Chapter 11

Negative Staining and Cryo-negative Staining: Applications in Biology and Medicine

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Abstract

Negative staining is widely applicable to isolated viruses, protein molecules, macromolecular assemblies and fibrils, subcellular membrane fractions, liposomes and artificial membranes, synthetic DNA arrays, and also to polymer solutions and a variety of nanotechnology samples. Techniques are provided for the preparation of the necessary support films (continuous carbon and holey/perforated carbon). The range of suitable negative stains is presented, with some emphasis on the benefit of using ammonium molybdate and of negative stain–trehalose combinations. Protocols are provided for the single droplet negative staining technique (on continuous and holey carbon support films), the floating and carbon sandwich techniques in addition to the negative staining-carbon film (NS-CF) technique for randomly dispersed fragile molecules, 2D crystallization of proteins and for cleavage of cells and organelles. Immuno-negative staining and negative staining of affinity labeled complexes (e.g., biotin–streptavidin) are presented in some detail. The formation of immune complexes in solution for droplet negative staining is given, as is the use of carbon–plastic support films as an adsorption surface on which to perform immunolabeling or affinity experiments, prior to negative staining. Dynamic biological systems can be investigated by negative staining, where the time period is in excess of a few minutes, but there are possibilities to greatly reduce the time by rapid stabilization of molecular systems with uranyl acetate or tannic acid. The more recently developed cryo-negative staining procedures are also included: first, the high concentration ammonium molybdate procedure on holey carbon films and second, the carbon sandwich procedure using uranyl formate. Several electron micrographs showing examples of applications of negative staining techniques are included and the chapter is thoroughly referenced.

Key words Negative staining, Cryo-negative staining, Carbon support film, Holey carbon support film, Immuno-labeling, Affinity-labeling, The single droplet technique, Floating technique, Negative staining-carbon film (NS-CF) technique, Dynamic negative staining, Uranyl acetate, Uranyl formate, Ammonium molybdate, Trehalose, Polyethylene glycol (PEG), Single particle analysis, 2D crystal, 3D reconstruction

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The authors dedicate this chapter to the late Robert (Bob) W. Horne (21st January, 1923–13th November, 2010).

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1 Introduction

The basic negative staining technique has stood the test of time, since its introduction as a laboratory procedure in 1959 by Brenner and Horne [1], as recently fully acknowledged by Harris and Munn [2] following the death of Robert (Bob) W. Horne in November, 2010. With variations the technique continues to be applied widely to thinly spread biological samples, ranging from viruses to single macromolecules and macromolecular assemblies, subcellular membrane fractions, lipid suspensions, toxin–lipid/membrane interactions, etc. The progression of fibrillogenesis of the range of amyloid-forming peptides and proteins, and collagen can readily be monitored by negative staining. Negative staining can also be applied to cells, and subcellular fractions where the material is sufficiently thin, permeablized or physically lysed, in particular to reveal cytoskeletal structures, then accessible for immunolabeling. The technique readily extends into nanotechnology, nanobiotechnology and polymer science for the assessment of particle size and structure [3]. In diagnostic virology negative staining made a significant early contribution which continues to this day, albeit to a lesser extent, alongside the more recently introduced molecular and immunological assays, *see* Mast and Demeestere [4]. For rapid assessment of biological extracts and purified proteins, often produced by bacterial expression, negative staining can provide useful information in a matter of minutes. This relates primarily to an immediate indication of sample purity, in addition to the dissociation and aggregation state of the aqueous sample. For those wishing to take sample material forward for higher resolution cryo-electron microscopy, either in the unstained vitrified state or as cryo-negatively stained specimens, conventional negative staining can provide valuable time-saving information, as indeed it can alongside 2D or 3D crystallization studies. Despite the limited resolution achievable from air-dried negatively stained samples (generally in the order of 2 nm), several groups continue to use this approach for producing low resolution 3D image reconstructions by single-particle analysis, in some cases with docking of higher resolution structures from NMR and X-ray crystallography [5–8]. Cryo-negative staining [9] can achieve a considerably superior resolution, in the order of 1 nm. The overall topic has been reviewed recently in depth by de Carlo and Harris [10].

It is of some technical interest to consider briefly the derivation of and modifications to the carbon support film procedures for negative staining that have occurred over the years, and how these impact upon present-day procedures. Pre-formed carbon supports, carbon–plastic support films, or holey carbon films across grids have found extensive usage for the thin spreading, adsorption and negative staining of biological samples, from droplets on Parafilm

or from a microspray/nebulizer with the sample and negative stain pre-mixed. The versatile droplet technique, which the authors favor, was initially introduced in 1965 by Ennio-Lucio Benedetti during his studies on liver cell membranes (*see* Subheading 3.1.1). However, an alternative approach was introduced in 1968 by the late Robin Valentine and colleagues [11], in that the carbon film can be released from mica, floating directly onto the surface of a solution of the biological sample in a small Petri dish, microwell or as a droplet on Parafilm, with subsequent sample adsorption onto the carbon film. This can then be transferred to grids, washed and negatively stained as required. Furthermore, this procedure can be readily extended to produce specimens between a carbon-sandwich on grids. A variant introduced by Horne and Pasquali-Ronchetti [12], termed the negative staining-carbon film (NS-CF) procedure, changed the order of carbon deposition, in that this was performed directly onto sample already spread in the presence of ammonium molybdate and air-dried on a mica surface, followed by release of the carbon film + adsorbed sample onto the surface of a second negative stain solution. A distinct advantage of this procedure has been for the production of 2D arrays and 2D crystals (even 3D microcrystals), when the drying of virus and protein samples onto the mica is performed in the presence of ammonium molybdate + polyethylene glycol (PEG). Removal of glycerol from samples in vacuo can also be performed using this technique and it can be extended as a "wet-cleavage" procedure for cellular samples that have been bound to Alcian blue-treated mica [13]. Extension of negative staining to holey carbon support films, where the biological material is freely suspended across holes, supported only by the surrounding negative stain and protective carbohydrate, provides conditions where the material is not adsorbed to carbon and thereby avoids possible adsorption-induced structural distortion and flattening. Essentially the same comment applies to the use of holey carbon support films for cryo-negative staining. Here, inclusion of PEG in the ammonium molybdate cryo-negative staining procedure also promotes the formation of 2D arrays prior to vitrification, indicating that fluid–air interface forces are involved in both techniques.

An important aspect of negative staining on carbon support films that need to be expanded upon, within the context of recent and older studies, is the possible benefits to be gained from adsorbing and negative staining samples on a single layer of carbon versus a carbon sandwich with the sample and stain layer trapped between the two layers. Negative staining of biological samples on a single layer of carbon has always shown regions of varying stain depth, essentially from almost unstained regions to regions with an excessive stain depth. The thinner partial-depth regions of stain may only generate images from the lower part of the structures under investigation, and excessive stain depth will mask structural detail.

To create a more even full-depth spreading of stain, inclusion of the disaccharide trehalose was introduced [14], and the problem is almost completely overcome by spreading and negative staining samples initially as a thin aqueous film in the presence of trehalose across the holes of holey carbon support films, followed by air drying (*see* Subheading 3.1.2). This latter approach is essentially equivalent to the procedure for preparing unstained or negatively stained frozen-hydrated/vitreous specimens, in that the mixture of negative stain and trehalose is a dried yet thin vitreous stain–sugar layer across the holes.

The creation of a double carbon layer with sample and stain trapped between was encountered and utilized many years ago, but at that time was generally thought to be unsatisfactory due to the likelihood of significant sample flattening/distortion. However, for rigid or glutaraldehyde-stabilized samples this approach has come into more recent use, both for air-dried negative staining and cryo-negative staining (*see* Subheading 3.1.4). The benefit here is that for single particle reconstructions, there is even coverage with stain all around the entrapped specimen particles. Thus, both approaches (i.e., single and double-layer carbon supports) are currently in use for negative staining studies of biological structures; each can claim to have some benefits and disadvantages. It is fairly clear that every laboratory performing negative staining will use slightly different in-house protocols; all will have been standardized locally, thereby generating reproducible data.

The currently used cryo-negative staining methodology developed from a productive collaboration between one of us (JRH) and the Lausanne-based cryo-electron microscopists Marc Adrian and Jacques Dubochet (*see* Adrian et al. [9]), in an attempt to produce superior sample preservation and image resolution, compared to the conventional negative staining technique with air-dried specimens. This topic was reviewed briefly by Harris and Adrian [15], and Harris et al. [16], and more recently by de Carlo and Harris [10]. From a methodological point of view, this sample preparation technique is very similar to the thin film vitrification technique for unstained specimens [17], with the exception that there is an additional step, a short time of contact between the biological sample and the negative stain prior to plunge-freezing (*see* Subheading 3.1.8). As a result, the biological sample is entrapped in a thin vitrified high concentration ammonium molybdate solution, essentially at a saturation concentration (~0.8 M). However, if this cryo-methodology is analogous to the initial rapid plunge vitrification procedure [17], the results obtained with this cryo-negative staining technique are completely different, in our opinion exceptional and strongly supported by recent applications [18–20].

Recent developments/variants of the negative staining technique are few, although Zhang and colleagues have made a significant contribution to the understanding of lipoprotein structure, using

an optimized negative staining protocol [21], extending from the approach presented by Ohi et al. [22]. An ambitious attempt to standardize and improve negative staining for TEM and STEM has recently been introduced by Kemmerling et al. [23], using robotic automated equipment with microfluidic-dialysis to desalt and mix the biological samples with negative stain. It is unlikely that this equipment and the detailed sequence that it provides, will immediately be widely available, but it provides strong evidence that further technical improvement is possible.

An alternative carbon sandwich cryo-negative staining method, using uranyl formate and glycerol, was developed by Stark and colleagues [24], as presented fully by Sanders and Golas [25]. A significant difference here is that the specimen, negatively stained with a low concentration of stain, is briefly air-dried before freezing in liquid nitrogen for cryo-negative staining EM study. Thus, this modified cryo-negative staining technique is not directly equivalent to the Adrian holey carbon technique [9], which has a closer parallel to the conventional vitrification procedure for unstained specimens.

Although some of the negative staining techniques included here have been presented by one of us (JRH) in a Royal Microscopical Handbook [26] and in the two earlier editions of this book (1999 and 2007), in this third edition some effort has been made to update these approaches and present the techniques in a new light. Nevertheless, all the techniques as previously and currently documented remain usable and reproducible. In addition, in Subheading 4 several new examples of EM data from the negative staining techniques will be presented here, but the examples used in the previous editions remain valid.

2 Materials

2.1 Equipment Required

1. The principal large item of equipment that is needed in order to prepare support films for negatively stained EM specimens is a vacuum coating apparatus (e.g., the Edwards model Auto 306, the Bal-Tec model BAE 080T, the Emitech model K405X, or Agar TEM Turbo Carbon Coater), together with facility to perform glow-discharge treatment. This latter may be an attachment within the vacuum coating apparatus or a separate item of equipment. Carbon coating, to produce thin continuous carbon, carbon–plastic, or perforated (holey) carbon support films (*see* Subheading 2.2 and also ref. 26), can be performed using carbon rods, carbon fiber, or a carbon electron beam source, as described by the equipment manufacturer concerned. A carbon thickness monitor can be useful, but is not essential. Brief (30–60 s) glow discharge treatment of the surface of support films is particularly useful to combat the inherent hydrophobicity of the carbon surface, which interferes

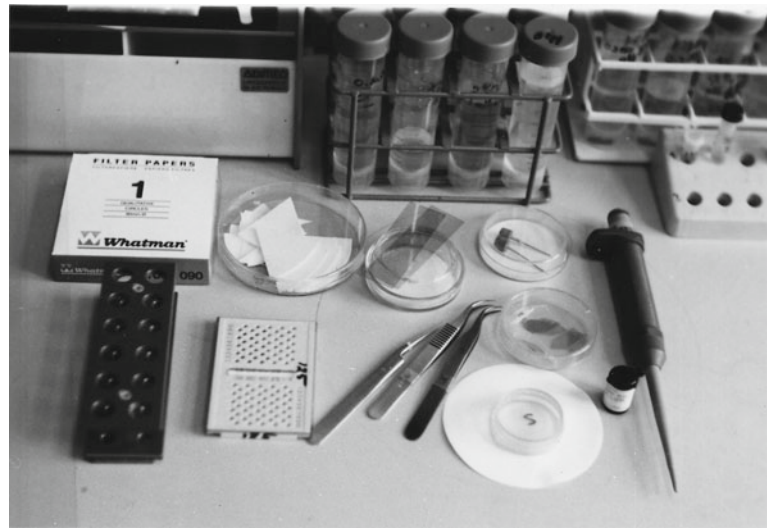

Fig. 1 An example of some of the small equipment and consumables needed for the routine production of negatively stained specimens

with the even spreading and adsorption of biological materials and the smooth spreading of a thin film of aqueous negative stain over and around the biological particles (i.e., satisfactory embedment in a high contrast medium) prior to air drying. The use of prolonged UV irradiation has also been shown to render the surface of carbon support films hydrophilic.

2. For cryo-negative staining a plunge-freezing apparatus and associated consumables (e.g., liquid nitrogen, ethane gas) will be required. Some time will need to be devoted in order to master the use of this equipment and the cryo-transfer of frozen specimens to a TEM [26].
3. Numerous smaller items of equipment are needed (*see* Fig. 1), such as Parafilm, fine curved forceps (with rubber or plastic sliding closing ring, or use reverse-action forceps), fine straight forceps, a range of lab. pipettes (e.g., 5, 10, 20, up to 1,000 μL), plastic tips, scissors, metal needle/ finely pointed probe, mica strips, filter paper wedges (e.g., cut from Whatman No. 1), petri dishes with filter paper insert, 300 or 400 mesh electron microscope (EM) specimen grids (usually copper, but nickel or gold for immunonegative staining), grid storage boxes, a microcentrifuge, tubes and tube racks, etc. Last and importantly, small Kleenex or other absorbant tissues need to be available to rigorously wipe the tips of the forceps and needles, immediately after use, to avoid sample cross-contamination.

2.2 Support Films

From the early days of TEM progress has depended upon the preparation of satisfactory support films for a range of different material science and biological samples, documented thoroughly

even in the 1961 edition of Kay's *Techniques for Electron Microscopy*. Although carbon support films can be readily purchased as consumables from the various EM supplies companies, it is still usual for individuals to prepare their own for immediate use. Some time needs to be devoted to the perfection of these additional techniques, to make available a ready supply of the necessary continuous or holey carbon support films for conventional negative staining or for the preparation of thin frozen-hydrated/vitrified cryo-negatively stained specimens.

Thin carbon support films can routinely be prepared by in vacuo carbon deposition onto the clean surface of freshly cleaved mica, with subsequent floatation of the carbon layer onto a distilled water surface followed by lowering onto a batch of EM grids (300 or 400 mesh) positioned beneath, i.e., in a Buchner funnel or glass trough with controlled outflow of the water [26]. The thickness of the carbon can be assessed by a crystal thickness monitor during continuous carbon evaporation, but this is not essential. With a little experience, repeated short periods of evaporation from pointed carbon rods readily enable the desired thickness (e.g., ~10 nm) to be achieved, based upon the faint gray color of a piece of white paper placed alongside the mica.

Carbon–plastic (e.g., collodion, Formvar, Butvar, and Pioloform) support films can be produced by first making a thin plastic film on the surface of a clean glass microscope slide, from a chloroform solution (0.1–0.5 % w/v). This plastic film is then released from the glass slide following scoring of the edges with a metal blade, with floatation onto a distilled water surface. An array of EM grids can then be positioned individually on the floating plastic sheet, or the plastic sheet can be lowered onto an array of pre-positioned EM grids at the bottom of the funnel or trough, when the water level is reduced. After drying, the batch of grids can be carbon-coated in vacuo.

