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Antibody-based affinity cryo-EM grid

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Abstract

The Affinity Grid technique combines sample purification and cryo-Electron Microscopy (cryo-EM) grid preparation into a single step. Several types of affinity surfaces, including functionalized lipids monolayers, streptavidin 2D crystals, and covalently functionalized carbon surfaces have been reported. More recently, we presented a new affinity cryo-EM approach, cryo-SPIEM, which applies the traditional Solid Phase Immune Electron Microscopy (SPIEM) technique to cryo-EM. This approach significantly simplifies the preparation of affinity grids and directly works with native macromolecular complexes without need of target modifications. With wide availability of high affinity and high specificity antibodies, the antibody-based affinity grid would enable cryo-EM studies of the native samples directly from cell cultures, targets of low abundance, and unstable or short-lived intermediate states.

Keywords

cryo-SPIEM; affinity grid; antibodies; single particle cryo-EM; 3D reconstruction; on-grid assays

1. Introduction

The antibody-coated grid technique for transmission electron microscope (TEM) imaging was originally established by Derrick (1973) [1, 2] for detection, identification, serotyping and quantification of plant viruses in crude extracts [3]. Briefly, a carbon-coated TEM grid was incubated with a given antiserum for the target viral particles to generate an adsorbed layer of antiserum. Alternatively, the TEM grid layered with carbon was first coated with protein A (or protein G) that binds the Fc region of antibodies, followed with antiserum incubation to selectively immobilize antibodies in the antiserum [4]. The use of such antibody-coated TEM grids resulted in about two orders of magnitude improvement of the number of particles attached to the grids [2–5]. Additionally, viral particles captured by the antibody-coated TEM grids were dispersed, which simplified efforts to reveal the morphology of pathogens. Due to the significant advantages of the antibody-coated TEM grid technique, it was further applied to the detection, diagnosis and serotyping of many

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human viruses and renamed as Solid-Phase Immune Electron Microscopy (SPIEM) [6–10]. For example, the SPIEM technique has been successfully employed in serotyping and detection of noroviruses directly using patient stool samples in gastroenteritis outbreaks [11].

Cryo-Electron Microscopy (cryo-EM) accompanied with single particle 3D reconstruction is an emerging technique for structural characterization of macromolecules at nanometer to atomic resolutions. To ensure that the electron beam can efficiently penetrate the frozen specimens embedded in vitreous ice to generate projections of target macromolecules with minimal background, only a very thin layer of the applied samples on TEM grid will be retained by blotting prior to plunge freezing [12]. Therefore, a highly concentrated sample (e.g., ~1 mg/ml for viral samples) is one critical prerequisite for efficient cryo-EM imaging. Unfortunately, there are some situations in which it is challenging to obtain highly concentrated specimens (i.e., low-yield viral system, low abundance and/or short-lived intermediates). To bring these low concentration samples within reach of cryo-EM, the Affinity Grids technique has been developed [13–15], which can concentrate samples on grids through an affinity layer coated on the grid surface. In contrast to conventional cryo-EM sample preparations (Fig. 1A), with the affinity grid target particles would be concentrated and immobilized on the grid surface. Higher surface concentrations of these targets are thus realized by the increased retention of particles after excess sample volume is removed by blotting (Fig. 1B). The net effect would be the removal of excess buffers instead of samples of interest and thereby reduce the required sample concentration of single particle cryo-EM by several orders of magnitudes [14, 16]. Application of such affinity grid technique can enable single particle cryo-EM studies of macromolecules of low-abundance and/or low-yield that have remained challenging. Moreover, by combining sample purification and grid preparation into a single step, use of the affinity grids will significantly expedite cryo-EM sample preparation and potentially maintain better sample integrity by skipping the stringent biochemical purification steps.

