The Virome Project, A "Manhattan Project" for Bioterrorism. III. Rapid Virus Isolation and Sequencing From Individual Plasma or Serum Samples

Version 1

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Norman G. Anderson
Viral Defense Foundation
And
N. Leigh Anderson
Plasma Proteome Institute

Note: Succeeding versions will cover the same subject material with additions or corrections.

Background

In a previous paper in this series we proposed as a goal the isolation and sequencing of viruses, known or unknown, from single patient-derived samples, within 24 hours. The rapid acceleration of sequencing proposed by Carlson (1) is now occurring and a complete human genome has now been sequenced in one week (2). To achieve the goal of patient-to-sequence in one day, the time required to complete each step between sample acquisition and vaccination must be determined experimentally and drastically reduced. Some of the methods required for doing this are proposed here.

As far as is currently known, all viruses can be stained with nucleic-acid-specific fluorescent dyes, and all viruses, so stained, can be individually visualized and counted. We do not know the purity required for direct counting, and whether all viruses from all sources can be prepared in a state of purity that allows detection and counting over a wide dynamic range. Other unknowns are the minimum number of virions required for counting and sequencing, and what losses that may occur during isolation and transfer.

We conclude, however, that one day sample-to-sequence is technically feasible, but will require extended research that can now be rationally planned.

S-ρ Separations

All classes of particles can be isolated on the basis of their size and density which are reflected in their sedimentation coefficients and isopycnic banding densities. This can now be done using two-dimensional zonal density gradient centrifugation.

The first objective is to isolate, by physical means, particles in the so called "virion window" shown within the circle in Figure 1. And then to isolate, if necessary, the smaller area the S-ρ plot shown in the red square which would include one or a few viral types.

In theory and in practice this plot allows one to organize separations over a wide or, as shown here, narrow *S* range, and then do the same thing in the ρ dimension.

While the basic techniques to be developed should be applicable to a wide range of sample sizes, and include body fluids and tissues, in this paper we are concerned only with plasma and serum. Our major interests are speed and efficiency of recovery.

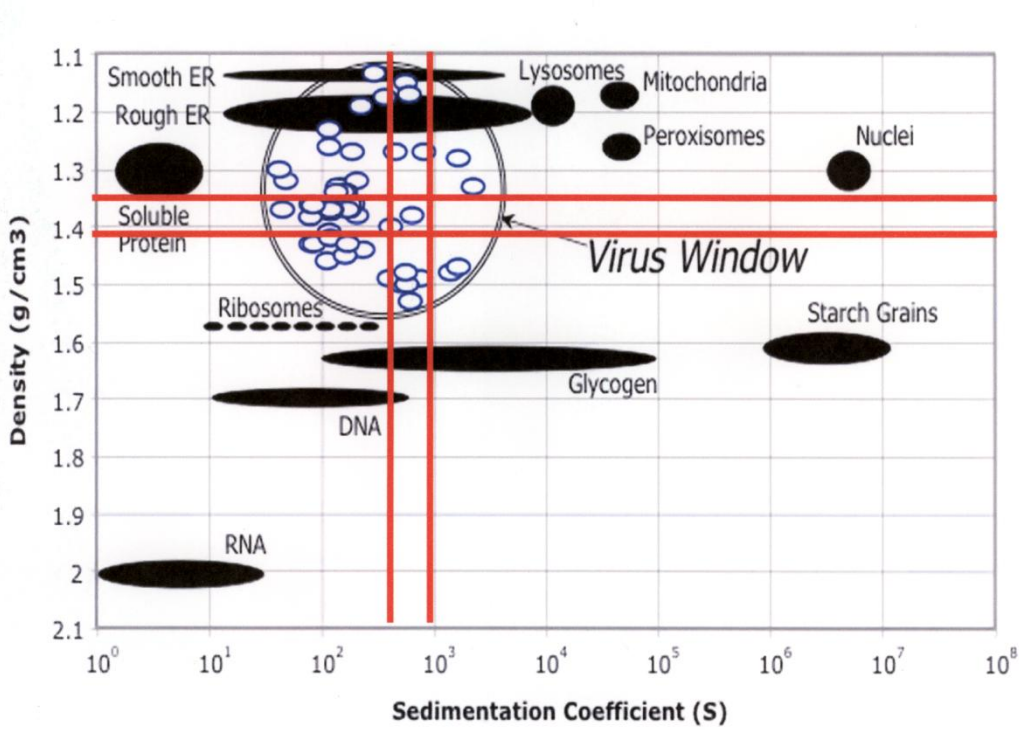

Figure 1. Plot of Sedimentation Coefficients of Viruses and Subcellular Particles Against Banding Density.