The presence of a thin plastic layer provides an increase in strength to the carbon support, which is desirable for the extended sequence of steps required for immunolabeling (*see* Subheading 3.1.6), but the extra film thickness does inevitably slightly reduce image detail and the maximal level of resolution. However, if desired, for both continuous carbon–plastic and holey/perforated carbon–plastic films, the plastic can be dissolved by washing grids singly by dipping on edge into an appropriate solvent such as chloroform or amyl acetate and touching to a filter paper wedge before use.

Most would agree that the production of holey/perforated carbon–plastic or carbon support films (also termed micro-grids) still remains something of a difficult art. Some therefore resort to the purchase of commercially prepared holey carbon films, or Quantifoil® and C-flat™ films with regularly sized and spaced holes, produced by semi-conductor lithographic techniques. The simplest

procedure is to initially perforate a drying film of plastic on the surface of a precooled (4 °C) clean glass microscope slide by heavily breathing onto the surface. The small water droplets in the breath perforate the plastic film during evaporation of the chloroform.

Alternatively, a more reproducible way of producing perforated plastic films is to use a glycerol–water (0.5 % v/v each) emulsion in chloroform containing 0.1 % or 0.2 % w/v Formvar. With vigorous shaking an emulsion of small droplets is readily produced. On dipping a clean glass microscope slide vertically rapidly in and out of the emulsion, allowing it to drain-off and dry, the thin plastic film so produced will be found to contain an array of small holes, of varying size. A phase contrast light microscope can be used to quality control the production of holes in the drying plastic film. After wiping the glass edges and the edge of the film on the surface of the slide (i.e., ~2 mm) with a paper tissue, the perforated plastic film can then released from the slide by floating the film onto a water surface and lowered onto a batch of EM grids previously placed beneath, as described above. After drying, the grids should be carbon-coated, with in vacuo deposition of an additional layer of gold or gold/palladium, if desired. The inclusion of the metal layer enables the quality of holey carbon grids to be more easily assessed by bright-field or phase-contrast light microscopy, but its prime advantage is for rapid TEM focussing at higher magnifications and the fact that the presence of a metal layer improves the spreading properties of the surface, so no glow discharge treatment is necessary before use. Attempts have been made by some researchers to standardize this procedure [27], but most do tend to include their own individual variations [26].

2.3 Reagents and Solutions

1. Buffer: 20 mM Tris–HCl at pH 7.0 or 8.0 is most useful for diluting biological sample solutions. 5 mM Tris–HCl can be used as an on-grid washing solution.
2. Glutaraldehyde: make a 1 % working solution in distilled water from the 25 % w/v stock solution. Add an aliquot directly to biological sample, usually to the concentration range 0.05–0.1 %.
3. Trehalose: make a 10 % w/v stock solution in distilled water. Add an aliquot to negative stain solution, to give 1.0 or 0.1 %, as desired.
4. Polyethylene glycol (PEG), Mr 1,000: make a 10 % w/v stock solution. Adjust to desired pH with a 0.01 N NaOH. Add an aliquot to negative stain and protein solution, as required.
5. Uranyl acetate/formate: use as 1 or 2 % w/v aqueous solution, without pH adjustment.
6. Ammonium molybdate, Na/K-phosphotungstate and -silicotungstate: use as 1 or 2 % (w/v) aqueous solution, or as 5 %

(w/v) solution when 0.1 or 1 % trehalose present. Adjust pH as desired with 1.0 N Na/KOH, usually to pH 7.0.

7. Methylamine tungstate: use as 1 or 2 % w/v solution, with pH adjustment as desired.
8. Sodium/methylamine vanadate: use as a 2 % w/v pH 7.0 solution. Useful for low contrast negative staining.
9. Preimmune rabbit serum: dilute to 0.1 % v/v with PBS.
10. BSA: make up 0.1 % w/v solution in PBS.
11. Rabbit anti-mouse IgG/Fab′: dilute with PBS, as required.
12. Protein A—5/10 nm gold conjugate: dilute with PBS.
13. Streptavidin—5/10 nm gold conjugate: dilute with PBS. Possible stability problem.
14. Ammonium acetate: use as 0.155 M solution.
15. Glycerol: add as a protectant to protein/viral solutions at 50 % v/v. Add to 0.155 M ammonium acetate solution at 30 % v/v.
16. Alcian blue: use as 0.01 % w/v aqueous solution.

2.3.1 Some Further Comments on the Use of Reagents and Solutions

Although prefixation/chemical cross-linking is not generally required for the negative staining of biological particulates, if it emerges that a sample is exceptionally unstable in the available negative stains, prior fixation with a low concentration of buffered glutaraldehyde (e.g., 0.05 or 0.1 % v/v) may be included. This can be performed in solution or by direct on-grid *droplet* treatment of material already adsorbed to a carbon support film. It must, however, be borne in mind that the chemical attachment of glutaraldehyde to the available basic amino acid side groups, producing intra-protein cross-linkage and stabilization, may at the same time produce structural alterations at the higher levels of resolution. Also, in solution, care must be taken to avoid protein–protein cross-linkage/aggregation induced by glutaraldehyde. A combined glycerol gradient purification and glutaraldehyde fixation procedure was introduced by Stark and colleagues [28, 29] prior to specimen preparation for unstable molecular complexes, but in practice glutaraldehyde can be added immediately following any purification procedure [30].

The more commonly used negative staining salts are listed in Table 1 (for a more detailed listing, including some of the less commonly used negative stains, *see* ref. 26). These negative stains are generally used as 2 % w/v aqueous solutions, but there is always the possibility of increasing the concentration to provide greater electron density for small proteins or reducing the concentration for excessively thick biological samples that retain a greater volume of surrounding fluid. If the stain concentration is too low, on air drying the thin layer of fluid that surrounds a biological

Table 1
Negative stain solutions

Commonly use negative stain solutions
These stains are generally prepared as 1 or 2 % w/v aqueous solutions[a,b], but for negative staining across holes and for cryo-negative staining a higher concentration is usually required (*see* protocols). All stain solutions except the uranyl stains can be neutralized
Uranyl acetate
Uranyl formate
Sodium/potassium phosphotungstate
Sodium/potassium silicotungstate
Ammonium molybdate
Methylamine tungstate
Methylamine vanadate (Nanovan)
Negative stain-carbohydrate combinations
All of the above negative stains can also be prepared as 2–6 % w/v aqueous solutions containing 0.1–1 % w/v carbohydrate (e.g., glucose or trehalose)[c]
Negative stain-PEG combinations
The inclusion of 0.1–0.5 % w/v polyethylene glycol (PEG) Mr 1,000 in 2 % w/v ammonium molybdate creates a solution that potentiates 2D virus and protein crystal formation[d]

[a]A low concentration (e.g., 0.1–1.0 mM) of the neutral surfactant *n*-octyl-β-d-glucopyranoside (OG) can be added to any of the above negative stain solutions to improve the spreading properties and assist permeation within biological structures

[b]The pH of negative stain solutions can usually be adjusted over a wide range; this does not apply to the uranyl negative stains, which readily precipitate if the pH is significantly increased above pH 5.0. By complexing uranyl acetate with oxalic acid, an ammonium hydroxide neutralizable soluble anionic uranyl-oxalate stain can be created, but this possesses an undesirable granularity after drying and increased sensitivity to electron beam damage

[c]Glucose and in particular trehalose can provide vitreous protection to the biological material during air drying. The presence of trehalose also creates a slightly thicker supportive layer around the sample, thereby reducing flattening. Electron beam instability of these carbohydrates necessitates minimal routine or low electron dose irradiation conditions, assisted by specimen cooling where possible. The inclusion of 1 % trehalose reduces the net electron density of the negative stain solution; this is why a higher stain concentration (e.g., 5 % w/v) is used

[d]When mixed with a purified viral or protein solution and spread as a thin layer on mica *or* across the holes of a holey carbon support film (also with trehalose present), this AM-PEG solution can induce 2D crystal formation (see text). Variation of the concentration of the PEG and the pH of the solution is always required, to obtain the optimal conditions for 2D crystal formation

particle may not leave a sufficiently thick layer of amorphous salt to completely embed and support the particle, thereby resulting in partial-depth staining and undesirable sample flattening. The adjustment of negative stain pH, to a value close to that of the sample buffer is standard. This is not usually possible for the uranyl negative stains, which precipitate at pH values above ca pH 5.5. It should also be borne in mind that the presence of even traces of phosphate buffer is incompatible with the use of uranyl negative stains.

Addition of a protective carbohydrate such as trehalose (e.g., 0.1–1.0 % w/v) to the negative stain solution has the advantage of creating a thicker supportive layer of dried stain, whilst at the same time helping to actually preserve the biological sample during air drying. Trehalose has uniquely beneficial properties in this respect. This is because of the retention of sample structure within a vitreous carbohydrate-negative stain-layer. Trehalose is thought to replace or protect protein-bound water and has been shown to have widespread value as a protectant in bioscience. Inclusion of a higher concentration of negative stain (e.g., 5 or 6 % w/v) is then usually required to create an optimal electron density [14, 31]. Specimen-bound water, with or without the presence of a carbohydrate, will be greatly reduced once a specimen is inserted into the high vacuum of the TEM unless direct cooling in liquid nitrogen is performed first and followed by cryo-transfer to the electron microscope (with the specimen then maintained and studied at low temperature). As specimen lability within the electron beam is initially related to the presence of vitreous water, the early and continued success of conventional negative staining would appear to be due to the rapid in vacuo removal of almost all loosely bound water from the biological material and amorphous stain, prior to electron irradiation. With the current increasingly widespread availability of TEM low dose systems, image recording from frozen-hydrated negatively stained specimens (produced following air drying or by rapid plunge freezing), can be successfully pursued, thereby creating the possibility of improved image resolution because of sample hydration maintained at low temperature. Although negative staining and cryo-electron microscopy do now appear to have some significant overlap [10, 26, 27], it is likely that the two separate technical approaches will often be maintained for the foreseeable future. Indeed, for high resolution low temperature negative stain studies the phrase *high contrast embedding media* has been introduced to avoid the *negative* connotations and undesirable limitations of conventional room temperature air-dry negative staining on continuous support films, that can at least in part be overcome when holey carbon support films are used (*see* Subheading 3.1.2).

A significant technical parallel exists regarding the use of PEG and ammonium molybdate to create 2D viral and protein crystals via the negative staining-carbon film (NS-CF) technique, during negative staining across holes and during the cryo-negative staining procedure (prior to freezing). In all three instances, it is thought that the 2D crystals form at the fluid–air interface, promoted by these reagents and surface tension forces [32, 33].

2.4 Sample Material

For conventional on-grid negative staining it is desirable to have purified sample material in the form of a free suspension (i.e., without any large aggregates) in water or an aqueous buffer solution, at a

concentration of ca 0.1–1.0 mg protein/ml. For negative staining on holey carbon support films [27, 34] and for the negative staining-carbon film technique [32, 35] (when used to produce 2D crystals) the optimal concentration will need to be, in the order of 0.5–2.0 mg/ml protein. For lipid suspensions, lipoproteins, nucleic acids, nucleoproteins and viral particles, these concentration figures provide only a general guide; the main aim in all cases is to avoid overloading the specimen with sample material since particle superimposition will always obliterate structural detail. The presence of a high concentration of sucrose, urea or other solute in the sample suspension will introduce some problem for negative staining, so these reagents must be removed. This can be done by prior dialysis or gel filtration using a dilute buffer solution, or by carbon adsorption and on-grid washing with distilled water or a dilute buffer solution, immediately before negative staining.

3 Methods

Protocols will be presented below for negative staining using the single droplet procedure, applied to samples adsorbed to a continuous carbon film or spread across the small holes of a holey/perforated carbon film [27]. The floating and carbon-sandwich methods will also be given, but the spray-droplet method will not be included, primarily due to present-day safety considerations. Although all the different approaches can generate satisfactory negatively stained specimens for TEM study, it is the considered opinion of the authors, that the use of holey carbon films with ammonium molybdate–trehalose as the negative stain, although technically slightly more difficult, is preferable for detailed molecular negative stain studies. For routine studies negative staining with uranyl acetate on continuous carbon support films is usually satisfactory.

The negative staining-carbon film (NS-CF) techniques, for 2D crystallization or proteins and viruses [26, 35], for randomly dispersed molecules and cleavage of cells and organelles [10] will be given. In addition, application of the droplet negative staining procedure for the production of antibody- or affinity-labeled specimens will also be given, together with comments on the possibilities for dynamic negative staining experiments (where the time-period is generally in the range of a few minutes to hours, rather than milliseconds or seconds). Finally, two procedures for the preparation of cryo-negatively stained specimens with be presented [9, 24].

Despite the procedural listings given below the user should, having gained some limited experience, be prepared to freely introduce small technical variations to suit any local requirements determined by the biological sample, the equipment available, and the

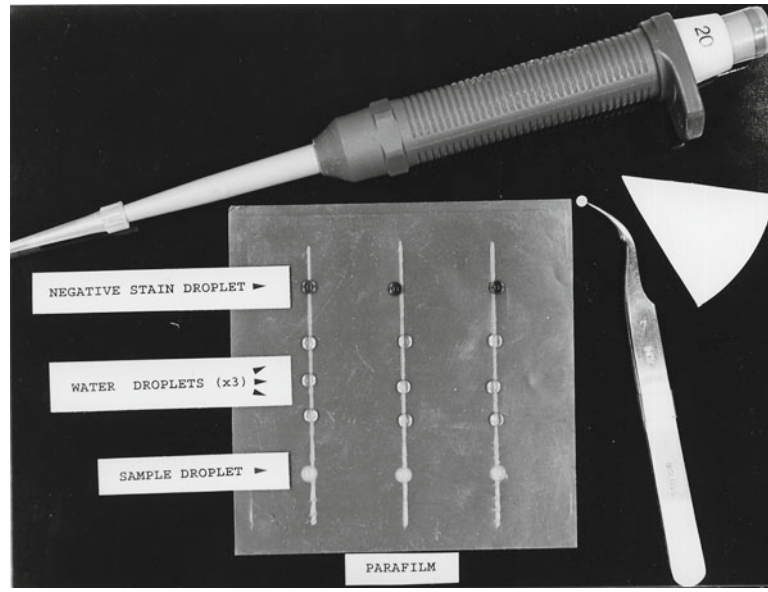

Fig. 2 A representative example of the layout of material for the single droplet negative staining procedure. The number of water-wash droplets is variable, depending upon the buffer and salt concentration in the sample

aims of any individual study. Thus, an overall scientific/technical awareness that improvements at the specimen *grid level* can continually be sought and included is highly desirable.

3.1 The Single Droplet Negative Staining Technique

This general and versatile procedure [26] is applicable to biological samples adsorbed to continuous carbon films and freely spread across the holes of holey carbon films. With slight modification, it is applicable to immuno-negative staining and dynamic negative staining experiments (*see* below, Subheadings 3.1.6 and 3.1.7).

3.1.1 Conventional Negative Staining on Continuous Carbon Films [26, 36]

1. Cut off a piece of Parafilm from a roll (length depending upon the number of samples and grids to be prepared), so that individual samples can be spaced by approx. 1.5 cm, as shown in Fig. 2.
2. Place the Parafilm, paraffin-wax-down, onto a clean bench surface and before removing the paper overlay produce a number of parallel lines by scoring with a blunt object across the paper. Then remove the paper overlay, leaving the Parafilm loosely attached to the bench surface.
3. Place 20 μL droplets of sample suspension, distilled water (or dilute e.g., 5 mM buffer solution), and negative stain solution onto the Parafilm, as in Fig. 2. The number of water droplets can vary, depending upon the concentration of reagent to be removed from the sample, with the proviso that each successive

wash may introduce additional breakage of the fragile carbon support film. (In general, it is best not to attempt to prepare more than 6–8 specimen grids as a single batch, unless this is absolutely necessary).