Multiple types of affinity grids have been developed, including those based on functionalized lipids [14], the one based on streptavidin-biotin interaction [17], and the functionalized carbon surface approach [18]. Inspired by the traditional SPIEM technique and also the frequent observation of the preference of protein samples for carbon bar regions when deposited to the holey carbon grids during sample freezing in cryo-EM, recently we have established another affinity grid method, the SPIEM-aided cryo-EM approach (cryo-SPIEM) [16]. Specifically, a TEM grid coated with holey carbon and continuous ultrathin carbon film was decorated with either purified antibody alone, or protein A (or protein G) and then antibody through non-specific protein adsorption to carbon film surface before applying samples [19]. Compared to other affinity grids methods [14, 17, 20], an extra benefit of the cryo-SPIEM technique is that neither genetic nor chemical modifications of the samples are needed, which makes the cryo-SPIEM technique an easier and more widely applicable type of Affinity Grids. In this review article, a detailed antibody-based affinity grid preparation protocol has been described. In addition, we also demonstrated that the antibody-based affinity grid coating procedure could be extended to other proteins and/or polypeptides to develop affinity grids based on interactions such as protein-ligand

interactions and charge-charge interactions. Potential applications and future improvements of the affinity grid technique have also been discussed.

2. Preparation of the antibody-based affinity grids: protocol and practical considerations

Holey TEM grids with additional layer of thin continuous carbon film for cryo-SPIEM can be either purchased (e.g., Ted Pella #01824) or prepared by layering a homemade thin carbon film onto holey grids [21]. As depicted in Figure 2, to coat the grids with antibodies, 5 μ l of 50 μ g/ml Protein A (or Protein G for some antibodies) was applied to the carbon surface and incubated for about 10 min at room temperature; The excess liquids were blot away, and 3 to 5 μ l of antiserum or purified antibody was quickly pipetted on the grid and incubated for 15 min at room temperature in a humid chamber. After blotting the majority of the residual antiserum or antibody, the grid was washed with 100 μ l of phosphate buffer for 10–30 seconds and now the target sample could be applied to the grid. Alternatively, when coating grids with purified antibodies, 3–5 μ l of the antibody can also be directly applied to the carbon side of a TEM grid and incubated for about 5–10 min at room temperature to allow direct deposition of antibodies to grid carbon surface. The excess liquid was then blot away and sample was applied to the antibody-coated grid quickly to reduce the potential denaturation of coated antibodies. The following sample incubation will be performed in a humid chamber at 4 °C or ambient temperature.

2.1 Treatment of TEM grids prior to antibody coating

In general, protein-carbon binding is not desirable for standard cryo-EM sample preparation, as it would make the majority of particles bound on carbon bars while few sample particles would remain in the open holes for imaging. However, this unfavorable phenomenon was turned into a precious asset in the antibody-based affinity grid approach. A pivotal step in the antibody-based affinity grid approach is the protein-carbon surface binding that is usually accompanied with partial unfolding of proteins [19, 22–24]. It has been indicated that the protein-surface adsorption is affected by both the nature of a given protein and the properties of the surface [23, 24]. To figure out what type(s) of carbon film surface would give the best antibody coating, different treatments of TEM grids were tested, including aging of grids by hexane to render it more hydrophobic and glow discharge treatment to make the surface more hydrophilic [25, 26]. Specifically, grids were aged by rinsing with hexane and dried for 1 hour; the glow discharge treatment was performed using the conditions: 0.2–0.15 Torr vacuum, 15 mA current, 30 seconds. As shown in Figure 3, both the aged grids and the glow-discharged grids resulted in good coating of the anti-His tag antibody, while the grids without any treatment had poor antibody coating. Considering that in general more protein denaturation will be induced on hydrophobic surface, glow discharge treatment instead of aging has been used. Therefore, the binding of antibody-based affinity layer to carbon film is presumably mediated by mixed electrostatic, hydrophobic and van der Waals interactions. These non-covalent interactions were found sufficient strong to allow affinity capture of target viruses or protein complexes onto TEM grids.

2.2 Concentrations of antibodies for TEM grids coating

Antibody concentrations need to be tested to find the best dilution factor of antibodies for a given system. For purified antibodies, such as the anti-His tag antibody we tested, concentrations between 1 µg/ml and 100 µg/ml have been tested, with 10 µg/ml a commonly used concentration. As aforementioned, coating of nonpurified antibodies (e.g., antiserum) was usually aided by protein A (or protein G). For protein A, the concentration of 50 µg/ml worked well for the majority of grids tested, which was consistent with the condition used in traditional SPIEM [4]. After protein A coating, serial dilution of given nonpurified antibodies was performed and evaluated for the ability to capture and concentrate target particles. As shown in Figure 4, the anti-phage T7 serum and the medium containing anti-Sindbis virus (SINV) antibody were diluted serially and tested with phage T7 infected cell lysate and the cell culture medium containing progeny SINV, respectively. The lowest antibody concentrations that gave a desirable amount of particles captured on grids could be used for subsequent grid coating.

