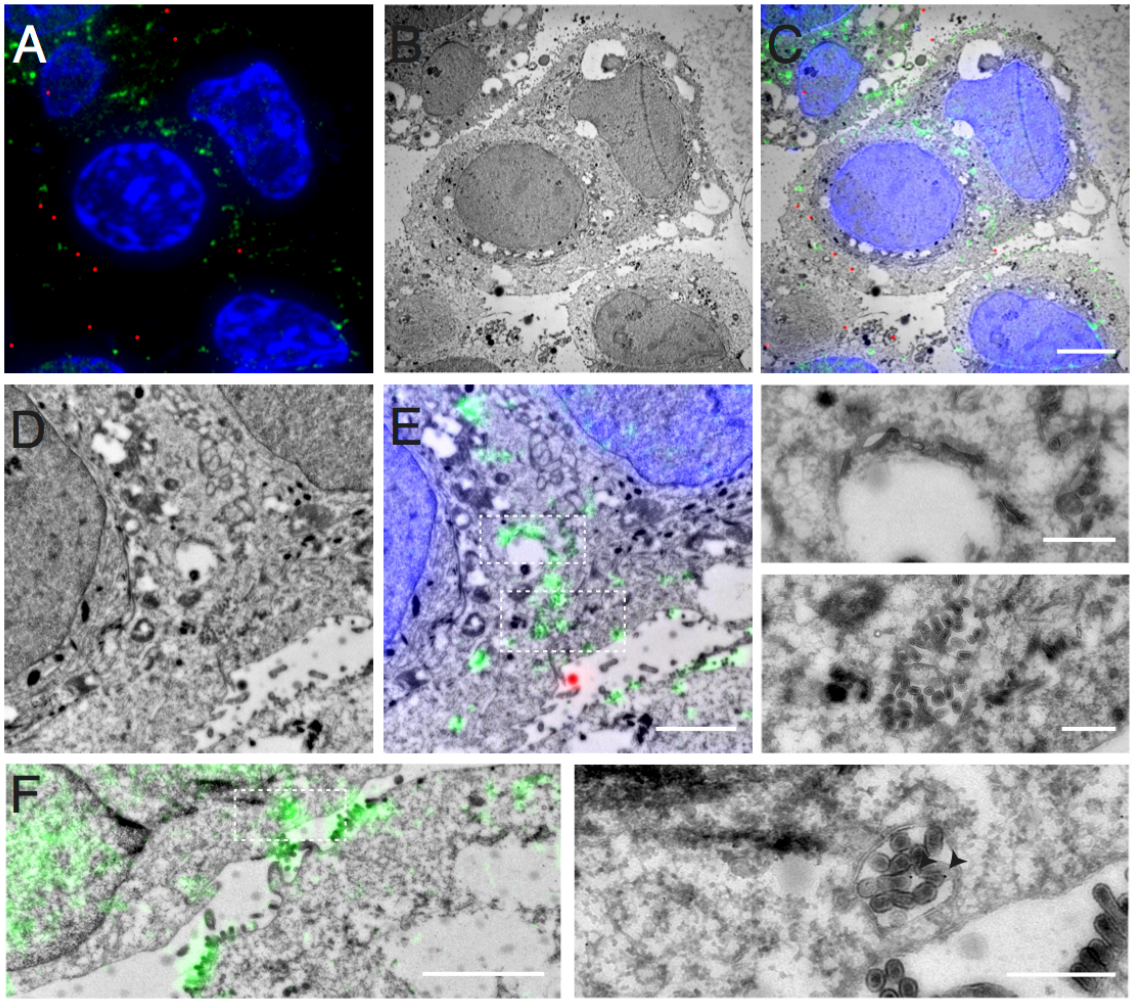

Figure 5: Correlation of light and electron microscopy images to visualise influenza virus budding in Tokuyasu CLEM samples. 90nm sections of IAV WSN PA-GFP infected A549 cells labelled with fluorescent beads (red) and nuclear dye (blue) were imaged by light microscopy (A) and transmission electron microscopy (B) and the fluorescent signal was overlaid onto the EM image using the signal from the fluorescent beads as fiducials (C). Higher magnification TEM image from B (D) with fluorescent signal overlaid (E) and zoom regions of boxed areas revealing viral fluorescence surrounding an endosome and budded virus particles between plasma membranes of cells. (F) Low magnification CLEM image of influenza virus budding from A549 cells and zoom region to reveal anti-GFP and 10nm gold labelling on virus particles.