4. Take in a pair of curved forceps an individual specimen grid coated with a thin continuous carbon support film. (Brief glow discharge treatment should be applied in advance to increase hydrophilicity of the carbon surface and thereby improve the sample and stain spreading). Touch the carbon surface to the sample droplet. After a period of a time, ranging from 5 to 60 s (*see* **Note 1**), remove almost all the fluid by touching the edge of the grid carefully to a filter paper wedge.
5. Before the sample has time to dry, wash the adsorbed sample with one or more droplets of distilled water, and each time carefully drain away the excess water from the grid, as in **step 4**.
6. Touch the grid surface to the droplet of negative stain and likewise remove excess fluid. Then allow the thin film of sample + negative stain to air-dry, before positioning the grid in a suitable container (petri dish or commercially available grid storage box; *see* **Note 2**).
7. After drying at room temperature, grids are immediately ready for TEM study, under either conventional electron dose conditions at ambient temperature or under low electron dose conditions at either ambient temperature or after specimen cooling in the TEM (e.g., to approx. –180 °C; *see* **Note 3**).

3.1.2 Negative Staining on Holey Carbon Support Films

This variant of the droplet negative staining technique is particularly useful for detailed studies on samples that have already been assessed by conventional negative staining on continuous carbon support films and justify more detailed investigation under conditions where carbon adsorption and specimen flattening are avoided. The protocol given in Subheading 3.1.1 should be followed, with incorporation of the following variations, as described by Harris and Scheffler [27]:

1. For holey carbon films, glow discharge is not usually necessary when a thin microcrystalline layer of gold or palladium metal has also been deposited.
2. It is necessary to use one of the negative stain-carbohydrate combinations (e.g., 5 % w/v ammonium molybdate or sodium phosphotungstate + 0.1 % w/v trehalose; *see* **Note 4**) since an unsupported air dried film of sample and stain alone breaks very readily. This situation is considerably improved by the presence of carbohydrate, but concentrations in excess of 1.0 % w/v trehalose lead to rapid damage in the electron beam unless low electron dose conditions are employed.

3. For specimens prepared across holey carbon films, sample concentrations of 1.0–2.0 mg/ml protein are generally desirable, as a greater quantity of free material is lost from the holes during the washing and staining steps. Biological material is, however, often retained at the fluid–air interface at the side of the grid *opposite* to where the filter paper is touched, due to surface tension forces.
4. The sample washing, to remove interfering buffer and saline, can be done either with 20 μL droplets of 0.1 % w/v trehalose or the 5 % w/v negative stain + 0.1 % w/v trehalose solution.
5. As there is a tendency for the film of stain across the holes to be too thick, at the final stage of the procedure the filter paper should be held in contact with the edge of the grid for approx. 10–20 s, to remove as much fluid as possible.
6. Inclusion of PEG Mr 1,000 (e.g., 0.1–0.5 mg/ml) in the specimen solution, the trehalose washing solution and/or in the negative stain–trehalose solution can promote 2D crystal formation of isometric viruses and macromolecules across the holes (*see* **Note 5**). In this case, longer times of incubation prior to the initial removal of excess sample and stain with the filter paper are desirable, to allow sufficient time for 2D crystal formation at the air–fluid interface, before drying.
7. Dry the grid at room temperature and study in a TEM, within ~ one day as recrystallization of the vitreous ammonium molybdate–trehalose film may occur.

3.1.3 The Floating Negative Staining Method [11]

This negative staining variant requires carbon-coated mica to be prepared in advance, Parafilm or more conveniently a series of micro-wells bored in a Teflon plate are also needed.

1. Pipette 20–60 μl droplets of samples, washing, fixing and stain solutions into the micro-wells, with adequate illumination available.
2. Using sharp scissors, cut off small pieces of carbon-coated mica and hold by one corner with fine forceps.
3. Carefully insert the mica into the sample droplet so that the carbon film starts to float onto the droplet surface, but do not completely release the carbon film. Allow the specimen to adsorb to the carbon and then slowly remove the mica, allowing the carbon to fall back onto the mica in its original position. Drain off excess fluid from the forceps and lower surface of the mica with a filter paper.
4. Using the same procedure, insert the mica + carbon into droplets of washing solution, usually distilled water, and if required a fixation step using 0.05–0.1 % glutaraldehyde.

5. Completely float off the carbon film from the small mica piece onto a negative stain droplet.
6. Using a blank, alcohol-washed EM grid, carefully insert the grid beneath the floating carbon film, and lift from the droplet. Alternatively, the carbon film can be recovered from above using a sticky grid. Remove excess negative stain with a filter paper and air-dry.

3.1.4 The Carbon Sandwich Negative Staining Method [24]

The carbon sandwich approach can be readily extended from either the droplet and floating methods, given above, the aim being to trap the specimen material in an even layer of stain between two layers of carbon. A risk of this procedure is that the force between the two layers of carbon may cause flattening or even disintegration of the sample, but this can usually be prevented by introducing a prior glutaraldehyde fixation step. Note that some scientists studying macromolecular structure rigorously avoid the use of glutaraldehyde fixation, because of the incorporation of additional mass and the possibility of introducing structural changes due to the chemical cross-linkages.

Using micro-wells in a Teflon plate as the final negative staining step, the grid + sample from the droplet procedure (*above*) a carbon-coated grid can (**a**) be inserted beneath a film of carbon released from a small piece of mica floating on negative stain, thus creating the carbon sandwich, or (**b**) with the floating method (*above*), a coated grid with a continuous or holey film is inserted beneath the floating carbon + adsorbed sample on negative stain and the two carbon layers brought together as the grid is carefully lifted out of the droplet. Excess stain should then be removed with a filter paper wedge, before air drying.

3.1.5 The Negative Staining-Carbon Film (NS-CF) Techniques

The NS-CF procedure was initially developed for the production of 2D arrays/crystals of viral particles [12, 35]. The technique has also been adapted for the study of randomly dispersed fragile macromolecules and wet-cleaved cells and organelles [13], with the samples dried in vacuo on mica from a protective glycerol–volatile buffer solution, prior to carbon coating and negative staining. It can also be used for the attachment of biological material such as cholesterol crystals spread on mica, for subsequent affinity or immunolabeling labeling techniques [37].

Two-Dimensional Crystallization of Protein Molecules and Virus Particles [12, 35]

1. Prepare small pieces of mica, ca 1.5 × 0.5 cm, with one end pointed (*see* procedural outline, Fig. 3). Cleave the mica with a needle point to expose two untouched perfectly clean inner surfaces.
2. On a small piece of Parafilm, mix a 10 µl volume of sample (e.g., purified virus or protein solution, ca 1.0–2.0 mg/ml) with an equal volume of 2 % ammonium molybdate (AM)

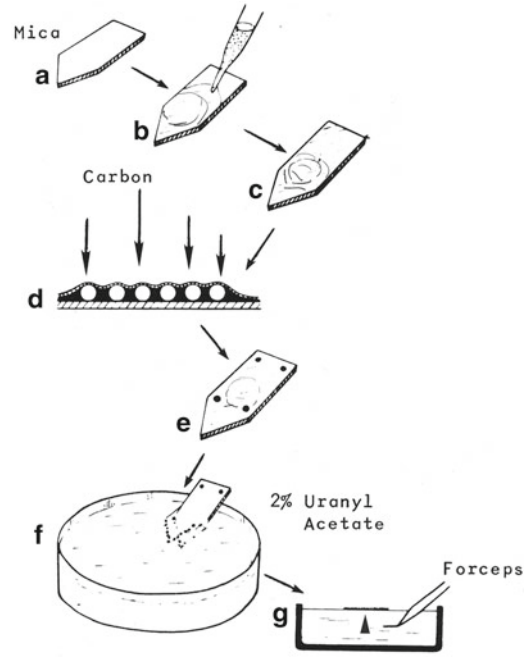

Fig. 3 A diagrammatic presentation of the successive stages of the negative staining-carbon film (NS-CF) procedure [12]

containing 0.1 or 0.2 % w/v PEG Mr 1,000. The pH of the AM solution can be varied between pH 5.5 and 9.0. (Some AM precipitation may be encountered during storage at pH 6.5 and lower, over a period of months).

3. Apply 10 μL quantities of the sample-AM-PEG to the clean surface of two pieces of mica (held by fine forceps) and spread the fluid evenly with the edge of a plastic pipette tip. Hold each piece of mica vertically for 2 s to allow the fluid to drain towards one end, remove most of the pooled fluid and then hold horizontally, creating an even very thin film of fluid. Allow the fluid to slowly dry at room temperature within a covered Petri dish (*see* **Note 6**). A clearly visible zone of progressive drying towards a final deeper pool can usually be defined. 2D crystal formation will occur at this stage of the procedure, in all probability at the fluid–air interface, since adsorption of biological material to the untreated mica surface does not occur.
4. Coat the layer of dried biological sample on the mica surface in vacuo with a thin film of carbon (5–10 nm).
5. Float off the carbon film + adsorbed biological material (randomly dispersed and as 2D arrays or crystals) onto the surface of a negative stain solution (e.g., 2 % w/v uranyl acetate or ammonium molybdate) in a small Petri dish (*see* **Note 6**). Recover pieces of the floating film directly onto uncoated 400

mesh EM grids from beneath, with careful wiping on a filter paper to remove excess stain and any carbon that folds around the edge of the grid. Often, the freshly deposited carbon film does tend to repel the aqueous negative stain, leading to under-staining rather than over-staining; attempt to avoid this if encountered, by a shorter contact time with the filter paper or inclusion of 0.1 % octyl glucoside in the negative stain. Allow the grid to air dry before positioning on a filter paper in a petri dish or placing into a grid storage box.

6. Study stained grids in a TEM.

Negative Staining of Glycerol-Containing Solutions of Fragile Proteins [13]

1. Prepare small pieces of freshly cleaved mica, as in Subheading "Two-Dimensional Crystallization of Protein Molecules and Virus Particles."
2. Pipette approx. 10 μL volumes of glycerol-containing solutions of protein or virus (0.1–0.2 mg/mL) onto the mica pieces. Remove excess fluid by touching to a filter paper (essentially as in Fig. 3).
3. Dry the glycerol–protein solution on the mica surface in vacuo at 10^{-5} Torr for 1 h, or longer (if a buffer is present, it should be volatile, i.e., ammonium acetate).
4. Coat the mica surface with a thin layer of carbon (10 nm) in vacuo.
5. Float off the carbon film + adsorbed protein onto either a negative stain solution or distilled water. Recover small pieces of the floating film with 400 mesh EM grids, from beneath the fluid surface. Remove excess stain solution by touching to the edge of a piece of filter paper. (If floated onto distilled water, finally add negative stain from a 20 μL droplet of stain on Parafilm).
6. Invert the EM grid (i.e., stain and protein then uppermost) and position horizontally on a filter paper to dry at room temperature.
7. Study stained grids in TEM.

Immobilized Cell and Organelle Wet-Cleavage by the Negative Staining-Carbon Film Procedure [13]

This NS-CF procedure can be utilized for the study of wet-cleaved red blood cells, white blood cells and cultured cells in suspension. It also has possibilities for the study of isolated cellular organelles, such a mitochondria, nuclear envelope, membrane vesicles, liposomes and reconstituted vesicular membranes (*see outline*, Fig. 4), and can be combined with immuno- or affinity-labeling. This procedure can be used for any biological sample spread on mica and subsequently adsorbed to a directly deposited carbon film [37].

1. Prepare small pieces of freshly cleaved mica, as in Subheading "Two-Dimensional Crystallization of Protein Molecules and Virus Particles."

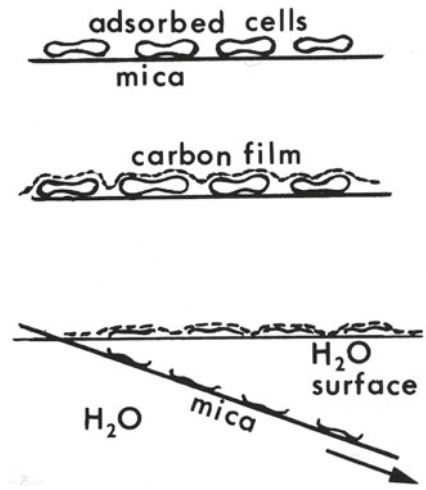

Fig. 4 A diagrammatic representation of the immobilized cell wet-cleavage by the negative staining-carbon film procedure [13]. The example shown uses erythrocytes, but cultured cells and isolated subcellular organelles can also be used

2. To promote cell attachment impart a positive charge on the mica surface by immersion in 0.01 % w/v aqueous Alcian blue solution for 30 s, followed by thorough washing in distilled water and air drying.
3. Pipette 10 µl volumes of cell suspension onto the mica surface and spread evenly over the whole area. Place the mica horizontally for a few minutes, for the cells to become attached.
4. Washed away excess unbound cells by vertical immersion in three changes of PBS. Attached cells can be monitored by light microscopy at this stage. Brief cellular fixation with 0.1 % v/v glutaraldehyde can be applied at this stage, if desired, but this may interfere with the cleavage **step 8**, below.
5. Remove the PBS by flooding the mica surface three times with 30 % v/v glycerol–0.155 M ammonium acetate.
6. Wipe the under surface of the mica with tissue and remove the glycerol–ammonium acetate on the upper surface by drying in vacuo at 10^{-5} Torr for 1 h, or longer.
7. Coat the dried cells with a thin layer (10 nm) of carbon in vacuo.
8. Float off the carbon film onto the surface of distilled water in a small petri dish (*see* Fig. 4). At this stage, the cells are physically split, by a wet-cleavage process, leaving half the cell attached to the mica, the other half being removed with the carbon film. Note that over-stabilization of the cells with too much glutaraldehyde will prevent this cleavage.

9. Pick up small pieces of the floating carbon film on 400 mesh EM grids, from beneath the fluid surface, and immediately touch the membrane-containing surface of the carbon onto a 20 μL droplet of negative stain solution (e.g., 2 % w/v uranyl acetate or ammonium molybdate) on a Parafilm surface.
10. Remove excess negative stain solution by touching the edge of the grid to a filter paper wedge, invert the grid and allow to dry at room temperature.
11. Study stained grids in TEM.

3.1.6 Negative Staining of Immunolabeled and Affinity Labeled Samples

The combination of immunological labeling of protein molecules, viruses, intact cytoskeletons, isolated cellular membrane fractions, fibrillar and cytoskeletal proteins in combination with negative staining offers considerable possibilities for antigen/epitope localization. For plant viruses this approach was elegantly demonstrated by Roberts as early as 1986 [38], yet the use of negative staining in combination with immunolabeling of proteins and isolated cellular components and animal viruses has been relatively small, when compared to postembedding immunolabeling of cells and tissues (*see* Chapters 13 and 14). All the negative staining protocols presented above can readily be adapted to study antibody- or affinity-labeled samples. Two main approaches can be followed:

1. The first approach requires prior preparation of the biological material in combination with a defined epitope-specific polyclonal antibody, monoclonal antibody (IgG or Fab' fragment) in solution, using antibody alone or antibody conjugated to colloidal gold or a smaller gold cluster probe. IgG molecules will cross-link soluble protein molecules, often creating specific linkage patterns that can assist the definition of epitope location. The use of Fab' fragments does in theory present the possibility for higher resolution definition of epitope localization, but TEM study is then inherently more difficult and does require some confidence that all available epitopes have a bound Fab'. With a satisfactory biological preparation, held in suspension as small immune complexes/immunoprecipitates (IgG) or soluble complexes (Fab'), the standard single droplet negative staining procedure can be followed (using gold or nickel grids), as described in Subheading 3.1.1 (*see* also ref. 38). An equivalent approach, using the biotin–streptavidin system can also be employed, when a component within the biological sample is biotinylated. Both these forms of in-solution labeling can also be used to prepare samples for cryo-negative staining.
2. The second approach utilizes the fact that particulate biological material can be adsorbed to a robust carbon-coated plastic support film to create an immobilized thinly spread layer,

which can then be allowed to interact successively with blocking solution, primary monoclonal antibody solution unlabeled or colloidal gold-conjugated (usually with 5 or 10 nm colloidal gold), wash solutions, colloidal gold-conjugated secondary polyclonal antibody or protein A-gold, wash solutions, distilled water and finally a negative stain solution (sequential labeling using a secondary visualization marker is usually best). This does, in general, follow the pattern of on-grid post-embedding immunolabeling of thin sectioned material, with the caution that somewhat greater handling precautions need to be adopted, particularly if the support film is carbon alone; carbon–plastic support films have superior handling properties. Brief fixation of the sample with 0.05 or 0.1 % glutaraldehyde can be useful, if dissociation is encountered during the prolonged incubation and washing sequence.