2.3 Sample incubation time and temperature

The incubation time of antibody-coated TEM grids with samples varies depending on the concentration and the volume of samples applied to the grid. Time ranging from 10 minutes to several hours has been tested for samples of different concentrations in our work. For some samples of very low concentrations (i.e., $<10^7$ /ml), active mixing on shakers with larger volumes (e.g., 0.5–1 ml) of samples or physical pre-enrichment of samples (e.g., using Microcon® Centrifugal Filters) will be needed. For example, the cell culture supernatants containing progeny SINV had a titer of $\sim 10^7$ plaque-forming unit/ml. Although some SINV particles were successfully captured by incubating ~ 70 µl of such samples with the antibody-coated grids at room temperature for about 2 hours without active mixing, the particle density on grid was not optimal for cryo-EM study yet (Fig. 5A). To further improve the particle density on grids, 750 µl samples were applied and incubated with the antibody-coated grids with active mixing (i.e., using laboratory shaker) at room temperature for about 3 hours, and as shown in Figure 5B, much higher particle densities were obtained. Similar results were also obtained by concentrating the SINV containing cell culture supernatant by ~ 15 fold using the centrifugal filters (Fig. 5C). Additionally, we found that after long incubation time at room temperature (i.e., > 3 h), the captured SINV particles showed obvious degradation and loss of integrity. Therefore, for samples liable to degradation, incubation at 4 °C should be performed.

2.4 Affinity grids based on other proteins and/or peptides

In addition to antibodies, other proteins and/or protein-conjugates could also be used to generate the affinity layer on grid surface through the generic protein-solid surface adsorption. For example, it has been reported that Tulane virus can bind the type B human blood group antigens (HBGA) [27] and therefore a bovine serum albumin (BSA)-conjugated type B HBGA (Glycorex AB) was tested for generating an affinity layer to concentrate/capture Tulane virus on grid surface. Coating of the BSA-type B HBGA to the grid surface was performed similarly as described in Figure 2. Briefly, 5 µl of 100 µg/ml BSA-conjugated type B HBGA was placed on TEM grids and incubated at room

temperature for 10 min; The excess liquid was then blotted thoroughly and 3 μ l Tulane virus sample was then quickly applied to the grid and incubated for 15 min in a humid chamber at room temperature. As demonstrated in Figure 6A–E, such an affinity layer formed by the BSA-conjugated type B HBGA could concentrate Tulane virus by $\sim$ 60 fold. As it has been reported that the univalent interaction of HBGAs with noroviruses (and most likely Tulane virus as well) was found to be weak with K_d values of several hundreds micromolar or worse [28], the effective concentration of Tulane virus particles on grid surface by the BSA-type B HBGAs presumably resulted from the multivalent binding of Tulane virus with HBGAs.

In addition to the specific interactions aforementioned such as antibody-antigen and protein-ligand interactions, nonspecific electrostatic interactions, for example, by a layer of polylysine adsorbed on grid, can also be used to concentrate and immobilize particles on TEM grids surface [29, 30]. As shown in Figure 6F, direct TEM imaging of bacteriophage T3 minor species that were sparsely located on the grid was challenging and inefficient. To tackle such samples of low concentrations and small sample quantity, the TEM grid carbon surface was coated with polylysine by incubating with a drop of polylysine solution (e.g., Sigma-Aldrich #P8920) for $\sim$ 10 min at room temperature. After blotting away the unbound polysine with filter paper, 3–5 μ l sample was applied onto the grid and incubated on-grid for $\sim$ 10 min in a humid chamber. After multiple on-grid washing to eliminate the gradient medium (e.g., Nycodenz), the sample was either negatively stained or plunge frozen into liquid ethane. As shown in Figure 6G/H, decent amounts of phage T3 particles were observed on both the negatively stained grid and the frozen grid, and a 20 Å structure was resolved using $\sim$ 400 particles captured on the frozen grid and imaged with a Phillips CM200 on photographic films. Moreover, we have solved viral structures of subnanometer [31] and near-atomic resolutions [32] using the polylysine-coated affinity grid.