The "virion window" is delineated by a circle, while the target for experimental resolution of S-ρ centrifugation is shown in the red square. Adapted from (3). Virus data supplied by Dr. Peter Spragg.

Initially, particles in freshly drawn blood having sedimentation coefficients greater than ~7,000 *S* can be removed by differential centrifugation while particles smaller than ~ 70 S are left suspended. by centrifugation These can then be sedimented or isopycnically banded. This is less sophisticated than using a gradient for the S separation, and may suffice for serum but not for tissue samples. For serum or plasma, this can involve a simple two-step procedure using a differential centrifugation followed by isopycnic banding.

Glass Microsedimentation Tubes

Single virions can be detected and imaged using epifluorescence microscopy. If a centrifuge tube could be made that came to a very sharp internal point, then very few, and possibly even one virion could be detected. We therefore asked whether delicate glass ultramicrobanding tubes that came to such fine point could be made that would withstand very high *g* forces. If the viruses were highly purified, then their packed mass could be determined volumetrically and one would have a "virocrit" analogous to the familiar hematocrit.

To explore the limits of detection of stained virions compacted into a very small volume or a narrow band in a gradient, we require tubes having as large a sample volume as possible, and which then taper down to a tip where virions may be either pelleted or banded in a steep density gradient. Using a vertical glass lathe we heated and pulled out tubes made of glass and of acrylic, as shown in Figure 2.

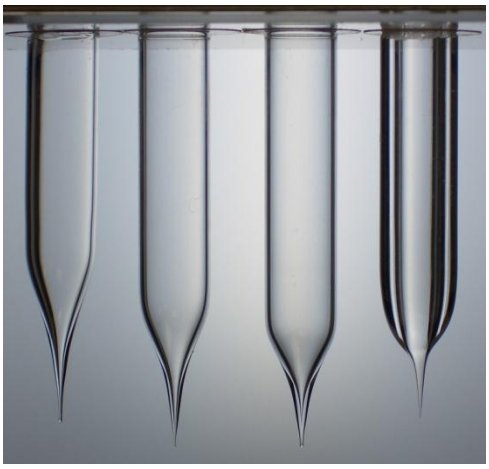

Figure 2. Microbanding Tubes Heat Drawn to Obtain a Sharp Internal point.

The first three tubes are of borosilicate glass, while the fourth, to the right, is of acylic. The wall thickness of the tapering glass can be controlled by the glass temperature when pulling, and by the rate of pulling. Acrylic tubes could be pulled out as shown but the tips were too thin and fragile to be useful and gas bubbles also occur in acrylic tubes in the heated area.

Since it proved difficult to make plastic tubes that come to very sharp points internally (a problem to be further considered), we worked initially with glass, delaying attempts to make such configurations in plastic. It is, at present, infeasible to support glass tubes with a plastic insert. However the glass tubes can be floated on ~80% RT saturated CsCl in Beckman Ultraclear plastic tube for an SW 41 Ti rotor.

The advantages of glass tubes are that they can be drawn out to sharp internal points, and have smooth internal surfaces. The disadvantage, to say the very least, is that glass tends to fracture (powder) at high centrifugal fields. Quite surprisingly, unscratched floating glass tubes will survive hours of centrifugation at 25,000 rpm floating on 80% saturated CsCl at RT as shown in Figure 3. This works because the effective density of the glass plus sample and step gradient is less than the density of the supporting liquid.

When filled with a gradient and sample, the glass tube should float about 3 mm above the floatation liquid.

Figure 4 shows a glass microbanding tube loaded with a density gradient. Since single virions can be detected by epifluorescence microscopy, it is of interest to see how small a viral load can be detected and defined in them.

A slowly pulled out glass tip is shown in Figure 5. We have not been able to reach the magnification necessary to show that it eventually terminates in a curved internal end, hence we do not know whether the internal diameter approaches that of one virion.

We have found that the lower limits of detection are largely set by the mass of extraneous material (dirt) present in sample and/or gradient solutions. Hence if this technology is to come into general use, particle-free reagents will be required.

Preparation of Particle Free Solutions.