The location of primary or secondary antibody-gold conjugates, rather than unlabeled primary antibodies alone, has been found to be most successfully pursued by this approach. Thus, with an unconjugated primary monoclonal antibody the reaction can be performed in combination with a conjugated secondary antibodies and/or the streptavidin/biotin reaction (with biotinylated primary or secondary antibody, followed by streptavidin-conjugated gold probe).

Site-specific labeling of functional groups using derivatized gold cluster probes (Nanogold and Undecagold) also presents increasing possibilities for the future. In this instance, because of the small size of the gold probe, the use of a low contrast negative stain such as 2 % w/v sodium vanadate or 3 % ammonium molybdate containing 1 % trehalose, is desirable. In general, with colloidal gold labeling, negative staining can be performed with any appropriate negative stain. As with immunolabeling of thin sectioned material, gold or nickel grids must be used.

An experimental lay-out of sequential 20 µl droplets on Parafilm can be adopted, for example: sample solution, blocking solution, primary monoclonal antibody (can be biotinylated), washing solution, secondary gold-conjugated polyclonal antibody (streptavidin-gold, if the first step uses a biotinylated antibody), washing solution or distilled water and negative stain, essentially similar to that shown in Fig. 2. For prolonged incubation times at room temperature, individual blocking or antibody droplets, or the whole Parafilm sheet, can be covered with a Petri dish or other container, containing a piece of water-moistened tissue paper to minimize evaporation.

A standard protocol is given below (after ref. 40), which can readily be modified to suit the requirements of almost any experimental conditions:

1. Take a glow discharge-treated carbon–Formvar/Butvar film on a nickel or gold 400 mesh EM grid and touch the surface to a

10 μL droplet of sample solution (usually glutaraldehyde-fixed). After a short time, remove excess solution by carefully touching to the grid edge to a filter paper. Select the sample adsorption time depending upon the concentration of material in solution. Avoid overloading the carbon surface with biological material.

2. Block nonspecific reactions by floating the grid for 10 min on a 20 μL droplet of 1.0 % preimmune rabbit serum in PBS. If Protein A-gold is to be used, avoid serum; use instead 0.1 % w/v BSA in PBS. Remove excess fluid with a filter paper wedge.
3. Float the grid on a 20 μL droplet of hybridoma culture supernatant or purified primary IgG antibody for 2 h (*see* **Note 7**), at room temperature in a moist environment.
4. Wash the grid surface with five successive 20 μL droplets of 10 % preimmune rabbit serum in PBS to remove free antibody (or 0.1 % w/v BSA in PBS) (*see* **Note 8**).
5. Incubate the sample for 2 h by floating the grid on a 20 μL droplet of secondary antibody (e.g., rabbit anti-mouse IgG- or Protein A—5 nm gold conjugate), at room temperature.
6. Wash the grid surface with three 20 μL droplets of PBS to remove secondary antibody and three droplets of deionized water.
7. Negatively stain the grid surface by touching to a 20 μL droplet of 1 or 2 % w/v stain solution. Ammonium molybdate or sodium phosphotungstate (pH 7.0) will generally be found to be superior to uranyl acetate (pH 4.5).
8. Air-dry the grid at room temperature and study in a TEM.

   The above procedure can be combined with cellular permeabilization procedures or surfactant extraction to reveal cellular cytoskeletons [40], *see* also **Note 9**.

3.1.7 Dynamic Negative Staining

The period of time required to produce conventionally negatively stained specimens, on continuous or holey support films, requires only approx. 30 s. The drying time for the thin aqueous film of stain, at room temperature, is two or three min. With the cryo-negative stain procedure, the situation is similar during the initial stages, but the plunge freezing is extremely rapid (msec). This provides the possibility that negative staining can be utilized to monitor progressive or dynamic biological events, as long as they occur at time periods in excess of ca 2 min. However, Zhao and Craig [41] have performed time-resolved studies using an accurate rapid grid application system, whereby prior stabilization of myosin filaments for only 10 ms with uranyl acetate or tannic acid influenced the effect of a variety of interactive solutions. This system could probably be modified to trap rapid structural changes in other biological systems directly "on the grid," by applying the uranyl acetate rapidly after a very short time after a biological agent

of functional interest. As some viruses react rapidly to a low pH and shed surface proteins, pH treatment of carbon-adsorbed viruses could provide a means to assess dynamic structural changes.

The PEG interaction time and drying time during the negative staining-carbon film procedure and during the holey carbon negative staining procedure greatly influences the nucleation of 2D crystals and molecular association to produce supramolecular assemblies. Although such times are likely to minutes rather than seconds, the procedure can provide useful molecular information.

3.1.8 Cryo-negative Staining

This procedure is a variant of the standard plunge freezing/vitrification procedure for the production of unstained cryo-specimens (*see* Chapter 19). In the present instance the technique is designed to produce frozen-hydrated specimens where a thin aqueous film of biological sample + negative stain is spread across the small holes of a holey carbon support and is rapidly vitrified, as presented by Adrian et al. [9] (*see* **Note 10**). The equipment and materials required are essentially the same as for the cryo-electron microscopy of unstained specimens, *see* Chapter 19. An additional procedural stage is included, within which the biological sample is mixed and incubated with a saturated ammonium molybdate solution prior to blotting and rapid freezing. The possibility also exists to create monomolecular 2D crystals of isometric viral particles and macromolecules immediately prior to freezing, by including polyethylene glycol (PEG) in the sample and negative stain solution [42] (*see* **Note 11**). A diagrammatic description of the overall cryo-negative staining procedure is given in Fig. 5 (*see also* ref. 10, for further detail).

Cryo-negative Staining Procedure on Holey Carbon Grids

1. Apply 5 µl of sample solution to a holey carbon support film held by a pair of straight fine forceps. This is followed by short period of time, ranging from 10 to 60 s, during which some concentration of sample at the fluid–air interface will occur.
2. Invert the holey carbon grid + sample solution onto a 100 µl droplet of saturated ammonium molybdate (~0.8M) (pH 7) solution and allow it to float for a period of time, usually ranging between 10 s and 3 min. The forceps + grid and sample mixed with ammonium molybdate are then positioned in the clamp of the plunge freezing apparatus.
3. Carefully blot the grid face-on with a filter paper, which is held in direct contact with the grid for approx. 1–2 s and gently lifted off. After a further 1–2 s, during which time some evaporation of water will occur to produce an optimal thickness of the aqueous film spanning the holes (achieved by personal experience only), the release catch of the manual *guillotine* is instantly activated (by a hand or foot mechanism) and the grid rapidly plunged into liquid nitrogen-cooled liquid ethane (*see*

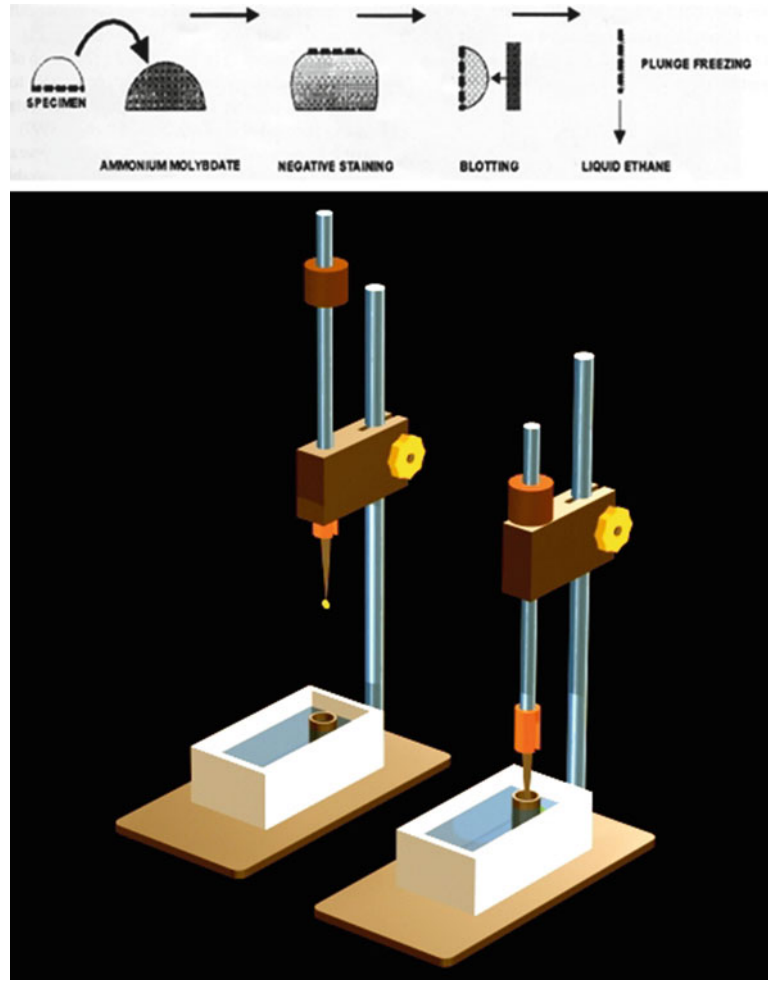

Fig. 5 An outline of the cryo-negative staining procedure [9] and manual plunge freezing equipment (courtesy of Marc Adrian). Immediately after the blotting stage, a short period of time (a few seconds) will enable some evaporation to occur with concentration of the sample and stain, prior to plunge freezing/vitrification. This procedure can also incorporate PEG within the sample prior to freezing

Fig. 5) (semi-automated plunge vitrification equipment is available, such as the FEI Vitriblot™ Mark IV and Gatan Cryoplunge™ 3, *see* **Note 12**). A thin vitreous layer is produced, containing negatively stained sample material, crossing the small holes and surface of the holey carbon support film.

4. The forceps are then released from the holding mechanism and very quickly lifted from the liquid ethane into the liquid nitrogen, with removal of as much adhering liquid ethane as possible (any remaining will solidify as a contaminant on the grid and can obliterate the specimen and/or interfere with the grid-holding screw or clip of the cryo-holder).

5. At this stage one or more grids can be stored in the small circular plastic transfer-holder. Such holders, with a screw-on cap, can be stored in ice-free liquid nitrogen, using a modification of the system routinely used for storing aliquots of viable cultured cells. Repeat specimens can be prepared, with the precaution that the tips of the forceps holding a new holey carbon grid must not contain any moisture, as this will prevent the easy removal of the grid following the rapid freezing sequence (light coating of the forceps tips with Teflon will help, if a problem is encountered).
6. The TEM cryotransfer holder should meanwhile have been precooled, either when within the electron microscope or when inserted into the cryo-workstation. The anti-contaminator system of the electron microscope must also be precooled. The small holder for the cryospecimens is then rapidly moved from the polystyrene box of liquid nitrogen to the liquid nitrogen within the cryostation. From there a grid is positioned in the space at the end of the cryoholder, the holding-clip or -screw inserted, the sliding protective shield moved over the specimen and the specimen holder rapidly transferred to the electron microscope. The exact sequence of actions relating to the individual electron microscope air-lock evacuation, insertion and rotation of the cryoholder need to be performed as described by the manufacturer.
7. Following insertion into the transmission electron microscope, the small Dewar flask of the cryoholder should be rapidly filled or topped-up as necessary and time then allowed for the specimen holder temperature to stabilize (usually –170 to –185 °C) and the electron microscope to achieve its high vacuum.
8. After removal of the sliding shield of the specimen holder, low electron dose TEM study and image recording can commence.

Cryo-negative Staining by the Carbon Sandwich Procedure [24]

This alternative cryo-negative staining procedure basically follows the one for floatation negative staining (*see* Subheading 3.1.4). After binding sample (often containing 30 % glycerol; *see* **Note 13**) to the carbon film by the floatation procedure, followed by incubation with a 2 % negative stain solution (usually uranyl formate) and blotting off excess fluid, grids are kept at room temperature for 1–2 min to air-dry (but retaining considerable sample hydration) before being manually frozen in liquid nitrogen or liquid ethane. Grids are then cryo-transferred to the TEM in a frozen-hydrated state. This procedure can be performed using a combination of small petri dishes and micro-wells in a Teflon plate, the latter being more economical with respect to the volume of negative stain solution.

1. A carbon-coated glass slide is inserted into sample-containing solution, with partial floatation of the carbon onto the fluid surface and withdrawal.

2. Sample adsorbed onto the carbon and is floated onto negative stain solution and EM grids then used to pick up pieces of carbon film + adsorbed sample from above.
3. A second carbon film is floated onto a well containing negative stain solution.
4. The grids with sample uppermost are then inserted beneath the floating carbon film, creating a carbon sandwich with the stained sample between the two layers.
5. After blotting the grid is allowed to air-dry for 1–2 min and manually frozen in liquid nitrogen.

4 Selected Examples of TEM Data Produced Using Some of the Negative Staining Protocols

Negative staining using the versatile single droplet procedure on continuous carbon support films is widely applicable, indeed it can be used for aqueous suspensions of almost any isolated subcellular fraction, viral, macromolecular, fibrillar, and biological sample. Numerous examples of data could be given; for further possibilities the reader is also directed to the extensive scientific literature, the two previous editions of this book and the author's book on negative staining [26].

As cellular and subcellular examples of negative staining we present our available data on the structural analysis of the apicomplexan parasite *Cryptosporidium parvum* (*see* Figs. 6, 7, 8, 9, and 10). The intact *C. parvum* oocyst is a large extremely robust ~5 μm spherical wall structure containing several sporozoites, residual body and other internal structures. Because of the thickness of the intact *C. parvum* oocyst, sporozoite and residual body, negative staining reveals rather little, apart from the overall dimensions (*see* Fig. 6). However, when these cellular structures are disrupted, by mechanical method, ionic treatment, ultrasonication, or other treatment, their substructure can often be revealed, including the sporozoite micronemes and amylopectin particles present within the residual body and oocyst wall fragments (*see* Figs. 7, 8, 9, and 10), in these instances revealed by ammonium molybdate negative staining by the droplet procedure. As a macromolecular example, the purified *Bacillus subtilis* stressosome RST complex is shown (*see* Fig. 11), revealed by negative staining with uranyl acetate. As fibrillar examples, the twisting fibrils formed in vitro by the amyloid-beta peptide and collagen type 1 are shown (*see* Figs. 12 and 13). Isolated membranes [36] and lipid suspensions (particulate and liposomal) can likewise be usefully studied by negative staining. Figures 14 and 15 show cholesterol microcrystals and cochleate cylinders [43] before and after interaction with the cholesterol-binding domain 4 of the bacterial toxin pyolysin.