In summary, through the generic protein/peptide-surface adsorption, affinity layers can be generated on the TEM grid surface to efficiently concentrate target samples by either specific (e.g., antigen-antibody, protein-receptor) or nonspecific (e.g., electrostatic) interactions, to provide cryo-EM grids with desirable particle densities.

3. Applications of antibody-based affinity grid method

With the ability of purifying, concentrating, and capturing samples of interest, the cryo-SPIEM affinity grid approach can be applied to nonpurified samples, and/or purified samples of low concentrations for single particle cryo-EM structural studies. This will make single particle cryo-EM a more generally applicable structural biology technique. Moreover, the antibody-based affinity grids can also be used to assist studies of macromolecular intermediates by efficient preparation and imaging of samples collected at different stages of a given biological process. Furthermore, for the first time we demonstrated the potential of correlating TEM imaging and structural studies with other biochemical measurements by taking advantages of the easier experimental operations allowed by the target macromolecules immobilized on the TEM grid surface.

3.1 Cryo-SPIEM can specifically capture target particles directly from cell culture without pre-purification

Highly purified samples are usually required for cryo-EM studies. With the help of the cryo-SPIEM technique, however, unpurified samples can be directly used for cryo-EM studies, by which the time-consuming and cumbersome sample purification steps are skipped. As demonstrated in our previous work[16], cell lysates of 6×His-tagged bacteriophage T7 (Fig. 7A), cell culture supernatant containing released mature SINV virions (Fig. 7B), and the crude lysates of cells with overexpressed ribosomal proteins (Fig. 7C), could all be directly used for cryo-EM imaging by using grids coated with corresponding antibodies. Moreover, multiple intracellular states of the bacteriophage T7 (capsid I, capsid II and mature phage), and the 70S, 50S, 30S ribosomal units, were imaged simultaneously and reconstructed, which indicated that the affinity grid technique could provide a convenient and practical tool to study the *in vivo* structural states of macromolecules.

3.2 Cryo-SPIEM helps to overcome low concentration problem

For some low-yield systems, obtaining samples of high enough concentrations for cryo-EM studies (e.g., 1 mg/ml for viruses) is challenging and costly. In addition, for highly contagious or dangerous viruses, it is also desirable to limit experiments to low concentrations. Tulane virus in the Recovirus genus of the Caliciviridae family, although it has been successfully propagated in a monkey kidney cell line [33], the yield of progeny Tulane virus is limited. As shown in Figure 8A, the amount of particles obtained using regular grids were at concentrations far below the desired density of cryo-EM. To work on such Tulane virus samples of low concentrations ($\sim 10^{8-9}$ particles/ml), the TEM grids were first coated with protein A as described in Figure 2, and then incubated with the anti-Tulane virus antiserum. After washing away the residual antiserum, 5 μ l Tulane virus sample was applied to the grid and incubated for 15min. As shown in Figure 8B, Tulane virus particle densities on the grid were improved significantly, which allowed efficient cryo-EM imaging and 3D reconstruction of Tulane virus (Fig. 8C). The cryo-SPIEM technique therefore provides a generic solution to the cryo-EM studies of samples of low concentrations.

3.3 Cryo-SPIEM assists study of macromolecular intermediates

Investigation of the macromolecular *in vivo* intermediates is important for establishing a comprehensive understanding of their functional mechanisms. Isolation and characterization of different intermediates require a large amount of efforts in general. With the help of the antibody-coated affinity grid technique, however, it was possible to use nonpurified cellular extracts to visualize the states of macromolecular complexes at different time points, which makes the kinetic and structural study of macromolecules intracellular development more rapid and efficient. For example, the antibody-coated TEM grid technique was applied to study the intracellular packaging and maturation of phage T7. As shown in Figure 9A, the *E. coli* cells at OD600 of ~ 1 were infected with His-tagged phage T7 at a multiplicity of infection of 10; After a short rise period, the density of infected cells went into a plateau during which progeny phages were produced, and finally most, if not all, the cells were lysed as indicated by the drop of the OD600 curve. To visualize the different intermediates involved in phage T7 packaging, cells at different time points in the plateau were collected,

lysed and visualized using the anti-His tag antibody-coated TEM grids. As demonstrated in Figure 9B–D, the evolution of phage T7 particles from empty precursor capsids to genome-filled virions was monitored successfully.