Obtaining particle-free solutions has always been a problem in light scattering studies, but is more serious here because we are concentrating contaminants from relatively large volumes into

Figure 3. Glass Heat-Drawn Micro-banding Tubes Floating in 0.8 RT Saturated CsCl in Beckman SW 41 Rotor Tubes. Scale Indicates Distance from the Center of Rotation.

Glass tubes are centered by O rings that float on the CsCl. Tubes shown are empty and will float at a lower level when filled with a density gradient.

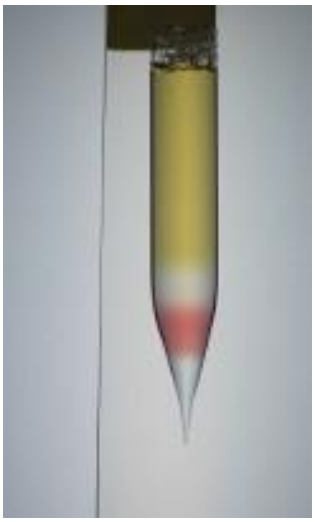

Figure 4. Glass Microbanding Tube With Frozen and Then Thawed Sucrose Gradient.

Sample is in upper yellow zone above three zones for reagents and washing sedimenting virions. Layers are 5, 10 and 15% w/v sucrose.

Figure 5. Borosilicate Glass Microbanding Tube Drawn to a Sharp Internal Point.

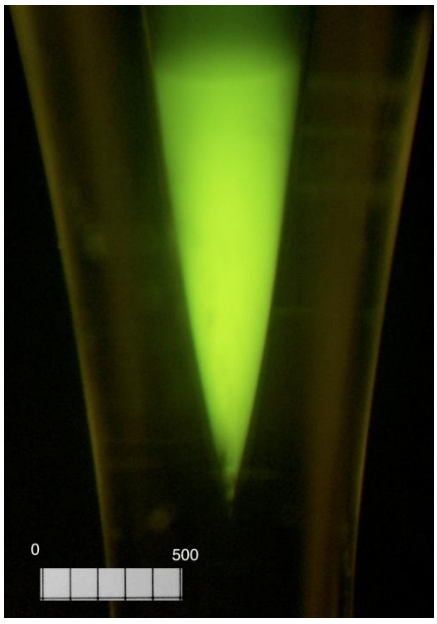

Figure 6. Sybr Gold Stained Lambda Phage Packed into Tip of Glass Microbanding Tube.

Estimated 10^{10} virions in pack. Scale is in microns.

very small ones. Filtration does not ordinarily remove all particles in a sample and may introduce some new ones, while storage and transfer may also introduce contaminants. The best way to remove contaminants is to use the same method that would concentrate them, namely ultracentrifugation, and not to transfer liquids except into microbanding tubes.

The beginnings of such a system are shown in Figure 7 which shows a relatively large volume polyethylene transfer pipette that will fit in a Beckman SW41 Ultraclear centrifuge tube. These pipettes may be easily be completely filled with an aqueous solution, heat sealed at the tip, and then centrifuged upside down, as shown, to sediment particles into the tips.

These pipettes are then removed from the Ultraclear tubes and, while still inverted, the bottom tip is cut off, the contaminants squeezed out, and the tip then resealed. Such a sealed container can be externally sterilized if necessary, and stored, preferably frozen. Polyethylene is less dense than water, and the solution being processed is heavier. By adjusting the density of the flotation medium, the pipettes can be made to just float without crushing the tip.

Different containers with individual solutions for making a step gradient may thus be prepared. These may be used to rapidly and conveniently make gradients as is illustrated in Figure 7.

The system of Figure 7 may be used in a different way. The tubes full of water or a light buffer may be compressed in an upright position to remove bubbles, and then inverted and filled by drawing in increasingly dense gradient steps. The tip may then be heat sealed using a special clamp, and then centrifuged in the position shown. In Figure 7 such a system is shown with one step stained. Single homogeneous solutions may be centrifuged supported in a solution having a slightly greater density to 41,000 rpm. In their present form, these tubes will deform or break at high speeds when they contain density gradients.

This development makes possible the manufacture and distribution of sets of density gradient steps, some of which may contain reagents including enzymes.

These tubes themselves provide a convenient method for doing low speed rate-zonal centrifugation, and may come into general use for that purpose. To recover a rate-zonal gradient, the inverted tip is cut off, and the gradient slowly squeezed out into a fraction collector using a small motor-driven device.