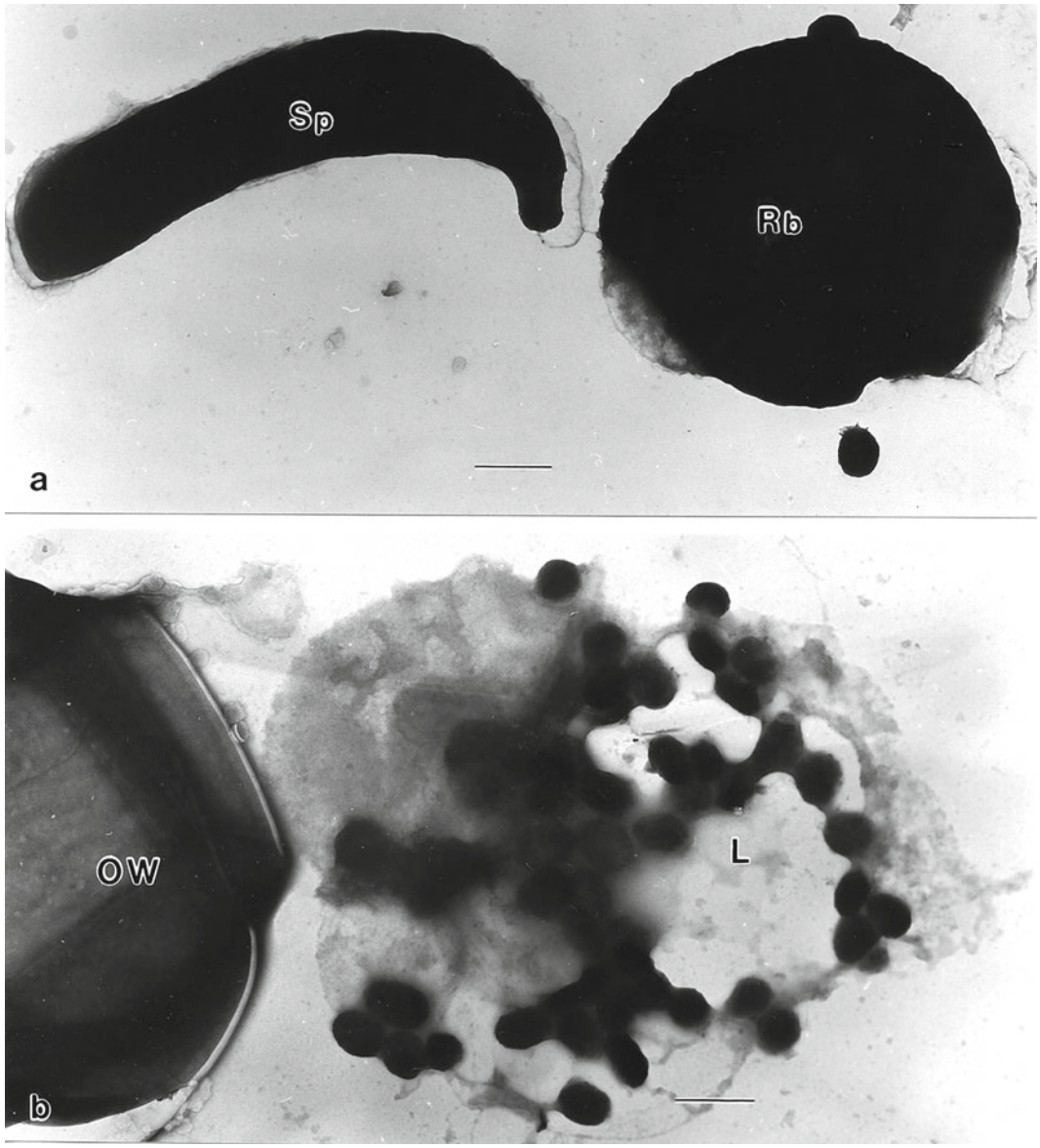

Fig. 6 Negative staining of a disrupted *Cryptosporidium parvum* oocyst preparation, showing released dense sporozoites and residual body (**a**) and oocyst wall and fragmenting residual body (**b**). Electron dense amylopectin granules are present within the residual body and a lipid body (**b**). This figure demonstrates the limit of negative staining for large/thick cellular and subcellular fractions. *SP* sporozoite, *Rb* residual body, *OW* oocyst wall, *L* lipid body. Negatively stained on a continuous carbon support film with ammonium molybdate (pH 6.9), using the single droplet technique [45]. Scale bars = 0.4 μm

Negative staining with the sample and stain spread across the holes of perforated "holey carbon" support films has often the potential to generate superior data compared to specimens adsorbed to continuous carbon support films. The freedom of particle mobility and orientation prior to entrapment in the dried negative

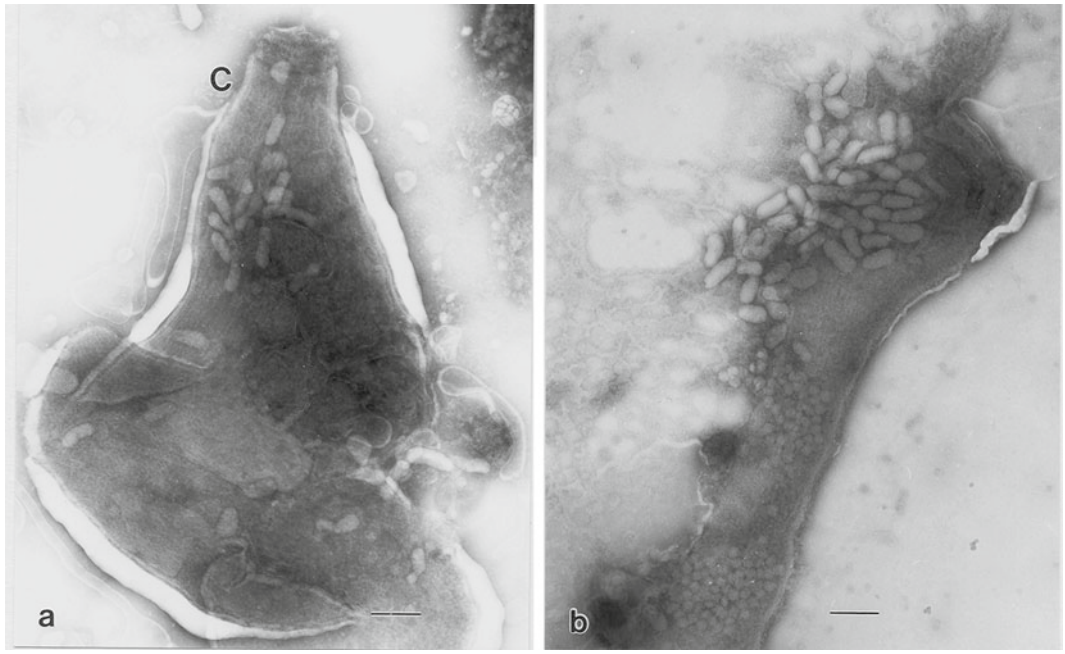

Fig. 7 Lysed *C. parvum* sporozoites showing the conoid (C) with polar rings at the apical end of the organism (**a**). In (**b**) the rod-like, stain excluding, micronemes have escaped from the damaged sporozoite membrane. Negatively stained with ammonium molybdate (pH 6.9) [45, 46]. Scale bars = 200 nm

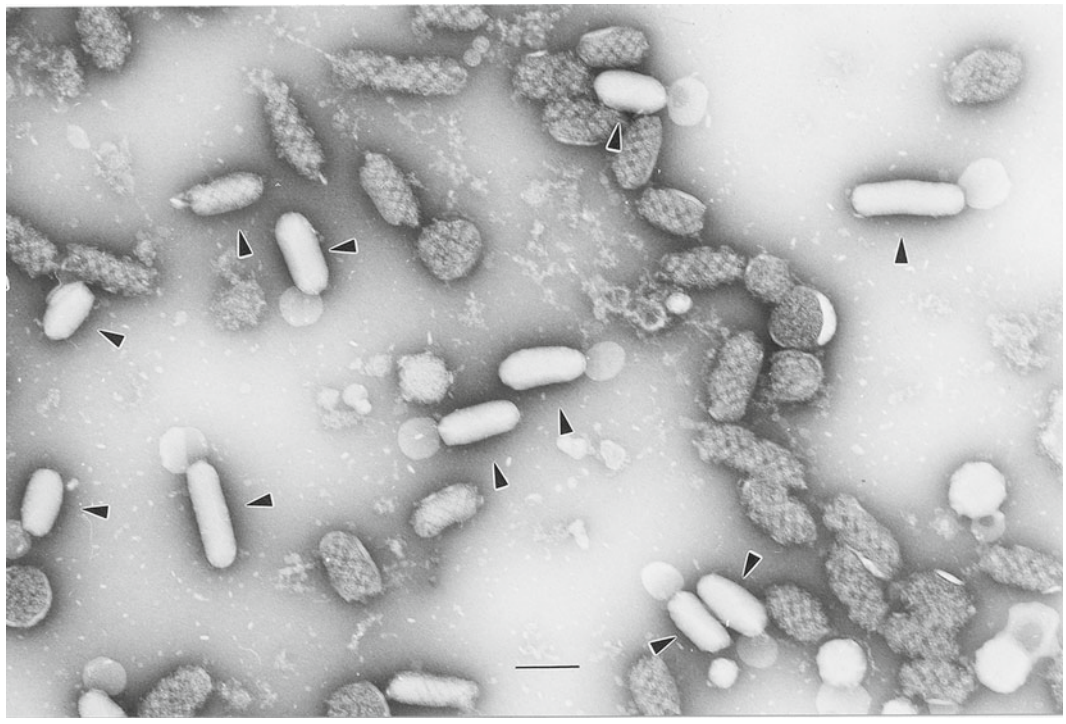

Fig. 8 A purified fraction of *C. parvum* sporozoite micronemes, showing intact (stain excluding) micronemes (*arrow heads*) and damaged micronemes, with internal particulate structure and surface membrane visible). Negatively stained with ammonium molybdate (pH 6.9) [46]. Scale bar = 100 nm

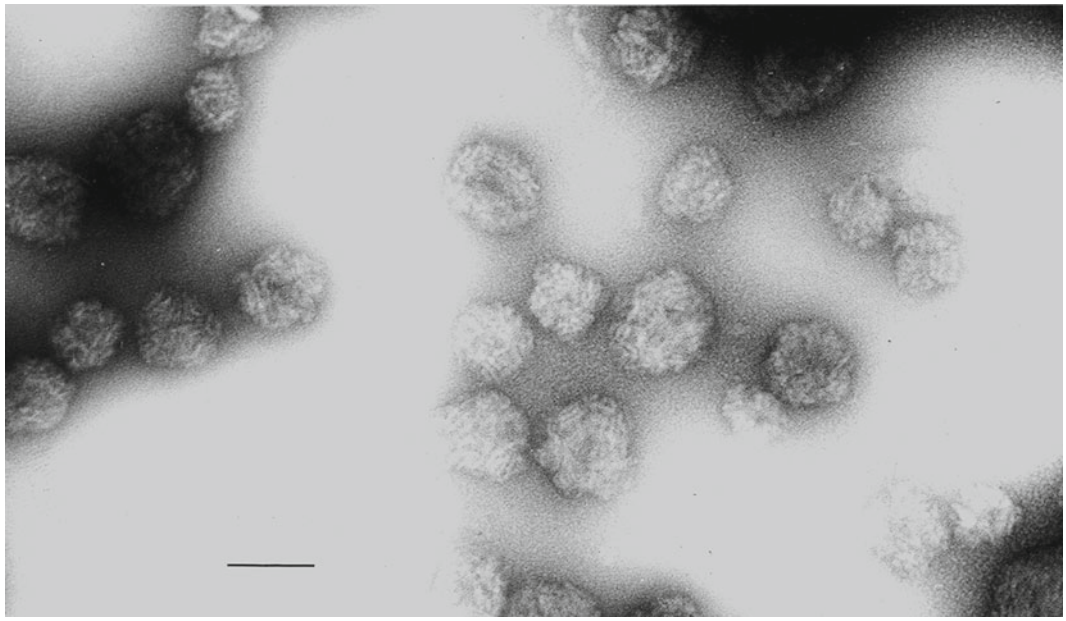

Fig. 9 A purified fraction of *C. parvum* residual body amylopectin granules. Note the interwoven rod-like substructure within the amylopectin granules [47]. Negatively stained with ammonium molybdate (pH 6.9). Scale bar = 200 nm

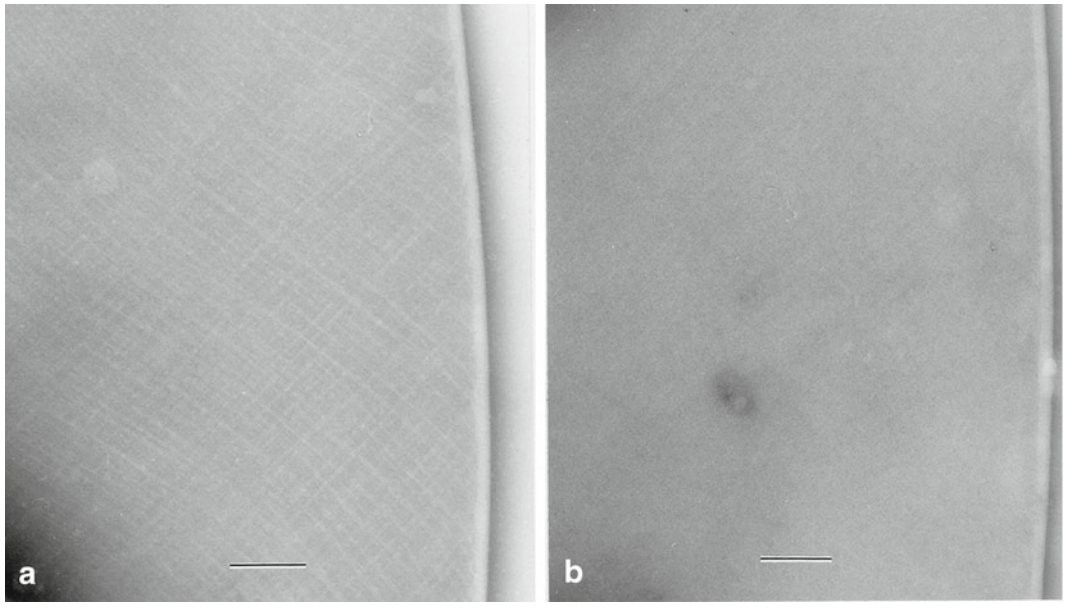

Fig. 10 Isolated oocyst wall from *C. parvum* oocysts following digestion with proteinase K for 2.5 h (**a**) and 16 h (**b**) [48]. Note the reduction in the fibrous array associated with the rigid wall following the longer proteinase digestion, with the . Negatively stained with ammonium molybdate (pH 6.9). Scale bars = 200 nm

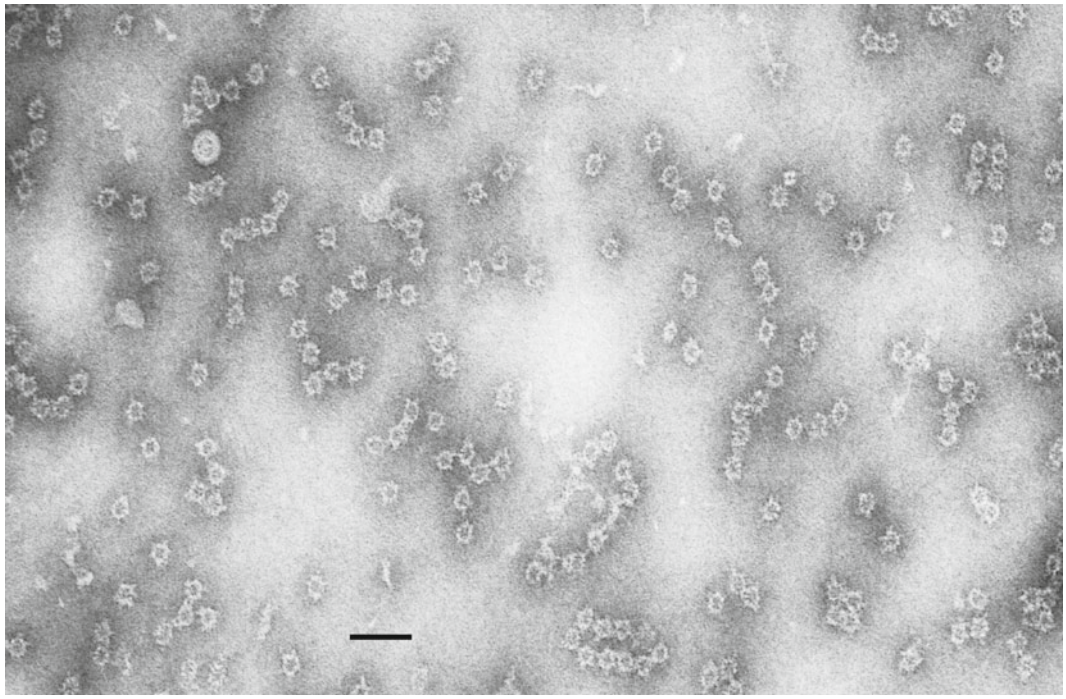

Fig. 11 *Bacillus subtilis* RST stressosome complex, negatively stained with uranyl acetate on a continuous carbon support film. Note the presence of clusters and the surface projections (turrets) extending from the central core (stressosome sample courtesy of Prof. R. J. Lewis, Newcastle University, UK). Scale bar = 100 nm

stain + trehalose, is essentially equivalent to the situation prior to plunge freezing for cryo-negative staining [5, 9] (*see* Fig. 16). Spreading in trehalose alone can also generate satisfactory specimens, where the sample has greater mass thickness than the surrounding thin dried yet vitreous sugar film [27]. Furthermore, inclusion of PEG prior to drying can potentiate the formation of 2D arrays or crystals, and even promote the formation of higher order macromolecular assemblies [44] (*see* Figs. 17 and 18). The same can occur with icosahedral viral particles (*see* Fig. 19).