3.4 Correlative on-grid assays and TEM imaging

The antibody-coated TEM grid technique shares similarities with some assays, especially the enzyme-linked immunosorbent assay that also involves protein-surface adsorption steps [34]. Such similarities indicate the potential of correlating TEM imaging and biochemical assays to obtain more comprehensive knowledge by combining information from different readout strategies. As a proof of concept, a preliminary study of heat-induced genome release of phage T7 was performed using both the SYBR safeTM (fluorescent DNA dye, Thermo Fisher Scientific) fluorescence to monitor the amount of released DNA and the TEM imaging of phage T7 particles to monitor the morphology of the capsids. Briefly, T7 phage was first captured on grid through the protein A-antiT7 affinity layer as mentioned above, and then the grids were floated on top of a drop of 10 mM ethylenediaminetetraacetic acid (EDTA) solution containing the DNA dye and incubated at 50 °C, under which phage T7 genome release was triggered. The fluorescence arising from the released double-stranded DNA bound with the dye was measured using the Spectramax microplate spectrophotometer (Molecular Devices), while the percentage of empty phage T7 particles was determined by TEM imaging. As shown in Figure 10, the measurement of the fluorescence signal and the TEM imaging could be performed using the same grid, and a good correlation between the amount of fluorescence and the relative amount of empty phage particles was observed successfully. This indicated that such a correlated characterization of macromolecules with biochemical assays and TEM imaging techniques would benefit cryo-EM structural studies involving intermediates by real-time monitoring for optimal timing of sample snapshots and correlate the structures with biological activities.

4. Determining near-atomic resolution structures using cryo-SPIEM

To obtain near-atomic resolution structures, one has to have good samples, and also collect high quality cryo-EM data (i.e., uniform view coverage, and high signal-to-noise ratio). With the affinity grid techniques, particles will be largely immobilized on the grid surface, and thus there has been a concern about restricting the views of particles when using the affinity grids for cryo-EM data collection. As demonstrated by the published work [14, 16, 17, 35], however, no significant orientation preference was observed, presumably due to the intrinsic flexibility of the affinity headgroups arising from a spacer, and/or tags with flexible linkers. Moreover, the application of polyclonal antibodies against multiple epitopes for the antibody-based affinity grids will also help to minimize the view preference. Another common concern about applying the antibody-coated grids for cryo-EM studies is the increased background noise arising from the antibody or protein A/G-antibody layers as well as the amorphous carbon underneath. As such background noises cannot be computationally removed, they potentially would limit the achievable resolution of single particle 3D reconstruction with data collected using the antibody-coated grids. On the other hand, however, the immobilization of macromolecular particles into a thin affinity layer could significantly reduce the Brownian motion of particles and therefore allow for thinner

vitreous ice without introducing more damage of particle structure at air-water interface [12]. We believe that by optimizing the ice thickness to the minimal, it is possible to largely balance out the noise introduced by the affinity layer of affinity grid approaches; therefore, high-resolution cryo-EM structures would be achievable using affinity grids. This is supported by our recent work of solving structures of multiple capsid states of phage T7 at ~ 7 Å resolutions using the lipid-based affinity grid approach [36]. Moreover, we have more recently solved the Tulane virus structure to ~ 2.6 Å using the antibody-based affinity grid approach (Yu et al., unpublished data). From these early successes, we think that the antibody-based affinity grid technique should possess great potential for solving high-resolution structures of many samples embedded in optimally thinned vitreous ice.

5. Future directions

Efforts are being devoted to further optimize the antibody-based affinity grid technique, including: 1) replacing the thin amorphous carbon film support with a thinner and more electron-transparent substrate (i.e., graphene); 2) employing electrophoresis/dielectrophoresis to actively concentrate samples towards the affinity grid surface for purification and further enrichment of targets. These efforts would potentially make the antibody-based affinity grid technique applicable to more samples of much smaller molecular mass than viral particles, and enable cryo-EM studies of samples at concentrations significantly lower than 10^7 particles/ml.