Scale bars: (C) 5 μm, (E and zoom) 200 nm and 500nm respectively, (F and zoom) 2 μm and 500nm respectively.

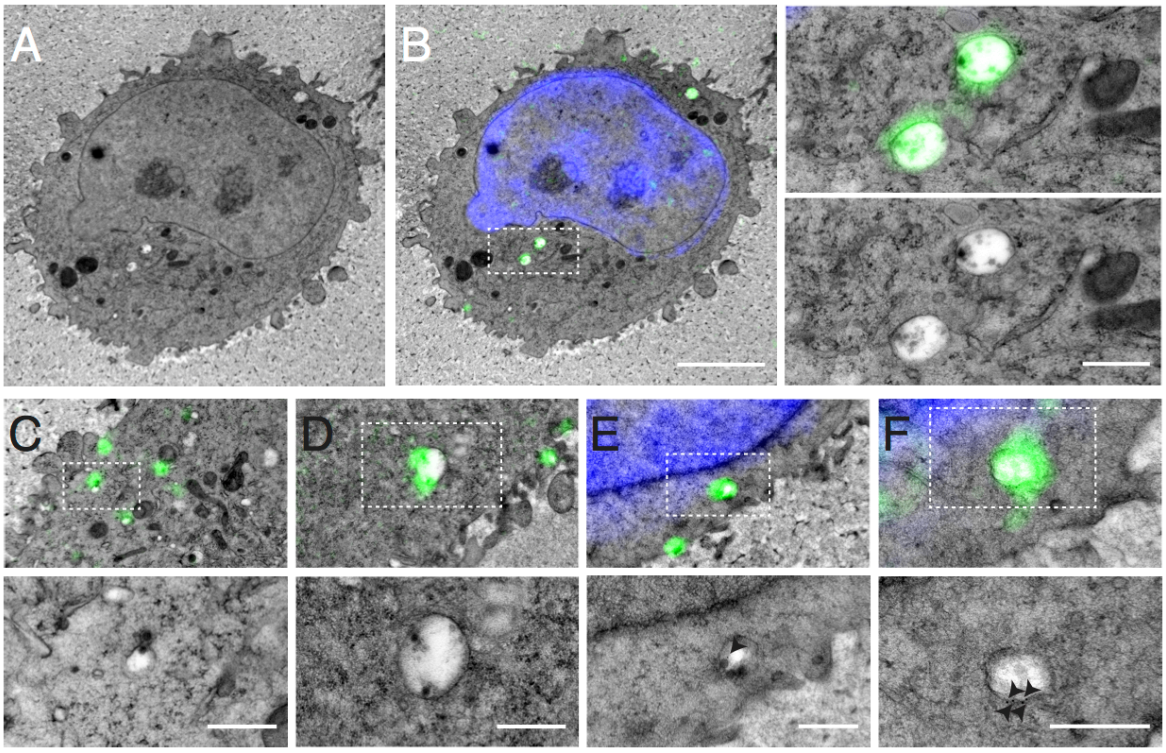

Figure 6: Influenza entry visualised using in-resin fluorescence CLEM. (A) Transmission electron micrograph of an A549 cell infected with IAV X31-Alexa Fluor 488 and (B) overlay of in-resin fluorescence. Higher magnification images of boxed region reveal that the fluorescence correlates with a viral particle within the endosome (top) and an endosome from which the virus has most likely already penetrated but the fluorescence is retained (below). (C, D) Representative correlation images of viruses in endocytic structures and (E, F) representative images of viruses labelled with anti-HA polyclonal Pinda antibody and 10nm gold, with their respective zoom regions. Gold labelling highlights a virus in an endosome (E) and fluorescence retained following fusion and penetration of virus (F).

Scale bars: (B and zoom) 3 μm and 500nm respectively, (C, D, E and F) 500 nm.