The mica-spreading negative staining-carbon film technique, has in the presence of ammonium molybdate and PEG the remarkable property of creating 2D crystals from regular viral particles and protein molecules. This directly parallels the influence of PEG creation of such monolayer 2D organization when similar samples are spread across holes for negative staining and cryo-negative staining. In all three instances it is considered that inter-particle association leading to 2D array and crystal formation occurs at the fluid–air interface. Figure 20 shows 2D crystals formed by the *Limulus polyphemus* C-reactive protein and the hollow cylindrical *Thermoplasma acidophilum* 20S proteasome, produced by the NS-CF procedure. Where sufficient order is present, the fast Fourier

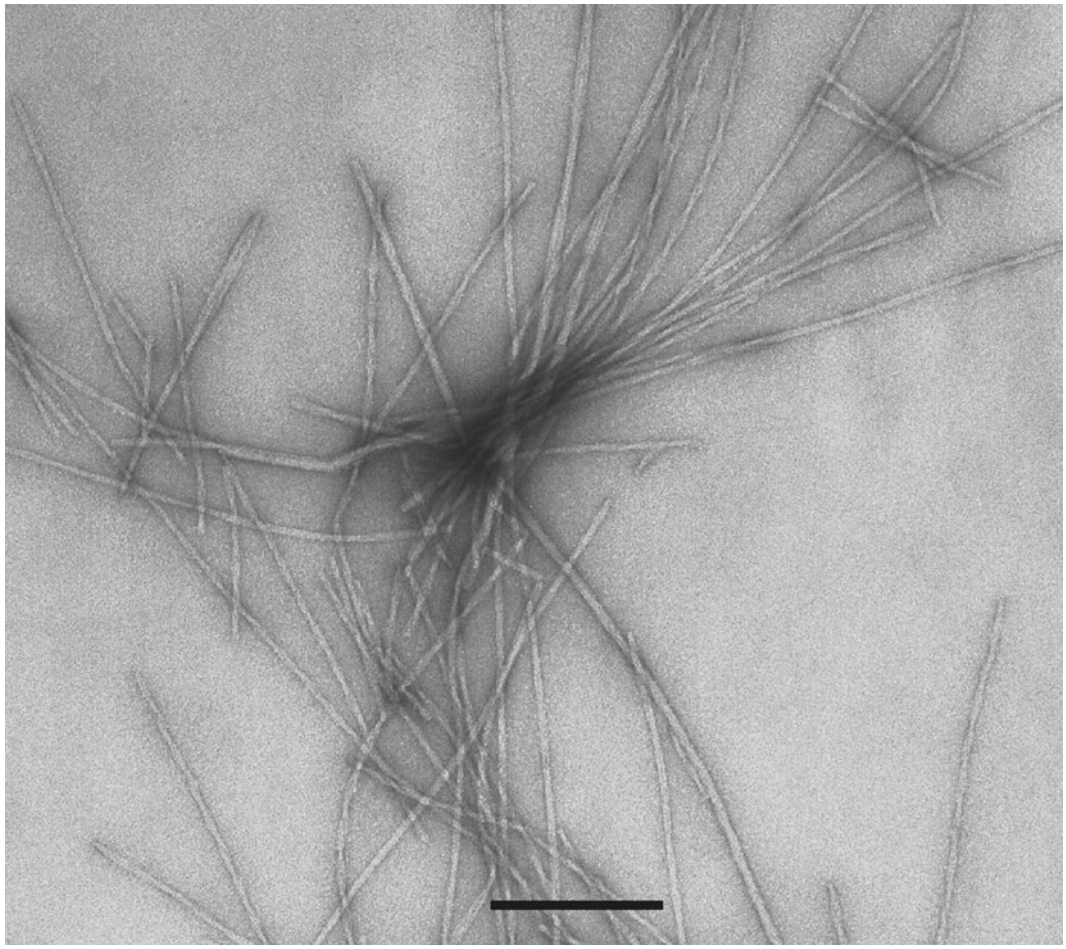

Fig. 12 $A\beta_{1-42}$ fibrils, formed in the presence of cholesterol microcrystals. Note that the fibrils radiate out from a *nucleating center* and that many possess a helical structure. Negatively stained with uranyl acetate. Scale bar = 200 nm

transform (FFT) can lead to a 2D image reconstruction; indeed from tilted images a 3D crystallographic reconstruction is possible.

In recent years cryo-negative staining has found use as a satisfactory intermediate between air-dry negative staining and unstained vitreous specimens. Indeed, single particle reconstruction at a resolution of 1.4 nm has been achieved by Schrive et al. [20] for the *Limulus* SAP-like pentraxin, shown in Fig. 21. Our own cryo-negative stain data has achieved a comparable resolution, as shown for the GroEL complex (*see* Fig. 22). For a cryo-negatively stained tobacco mosaic virus sample a resolution of 1.15 nm was produced (*see* Fig. 23). Inclusion of PEG during cryo-negative staining can lead to the production of 2D crystals, as shown for apoferritin and brome mosaic virus [42] (*see* Fig. 24). This

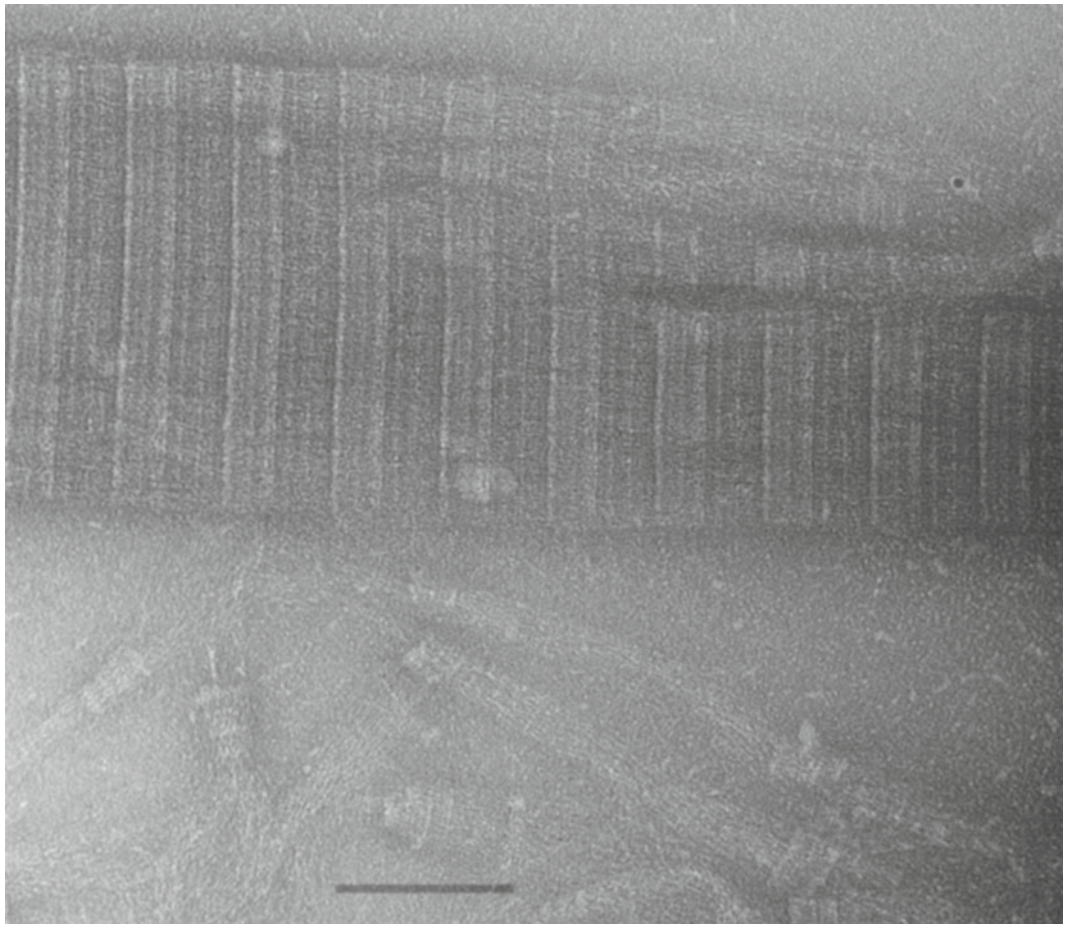

Fig. 13 Fibrils of rat tail collagen type I, reassembled from the acid-soluble heterotrimer at a sub-optimal NaCl concentration. Partly assembled fiber with individual fibrils are present (*upper region*) with several pre-fibrillar assemblies (*lower region*) showing the early establishment of the collagen banding pattern from heterotrimer bundles. Negatively stained with uranyl acetate (JRH, previously unpublished data). Scale bar = 100 nm

crystallization technology has not been extensively exploited, but the data shown indicates the future potential of this approach.

5 Notes

1. Specimen grids prepared from a low concentration of biological material require a longer carbon-adsorption time and those with high concentration of material require shorter time. Overloading of the carbon surface with sample should be avoided.
2. Negatively stained specimens can usually be stored for many weeks. However, for specimens containing a mixture of trehalose and ammonium molybdate negative stain, after a period of

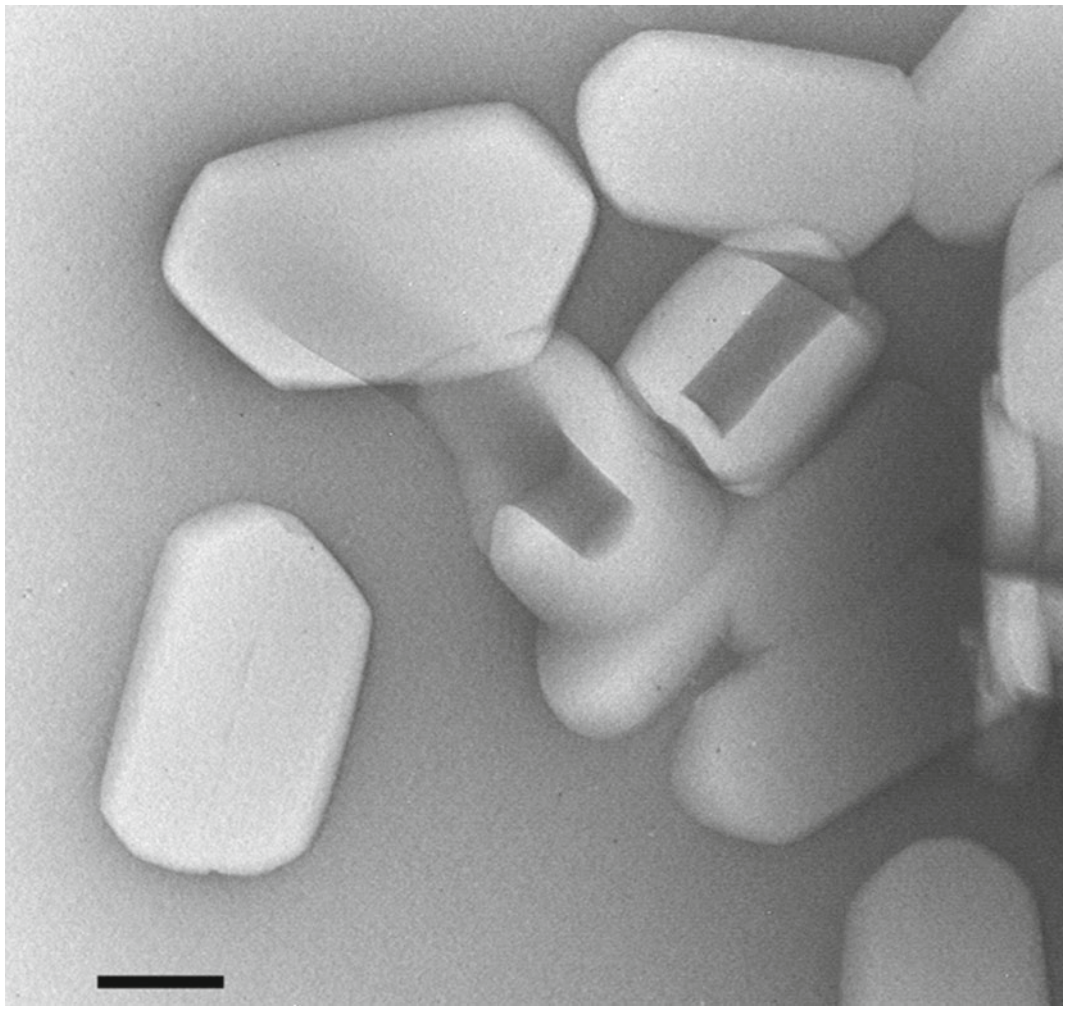

Fig. 14 Cholesterol microcrystals and cochleate cylinders prepared by injection of an ethanol solution into distilled water [43]. Note the hexangular facets of the stacked cholesterol bilayers and the stain penetration with the hollow cochleate cylinders. Negatively stained with uranyl acetate. Scale bar = 100 nm

a few days the initially amorphous/vitreous glass from the air-dried mixture of stain and carbohydrate (which still contains a considerable quantity of bound water), will exhibit signs of undergoing recrystallization of the negative stain. Such specimen grids should therefore be studied in TEM as soon as possible after preparation (i.e., within ~2 days) or stored with desiccation.