To use graphene as the substrate for the affinity layers and the specimens

Although amorphous carbon film is currently the dominant sample support for cryo-EM, it is not a desirable sample support material for cryo-EM imaging because of its poor electron conductivity and increased background. Therefore, a better sample support than amorphous carbon for cryo-EM imaging is desirable, and graphene has been indicated as a superior support substrate due to its preeminent properties, including its electron transparency, excellent electrical conductivity and mechanical strength [37, 38]. Given these favorable properties of graphene and growing knowledge on protein-graphene adsorption [38–40], it is of interest to explore functionalization of graphene with antibodies or other affinity ligands for sample capturing and concentration in cryo-EM sample preparation, which is highly likely to help bypass the poorer contrast issue for the affinity grid technique.

On-grid active concentrating with electrophoresis and dielectrophoresis

As aforementioned, the incubation of samples with affinity grids involves a long passive diffusion process for the target macromolecules from the entire solution volume to reach the grid surface. This can take tens of minutes to several hours depending on the volume of the sample applied. To speed up this process, minimize the potential sample degradation during incubation, and also make samples of very low concentrations (i.e., $\ll 10^7$ particles/ml) usable, we suggest enhancing the affinity grids with on-grid electrophoresis or dielectrophoresis techniques. Most macromolecular complexes have net negative charges at neutral pH and can be attracted towards the anode in electric field, as utilized in the native gel methods [41]. For neutral macromolecules in given buffers, dielectrophoresis can be utilized to actively transport and concentrate the proteins towards grid surface by employing

the polarizability of the molecules [42]. For example, the TEM metal grids coated with affinity layers can be used as the electrode that the macromolecules would migrate towards, and thereby a local higher concentration of samples at the TEM grids will be created, which will facilitate the capture of target proteins by the affinity grid.

In summary, application and further optimization of the affinity grid technique will grant single particle cryo-EM technique access to more biological samples including those challenging ones (e.g., low-concentration samples, transient intermediates). In addition, the simplified cryo-EM sample preparation procedure by merging purification and cryo-EM grid preparation into a single step using the affinity grids based on specific interactions, will markedly expedite single particle cryo-EM studies using less amounts of given nonpurified materials, which would potentially help to develop single particle cryo-EM into a high-throughput technique.

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Research highlights

- Cryo-SPIEM affinity grid method enables cryo-EM studies of many diluted specimens.
- Different affinity layers can be generated through the protein-surface adsorption.
- The affinity grid technique would assist studies of macromolecular intermediates.
- Affinity grids will make cryo-EM a widely applicable structural biology technique.
- Use of affinity grids would help develop cryo-EM into a high-throughput technique.

Figure 1. Sample blotting on regular and affinity TEM grids before plunge freezing
(A) Sample of high volume concentration on a regular TEM grid. (B) Sample of low concentration was concentrated on the surface of an affinity TEM grid.

Figure 2. Schematic diagram of SPIEM-aided sample preparation for cryo-EM

(1–5) describe the protein A or protein G aided coating of antibodies (nonpurified antibodies or antisera) to TEM grids. (1'–3') depict the procedure of direct coating of purified antibodies to TEM grid carbon surface. **1.** Incubate protein A or protein G on TEM grid carbon surface at room temperature for ~10 min. **2.** Blot away as much as possible residual liquids on grid using filter paper. **3.** Quickly apply 5 μ l of a given antibody or antiserum on the grid and incubate for ~15 min at room temperature in a humid chamber. **4.** Blot most of the excess liquid off the grids, and then wash the grids with 1 $\times$ PBS, pH 7.4 for about 30 s. **5.** Incubate samples with the antibody-coated grids, by either placing the grid facing up and applying a small volume of samples (e.g., 10 μ l) to the grid, or having grids facing down and floating on a large volume of samples (e.g., 30–100 μ l). **1'.** Incubate antibodies on TEM grid carbon surface at room temperature for ~10 min. **2'.** Blot away as much as possible residual liquids on grid using filter paper. **3'.** Incubate samples with the antibody-coated grids, by either placing the grid facing up and applying a small volume of samples to the grid, or having grids facing down and floating on a large volume of samples.

Figure 3. Effects of different surface treatments of TEM grid on capture efficiency of antibody coated affinity grids

Negative staining images of phage T7 particles captured with anti-His tag antibody coated TEM grids filmed with carbon of different properties: A. Hexane-aged grids; B. Glow-discharged grids; C. Original grids without any treatment. The arrows point to representative phage T7 particles.