Thus tubes of this design may be used for preparing particle-free reagents, and as a convenient means for doing both rate-zonal separations and isopycnic banding, and in addition, reagents may be lyophilized in them, and reconstituted using particle free water.

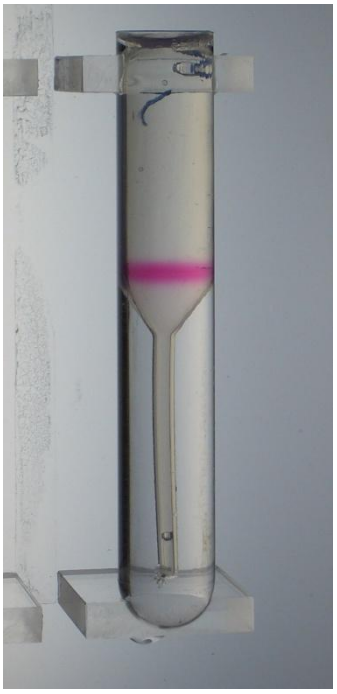

Figure 7. Use of Sealed Transfer Tubes to Remove Contaminants from Reagents.

Microbanding Tubes of Soft Plastic

With the discovery that plastic tubes, supported in liquid slightly denser than the averaged density of the tubes + gradient, could be centrifuged at high speed, we began to explored larger and more complex micro-banding tubes made of soft plastics such as polypropylene, as illustrated diagrammatically in Figures 8.

This is a very versatile design and may be used in several different ways. These include making rate-zonal separations to purify and separate viruses having different sedimentation rates and recovering them in a microgradient at the bottom, banding viruses isopycnically in the bottom microbanding section of the tube, or sedimenting viruses into the conical section in the bottom for observation by fluorescence or by epifluorescence, or sedimenting the viruses against a very small volume or cushion of a fluorocarbon, as described below, that is denser than any volume element or particle in gradient.

In all studies to date, these tubes of this design have withstood 41,000 rpm and 288,000 x g, Aqueous gradients may be prepared by loading gradient steps manually or automatically one at a time, and freezing after each addition. Such tubes may be stored frozen. Note that all solutions should be deaerated before freezing or bubbles will form on thawing.

Polypropylene has a semi-crystalline structure as evidenced by the translucence of the solid structure. Hence crystals appear on the surface during cooling producing a rough surface which may trap sedimenting virions. An ongoing problem is to find a relatively soft plastic that can withstanding high g forces, is completely transparent, yields very smooth surfaces on injection molding, can be readily sectioned, and which does not bend the mold pin defining the capillary section.

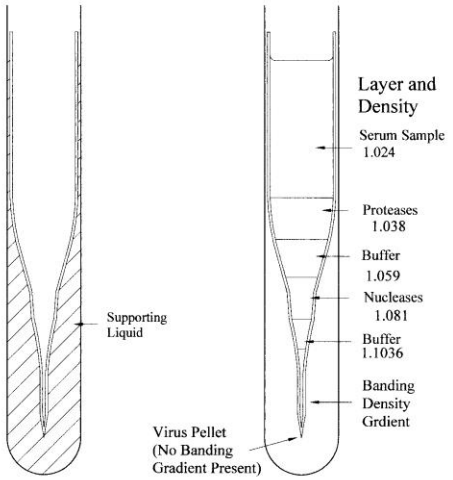

Figure 8. Proposed Design of Intermediate Size Polyproplylene Floating Microbanding Tubes.

Our initial attempts to make microbanding tubes of polypropylene are shown in Figures 9 and 10. These tubes may be unloaded in several different ways. Long thin syringe needles may be used to draw out liquid to the level to be recovered, the entire microbanding region may be cut off and sectioned as described below, or the very tip may be cut off and the gradient extruded using a microsyringe.

Thus injection molded polypropylene tubes may be used for combined gradient preparation (fresh or frozen) for rate-zonal separations with viruses exposed transiently to enzymes and other reagent, washed, and then either sedimented against a cushion, or into the conical section at the bottom. Alternatively isopycnically banded viruses in a very dense gradient in the extreme tip may be recovered. The tip of one of these tubes is shown in Figure 10. The use of wax closures with such tubes is described below.

Figure 9 Injection Molded Polypropylene Microbanding Tubes

Injection molded polypropylene tubes in which a series of different operations may be made. These tubes will float in SW 41 Ti ultraclear centrifuge tubes in water or dilute salt solutions, depending on the gradient used for microbanding.
(Prototypes kindly provided by Thermo Fisher).