3. If air-dried negatively stained specimens are subjected to rapid freezing and cryo-transfer, the remaining specimen-bound water will be retained within the thin film of sample + stain. They must then maintained at low temperature (−180 °C) and

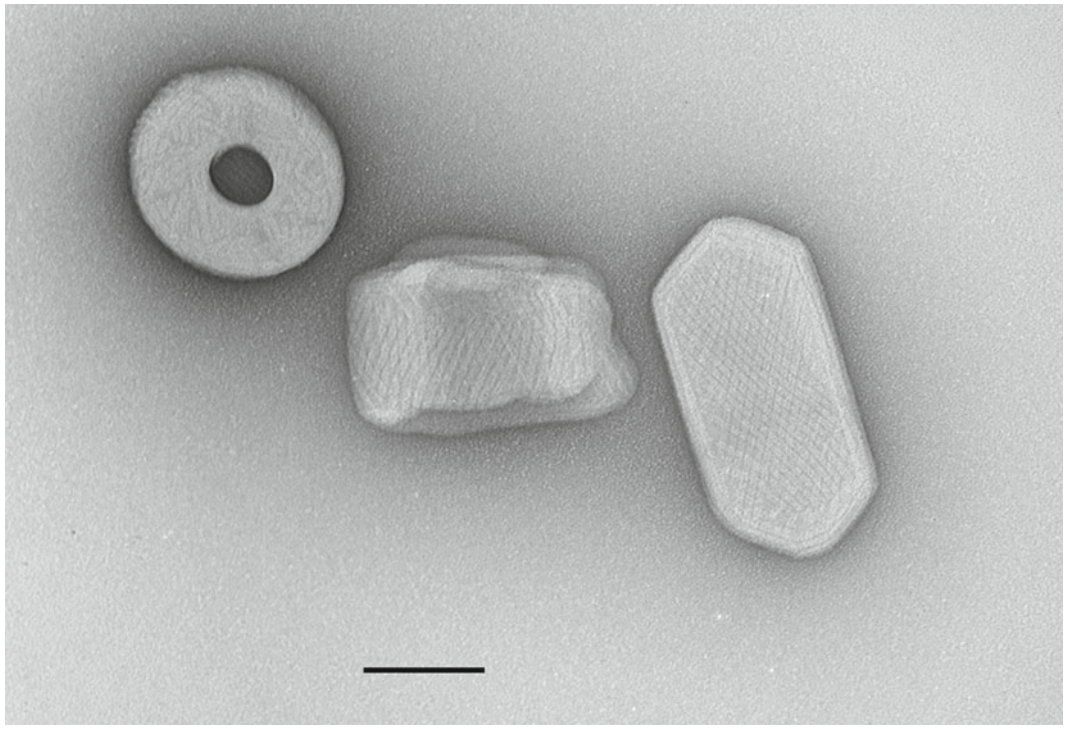

Fig. 15 A cholesterol microcrystal and two cochleate cylinders with surface-bound Pyolysin domain 4 (the cholesterol-binding domain). Note the ordered binding of the protein, determined by the underlying crystallinity of the cholesterol bilayers [43]. Negatively stained with uranyl acetate. Scale bar = 100 nm

require low electron dose study. Specimens can also be cooled following room temperature insertion into the TEM; this will produce maximal dehydration of the specimen prior to cooling to a low temperature. In general, for all specimen conditions, low dose study is desirable to obtain the best quality data, particularly for those specimens containing carbohydrate and is essential for those containing vitreous water.

4. Although the use of ammonium molybdate is described, there is also the possibility of using an alternative negative stain solutions such as sodium phosphotungstate, silicotungstate or methylamine tungstate in combination with trehalose for negative staining across holes. Uranyl acetate/formate solution is generally less suitable in this instance, because of instability in the electron beam and excessive image granularity, but is satisfactory when grids are studied under low electron dose conditions.
5. The PEG concentration and molecular weight of the PEG can be varied within a reasonable range, e.g., the concentration can

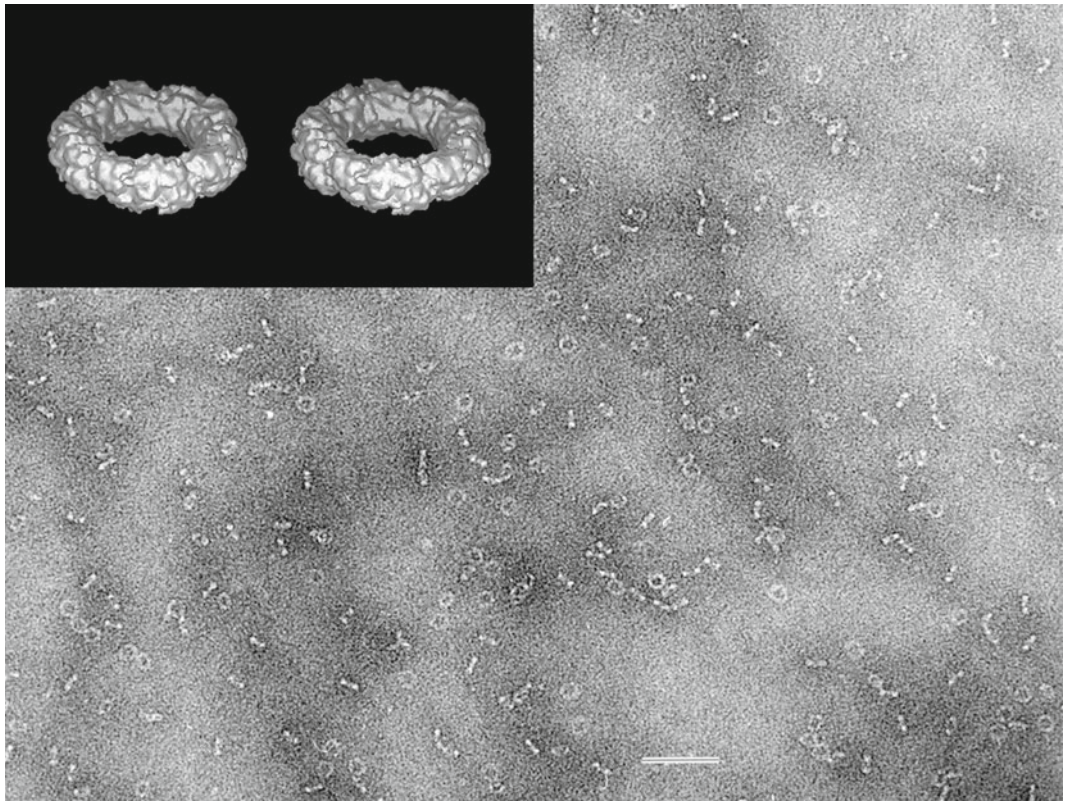

Fig. 16 Human erythrocyte peroxiredoxin-2 imaged by negative staining across a hole in a perforated carbon support film. Note that the freely spread ring-like (decameric) molecules are orientated at varying angles, enabling a 1.9 nm 3D reconstruction to be produced (*inset*), that correlates well with the X-ray structure of this molecule [5]. Negatively stained with 5 % ammonium molybdate (pH 6.9) containing 0.1 % trehalose. Scale bar = 100 nm

range from 0.05 to 0.5 % w/v and the molecular weight of the PEG from Mr 1,000 to Mr 10,000. If protein or viral aggregation is encountered, this will usually indicate that the PEG concentration is excessive or that the pH is too low. At higher PEG concentrations, individual microcrystals of PEG may be produced during air drying.

6. One possible disadvantage of the NS-CF procedure is the fact that the biological sample is air-dried twice and subjected to direct in vacuo carbon-coating, which might then restrict the penetration of negative stain at the zones of carbon–protein

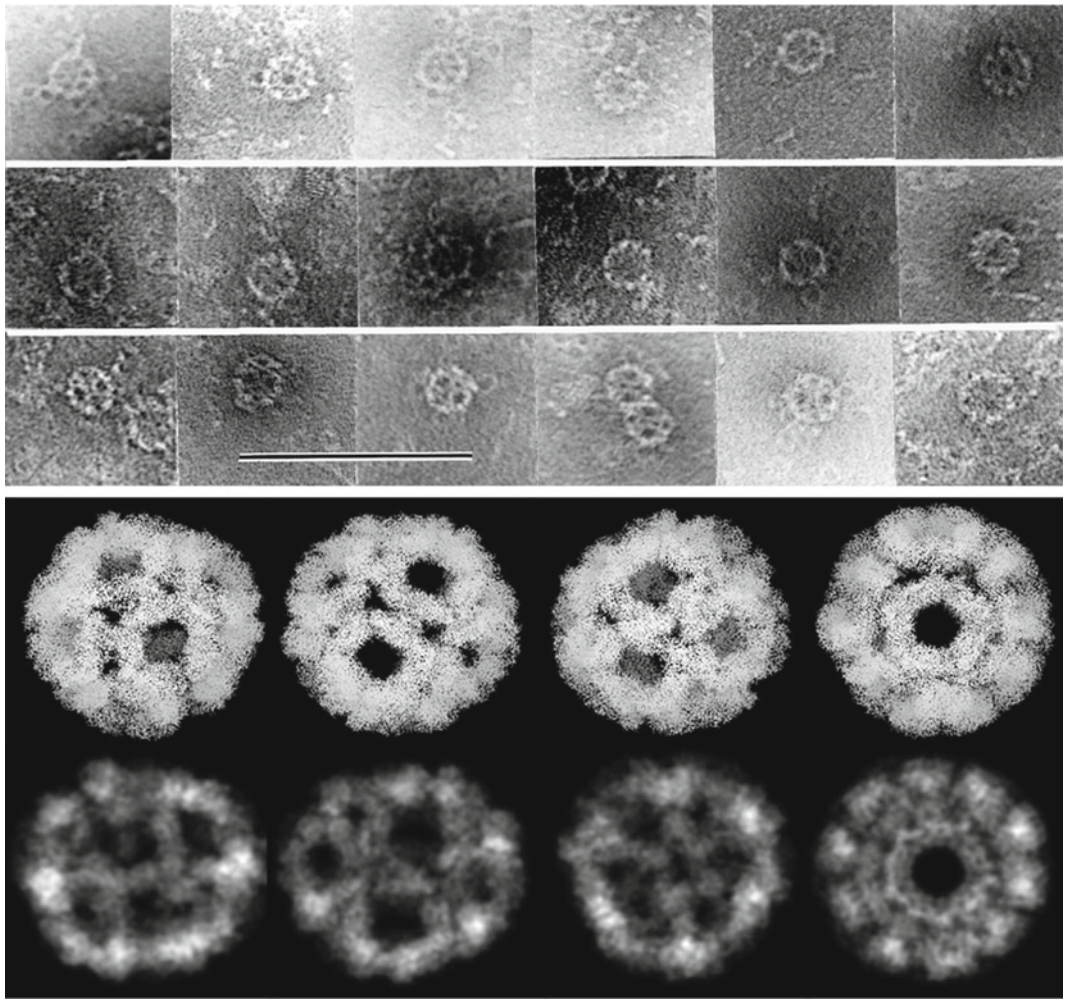

Fig. 17 The dodecahedral higher order assembly produced by human erythrocyte peroxiredoxin-2 following 3 h incubation in the presence of 0.2 % (w/v) PEG followed by negative staining with 5 % (w/v) ammonium molybdate, 0.1 % (w/v) trehalose, 0.1 % (w/v) PEG (pH 6.5) across a holey carbon support films [44]. The *upper region* (**a**) shows a gallery of dodecahedral images with image reconstructions and molecular fitting below (**b**). Scale bar = 100 nm

attachment. In general, this does not appear to be a problem, but some tendency for the final negative stain film to be rather too thin may be encountered, due to the inherent carbon hydrophobicity. The drying time on the mica during 2D crystal formation can be varied by placing in a humid environment or a refrigerator. At the final stage any of the conventional negative stain solutions, with or without the carbohydrate trehalose, can be used. If a very thin carbon film is deposited in vacuo to obtain best possible resolution, this fragile carbon

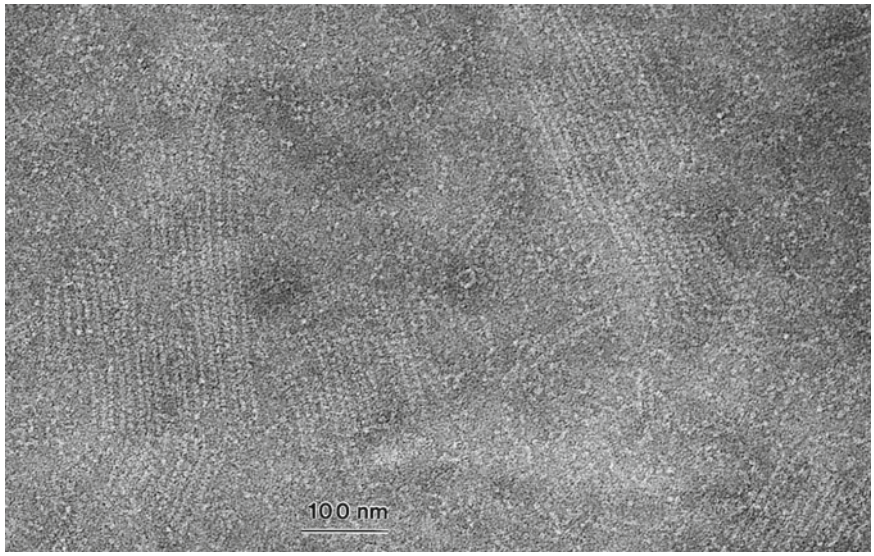

Fig. 18 2D arrays of the ring-like decameric peroxiredoxin from *Thermus aquaticus* (courtesy of Stephen G. Mayhew) spread across a holey carbon support film in the presence of 5 % ammonium molybdate (pH 7.0), 1 % trehalose, and 0.1 % PEG (Mr 1,000). Similar 2D molecular arrays, but adsorbed to a carbon film, can be produced by the negative staining carbon film technique [5]. Scale bar = 100 nm

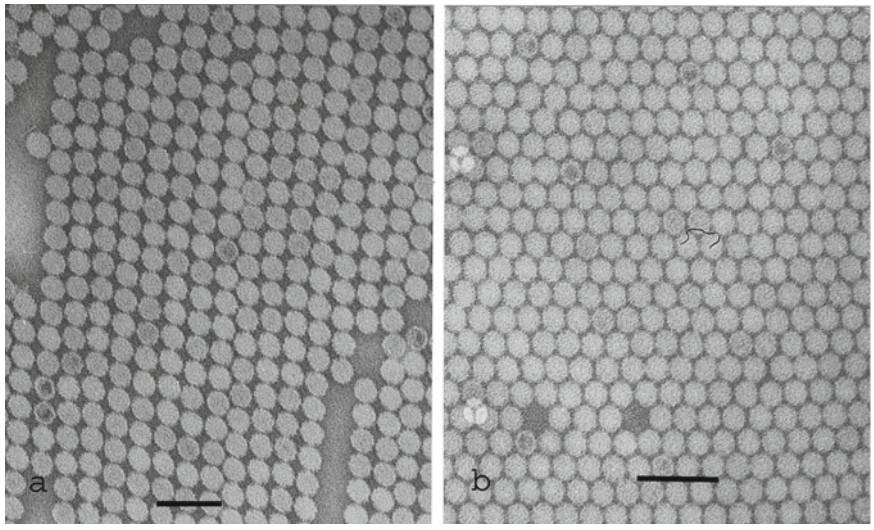

Fig. 19 Tomato bushy stunt virus spread across a holey carbon support film and negatively stained with 5 % ammonium molybdate (pH 6.9) solution containing 1 % trehalose and 0.1 % PEG (Mr 1,000). A partly ordered linear 2D array is shown in (**a**) with a superior hexagonal array in (**b**). Scale bars = 100 nm. (Note that to produce 2D arrays and crystals across holes it is essential that a cryo-negatively stained sample be blotted from one side only, as arrays form at the untouched fluid–air interface, cf Fig. 24) [9, 27]. Scale bars = 100 nm

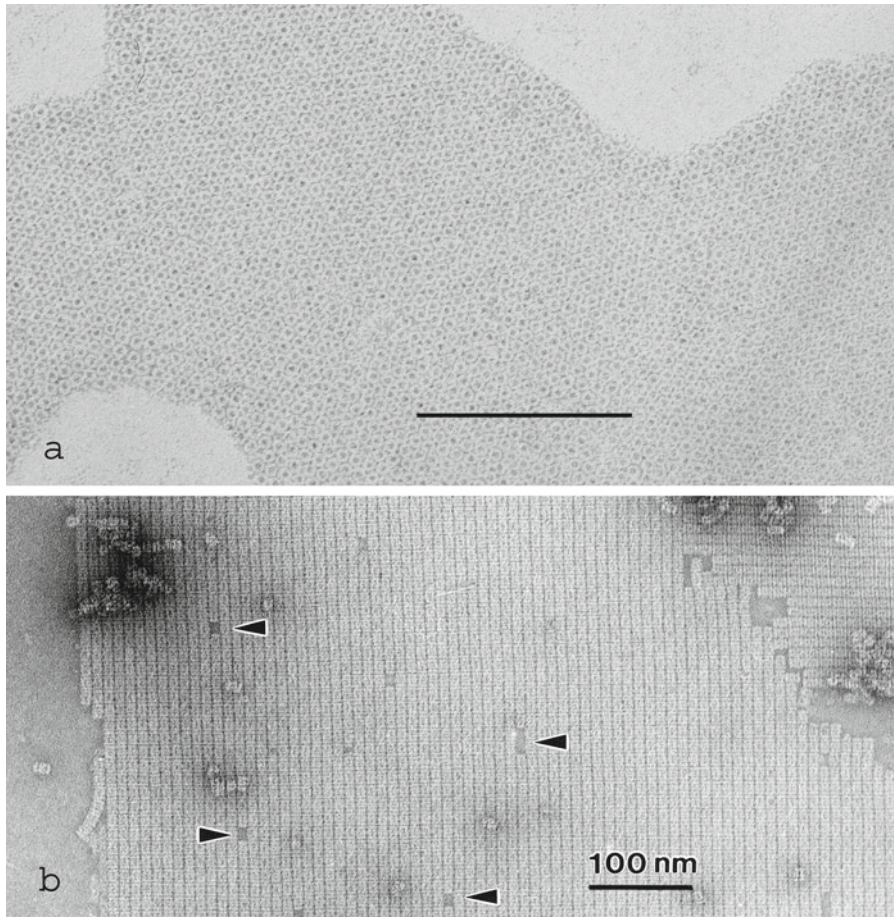

Fig. 20 The *Limulus polyphemus* C-reactive protein (equivalent to serum amyloid P component) (**a**) and the *Thermoplasma acidophilum* 20S proteasome (**b**), both prepared by the negative staining-carbon film procedure in the presence of ammonium molybdate and PEG, with uranyl acetate as the final stain. The C-reactive protein has produced a *quasi* 2D array, with all the molecules orientated in the top view, and the proteasome molecules are all orientated on-side, displaying a superior 2D crystal. Scale bars = 100 nm

layer can be supported on a holey carbon film. It is also possible to float the carbon film + attached sample onto distilled water, with recovery and removal of excess water, followed by rapid plunge-freezing, as for the preparation of unstained or negatively stained vitreous specimens.