Figure 4. Dependence of particles capture efficiency on antibody concentrations

Different concentrations of antibodies (anti-T7 phage antiserum, nonpurified anti-SINV antibody) were used to coat grids pre-coated with protein A as described in figure 2. (A) A serial dilution of anti-T7 phage antiserum for grids pre-coated with protein A. The sample is a lysate of T7 infected *E. coli* cells. (B) A serial dilution of anti-E2 of SINV for grids pre-coated with protein A. The sample is cell culture medium of SINV infected BHK-21 cells. Both the phage T7 particles and the SINV particles were stained with 2% phosphotungstic acid.

Figure 5. Enhanced capture efficiency by active mixing and pre-concentrating large volume of low abundance samples

Negative staining images of SINV particles captured by the antibody coated grids from nonpurified samples. (A) Incubation with 70 µl nonpurified SINV supernatant at room temperature for 2 hours. (B) Incubation with 750 µl SINV supernatant at room temperature for 3 hours with active mixing using a laboratory shaker. (C) Incubation with 50 µl of ~15 fold concentrated SINV supernatant.

Figure 6. Affinity grids based on other proteins and/or peptides

(A) Negative staining image of Tulane virus sample on regular Formvar/carbon coated TEM grid. (B) Tulane virus sample on BSA-type B trisaccharides coated TEM grids. (C) Tulane virus sample on BSA-coated TEM grid (negative control). (D) and (E) show the cryo-EM images at different magnifications of Tulane viruses on BSA-type B trisaccharides coated lacy/continuous carbon grids. (F) and (G): Negative staining image of a minor capsid band (1.073 g/ml) in a Nycodenz gradient of phage T3 mutant (T3^{SR3-1}). (F) A regular Formvar/carbon coated TEM grid. (G) Polylysine-coated Formvar/carbon TEM grid. (H) Cryo-EM image of the phage T3^{SR3-1} capsid on polylysine-coated lacy/continuous carbon grids. The arrows point to the representative particles.

Figure 7. Examples of images and 3-D reconstructions of unpurified samples using cryo-SPIEM method

(A) Three major states of phage T7 particles (procapsid (CI), empty capsid (CII) and genome-filled mature phage) captured on anti-His tag antibody coated grids, and reconstructed to 22, 24 and 17 Å using about 300, 258 and 900 particles, respectively. (B) SINV particles captured on protein A-antiE2 coated grids and reconstructed to 25 Å using ~1600 particles. (C) His-tagged ribosomal particles captured on anti-His tag antibody coated grids. The insets show the reconstructed 3-D maps of 70S, 50S and 30S ribosomal particles at 38, 35 and 41 Å resolutions using the 1663, 2213 and 270 captured particles, respectively. Image processing details could be found in reference 16.

Figure 8. Cryo-SPIEM for Tulane virus sample of low concentration

Negative staining of Tulane virus of low concentration on a regular TEM grid (A) and an anti-Tulane virus antiserum coated affinity grid (B). The arrows point to the Tulane virus particles. (C) A cryo-EM image of Tulane virus particles captured on an anti-Tulane virus antiserum coated affinity grid. The inset shows the reconstructed 3-D map of Tulane virus at 19 Å using ~900 of the captured particles. Image processing details could be found in reference 16.

Figure 9. Using antibody-coated grid to capture different states of phage T7 during the intracellular evolution

(A) The cell density (OD600) of *E. coli* cells infected by His-tagged phage T7. At the end of the infection by bacteriophage T7, cells were lysed as indicated by the drop of the OD600 curve. (B–D) T7 particles captured using the anti-His tag antibody coated grids at post infection time points 5min, 8min and 11min, respectively.

Figure 10. Correlative on-grid assay and TEM imaging of heat-induced genomic DNA release of phage T7

T7 phage was first captured on grid with Protein A-AntiT7, and then the grids were floated on top of 10 mM EDTA solution containing the DNA dye SYBR safeTM. Incubation was performed at 50 degree. (A) A negative staining image after 20 min incubation. The corresponding fluorescence resulted from DNA-binding was about 8 relative fluorescence unit. (B) A negative staining image after 40 min incubation, with corresponding fluorescence of 20 relative fluorescence unit.