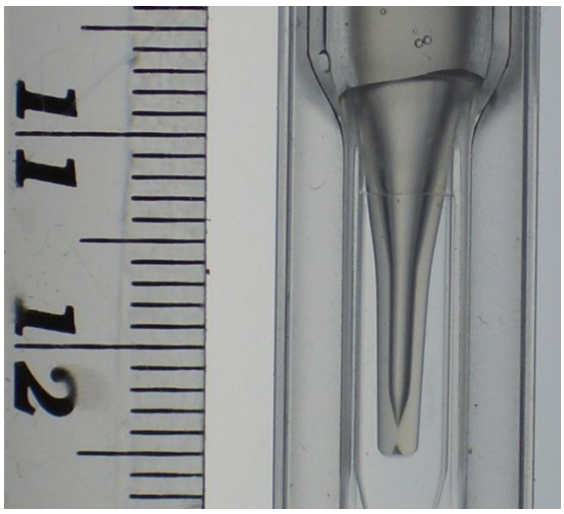

Figure 10. Conical Tip of Polypropylene Microbanding Tube.

Microbanding section is ~5 mm long, and ends in a sharp tip. Tube is shown supported in water in a small cuvette.

The polypropylene micobanding tubes are also designed to fit on an adjustable micro-pipetter as shown in Figure 11.

Unfortunately, when pipetters such as shown in Figure 11 are used with very small volumes, it is discovered that the compressibility of the air used to drive liquid movement results in unpredictable movement of the liquid to be ejected, partially due to surface tension at the tip. If very much smaller designs are to be considered, they must be designed without air between liquid and the piston, i.e., they must be completely full of liquid as described below.

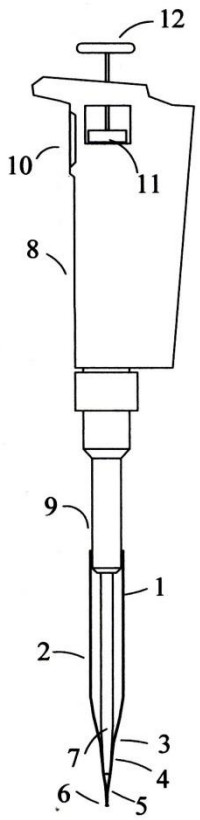

Figure 11. Adaptation of Microbanding Tube to Adjustable Pipetter.

1. Polypropylene tube. 2. Sample zone. 3. Tapered portion of tube. 4. Section that exactly fits adjustable pipetter. 5. Floating pellet on fluorocarbon. 6. Tip that can be cut off. 7. Tip of adjustable pipette with the tip expeller removed. 8. Adjustable pipetter. 9. Section of piper that helps center tube. 10. Dial indicating volume. 11 Adjustment wheel.

UltraMicrobanding Tips for Nanoliter Volumes

While methods have been developed for measuring and transferring very small amounts of liquids using adjustable micropipettes, there has been no comparable advance for handling measuring and transferring particulate or viscous material.

For example, ug to ng quantities of viruses and sub-cellular particles cannot be readily concentrated to pellets, their mass measured, and then transferred to other spaces, including MALDI targets, without appreciable losses. As mass spectrometric methods for protein analysis now extend into the attomole range there is a requirement for sample handling technologies that begin to match the such sensitivity. We describe here an approach to solving this problem.

Very small polypropylene centrifuge tubes may be made by heat sealing the tips of conventional electrophoresis gel-loading pipets (GLPs) as shown in Figure 12. These may be stored lint-free by suspending these from double-sided foam tape as shown.

These tips are 65 mm long, hold as used 300 ul of which ~9 ul are in the 23 mm long capillary section at the end. The density of polypropylene is 0.89 hence the tubes will float on water. If the ends are sealed against molten polypropylene held in a small cup attached to the tip of a small soldering iron, and quickly pulled out to avoid making a small plastic ball at the end, and if they are filled with water or serum to within ~5 mm of the top, they can be centrifuged at 41,000 rpm floating in Beckman SW 41 Ti rotor tubes at 20^0C without deformation.

The useful capacity of the narrow tip section is ~4 ul, presenting an interesting problem if step gradients are to be made and recovered, or if very small pellets are to be considered.

Our first approach was to investigate pelleting against a dense immiscible liquid of high physical density. Saturated fluorocarbons are among the least toxic liquids available, are immiscible with water, and can be dense enough to stop viruses.