7. The necessary dilution of antibody and gold probe solutions and adequacy of blocking can only be determined by the individual experimenter, with the comment that the higher the antibody dilution and more efficient the blocking, the greater the labeling specificity is likely to be and the lower background nonspecific labeling. Generally, prefixation of the biological sample should not be necessary for immunonegative staining. Thus, loss of sample antigenicity should not apply within this

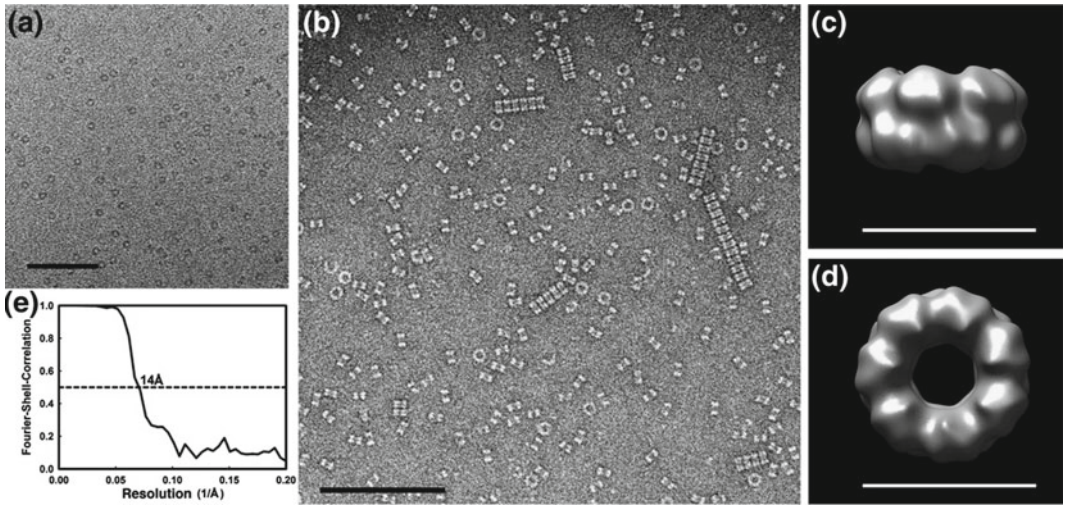

Fig. 21 Transmission electron microscopy imaging of serum amyloid-P (SAP) oligomers. (**a**) Cryo-EM image of a nonstained frozen hydrated preparation. (**b**) Cryo-negative stain EM image. Stacks of ring-like particles were frequently observed under all preparation conditions. Scale bars in (**a**) and (**b**) = 100 nm. (**c**) The side view of the 3D reconstruction from cryo-negative stain EM. (**d**) The *top view* of the model. Scale bars in (**c**) and (**d**) = 11.8 nm. (**e**) The FSC plot of the final reconstruction showing a resolution of 1.4 nm (from Schrive et al. [20], with permission of Elsevier Science)

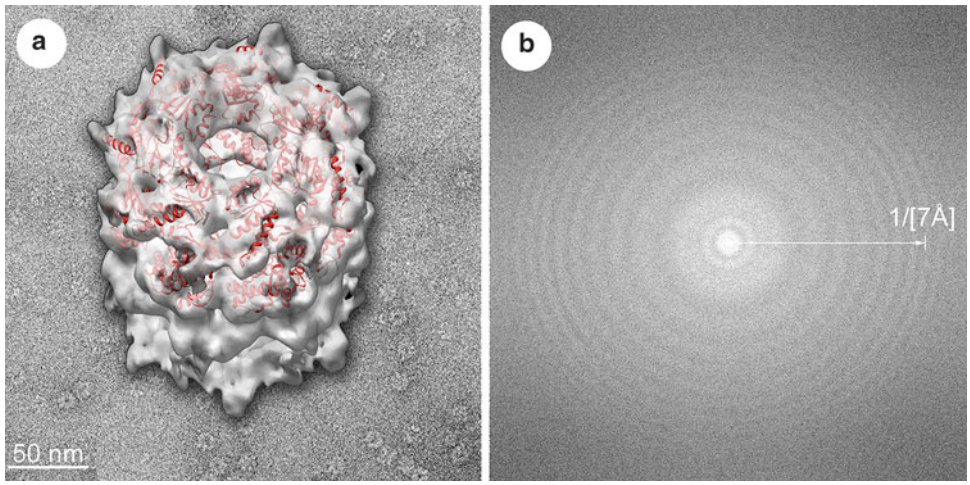

Fig. 22 (**a**) An *Escherichia coli* GroEL 3D molecular reconstruction at 1.0 nm resolution with atomic structure fitted (*red ribbons*). The background is the original cryo-negative staining data (saturated ammonium molybdate) as collected on a FEI CM200-FEG electron microscope (EMBL). (**b**) Power spectrum calculated over the area shown in (**a**). Note that Thon rings are clearly visible to about 0.5 nm in the absence of a carbon film support underneath the hole

system. However, a brief on-grid fixation with a low concentration of glutaraldehyde (e.g., 0.05–0.1 %) can be included, if sample instability is encountered during the blocking, labeling, washing and negative staining sequence, or there is prior

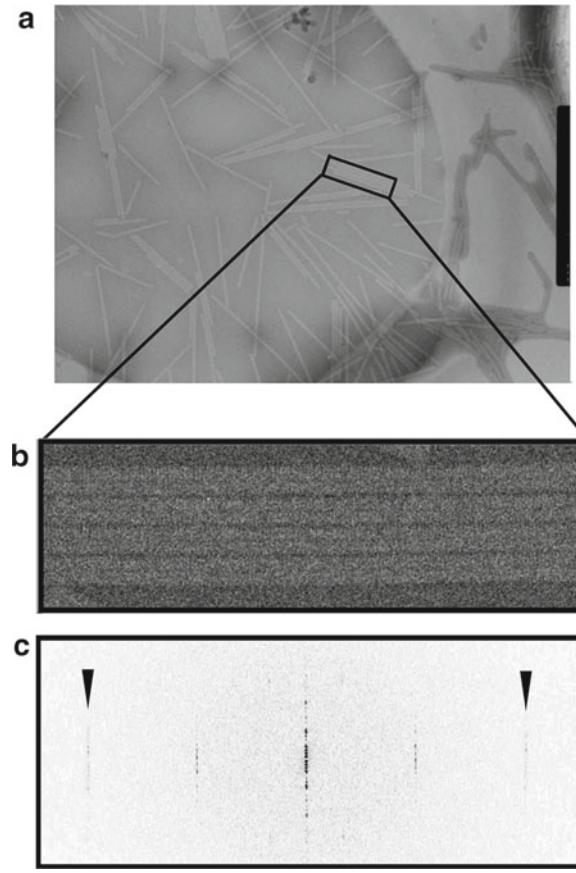

Fig. 23 (**a**) Tobacco mosaic virus (TMV) shown by cryo-negative staining with saturated ammonium molybdate. Scale bar ¼ 0.5 mm. (**b**) *Inset*: selected TMV rods that were used to calculate a power spectrum (shown in reversed contrast in (**c**). The 11.5-A° layer lines are faint but still visible (*arrowheads*). Defocus = 700 nm (*from* De Carlo and Stark [49], with the permission of Elsevier Science)

knowledge regarding sample instability in-solution. Appropriate controls should always be included in all immunological studies.

8. With Protein A-gold, it is be recommended that blocking with rabbit preimmune serum should not be used, instead use 0.1 % w/v bovine serum albumin or casein in PBS.
9. A more detailed presentation of immunogold labeling techniques in relation to negative staining can be found in ref. 40 and useful information on the use of gold cluster probes can be obtained in the promotional literature from Nanoprobes Inc.
10. The ammonium molybdate solution used for cryonegative staining may induce biochemical instability with some samples. pH variation should then be explored, as can prior fixation with a low concentration of glutaraldehyde (e.g., 0.05–0.1 % v/v).

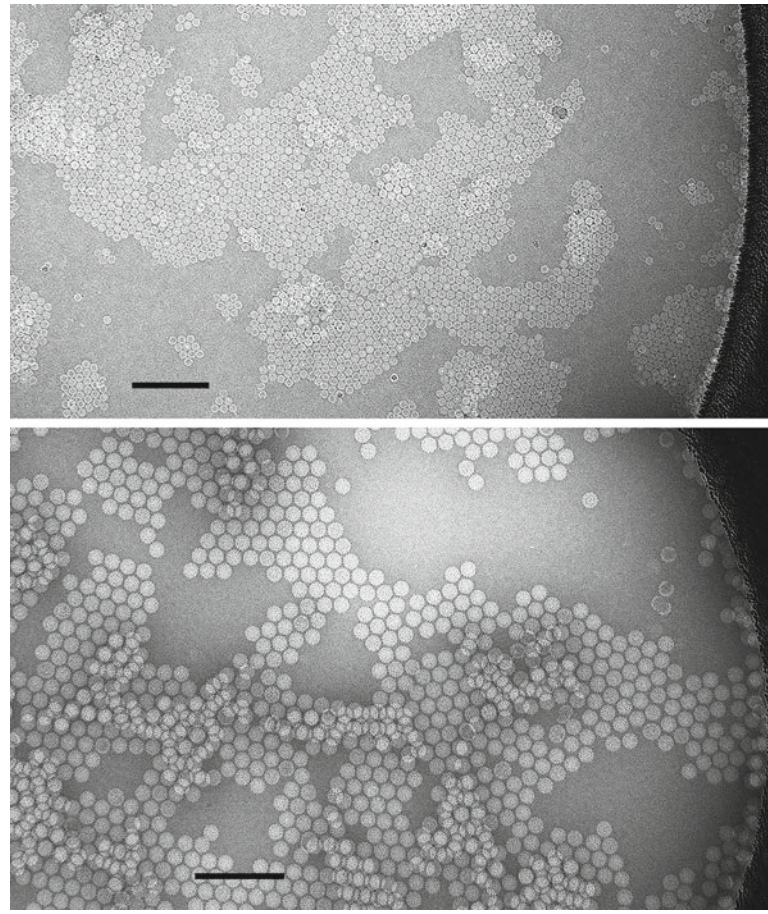

Fig. 24 Horse spleen apoferritin (*top* **a**) and brome mosaic virus (*bottom* **b**) prepared by cryo-negative staining with 16 % ammonium molybdate in the presence of 2 % PEG (images courtesy of Marc Adrian). In both cases formation of 2D crystals is apparent, cf Fig. 19

The higher than usual concentration of ammonium molybdate (e.g., ~0.8M here, compared to 2–5 % w/v for air dry negative staining) is necessary because a very thin aqueous film of sample + stain, across the holes of the grid, is produced by direct blotting. Note the similarity of this cryo-procedure to air drying of negatively stained specimens across holes.

11. For 2D crystallization of icosahedral and filamentous viral particles, macromolecules and enzyme complexes during the cryo-negative staining procedure, the optimal concentration of the start material, the pH of the ammonium molybdate solution, the concentration of PEG and the time period of incubation of the sample with PEG and ammonium molybdate all need to be determined by the experimenter for each sample under investigation [42].

12. We usually follow the cryo-negative staining protocol of Adrian et al. [9], using lab-made holey carbon films on 300 or 200-mesh copper grids (*see* Fig. 5). The best results can be obtained after evaporating a thin layer of Au/Pd on one side of the holey carbon grid to assist sample spreading, a useful innovation introduced by Marc Adrian. Glow discharge may be used to improve the hydrophilicity of an untreated carbon surface. Commercial Quantifoil® or C-flat™ holey grids can also be used. The negative staining solution consists of ammonium molybdate at saturated concentration. Briefly, add 1.2–1.3 g of ammonium molybdate tetrahydrate to 0.875 ml water, neutralized to pH 7.2–7.4 with the addition of 0.125 ml of 10 M NaOH at room temperature. This forms a saturated slurry that can be stored as such for some time. Immediately before the use we shake the slurry quickly again, let it sediment for a few seconds and take the supernatant as the negative staining solution. At this point we have measured a solution density of 1.45 ± 0.05 g/cm^3. Under these conditions, the vitrified specimens are almost fully hydrated (in terms of protein-bound water), as the vitrified medium contains a saturated heavy-metal salt with approximately 30 % water by volume [9]. Nevertheless, the results obtained through the past decade and more recently have clearly demonstrated that although not in their fully hydrated state, samples prepared with the Adrian cryo-negative staining technique are well-preserved in a vitreous/frozen-hydrated state.

    An advantage of using anionic molybdate over the commonly used cationic uranyl salts, is that even at saturated concentrations it can be buffered to any desired physiological pH by the addition of a very small volume of strong base, although this comment could also apply to the phosphotungstate/silicotungstate negative staining salts. Cryo-negative staining can readily be performed in a semi-automated fashion inside a commercial blotting and vitrification device that is fully computer controlled. It should be noted, however, that there can be a difference between specimens prepared by single versus double-sided blotting, particularly if it is desirable to induce 2D crystal-formation at one *untouched* fluid–air interface prior to plunge freezing. Special precautions may need to be taken if the blotting chamber is used at 100 % relative humidity. In our experience it is better to keep the sample environment relatively dry immediately before blotting. If the sample buffer concentrations need to be maintained at defined humidity values the blotting parameters on the automated vitrification device will need to be changed accordingly.

13. The carbon sandwich cryo-negative staining procedure has the advantage that sample material initially in the presence of a

high concentration of glycerol, sucrose or other solute can be processed, with removal of the solute. The original procedure [24] has been linked to a glutaraldehyde-containing glycerol gradient procedure for the purification and stabilization of fragile/unstable proteins prior to EM specimen preparation [28, 29].

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