The problem is how to introduce a very small volume of a fluorocarbon, followed by an aqueous step gradient, into the bottom of these tubes in a reproducible manner.

Centrifugal force can move very small volumes quantitatively, and forces up to a half million x gravity are available. Unfortunately, when micropellets are to be recovered, they are often diluted by liquid remaining on the wall.

Viruses can be "pelleted" onto fluorocarbon surfaces in capillaries, Hence such a procedure can give the optimal concentration for detection.

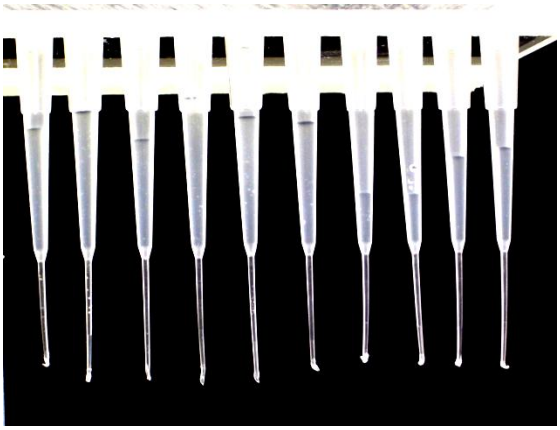

Figure 12. Heat-Sealed Ultra-Micro Banding Tubes Made From Gel- Loading Pipets (GLPs).

The interface between fluorocarbon and the aqueous overlay is barely visible.

Inert fluorocarbons do not affect poly-propylene, will volatize in a mass spectrometer, are non-toxic, and a few microliters at the tip of the GLP can stop the densest known biological particles. Fluorinert PF 5050 has a density of 1.63 and boils between 26-36^0C. Microliter amounts of such a volatile solvent cannot be readily pipetted into a GLP, however if a few drops of cold PF 5050 are introduced into the top of a GLP, and are shaken down to the tip, then most of the excess may be easily shaken out.

If the tip is then placed briefly in a small beaker of water at 35^0C, the fluorocarbon evaporates from the top, and, when only a few millimeter of fluorocarbon remain, the tip is cooled in ice water, and a few drops of the aqueous solution used to fill the capillary are introduced. The air bubble between the fluorocarbon and the aqueous overlay can then be floated up by brief centrifugation.

Table 1 Shows the relevant properties of several different fluorocarbons. PF 5050 was chosen initially because of its low boiling point. If denser particles are to be stopped then a denser fluorocarbon can be used. Additionally, the four fluorocarbons listed may be arranged to form a step gradient to band particles denser than 1.62 g/ml.

Table 2. Relevant Properties of Fluorocarbon.

Trade Name	Chemical	B.P. ^{0}C	Density
Fluorinert PF-5050	Predominantly perfluoro(dibutylmethyl) amine	26-36	1.62
Fluorinert FC-77	Pefluoro compounds, primarily with 8 carbons	97	1.78
Fluorinert FC-40		97	1.85
Fluorinert FC-70	Perfluorotripentylamine	215	1.93
FC-5080			1.76

After the fluorocarbon is in place, has been introduced and either shaken down or lighly centrifuge, a very small amount of the dense end of an aqueous gradient may be introduced from the top and centrifuges down in a clinical centrifuge using the adapters and holders shown in Figure 13. The black holders serve to sets of 12 tubes, and the round ones, each holding six ultramicrobanding fit in a 50 ml plastic tube to centrifuge down each addition, and to removing air bubbles. The sample volume is ~200 ul.

Note that one set of ultramicrotubes has red tops, which are of a special soft red wax that seals the top during high speed centrifugation, and still allows small air bubbles to escape. As later shown, these wax plug are essential to precision recovery of samples and gradients.

Tubes are either photographed in a fixture which also shows the radius in the rotor at different levels in the tube, or under an epifluorescence microscope at 4X objective magnification.

The accessories shown in Figure 13 are essential to the system.

Figure 14 shows two modified GLPs (ultramicrobanding tubes) floating in water in SW 41 Ti Ultracear centrifuge tubes. Approximately 10^9 lambda phage virions were added to each tube and centrifuged for 4 hours at 41,000 rpm at 25^0C against the fluorocarbon PF 5050.

A section of a tube containing the virus pellet barely visible in Figure 28 was examined in the epifluorescent microscope as shown in Figure 15.

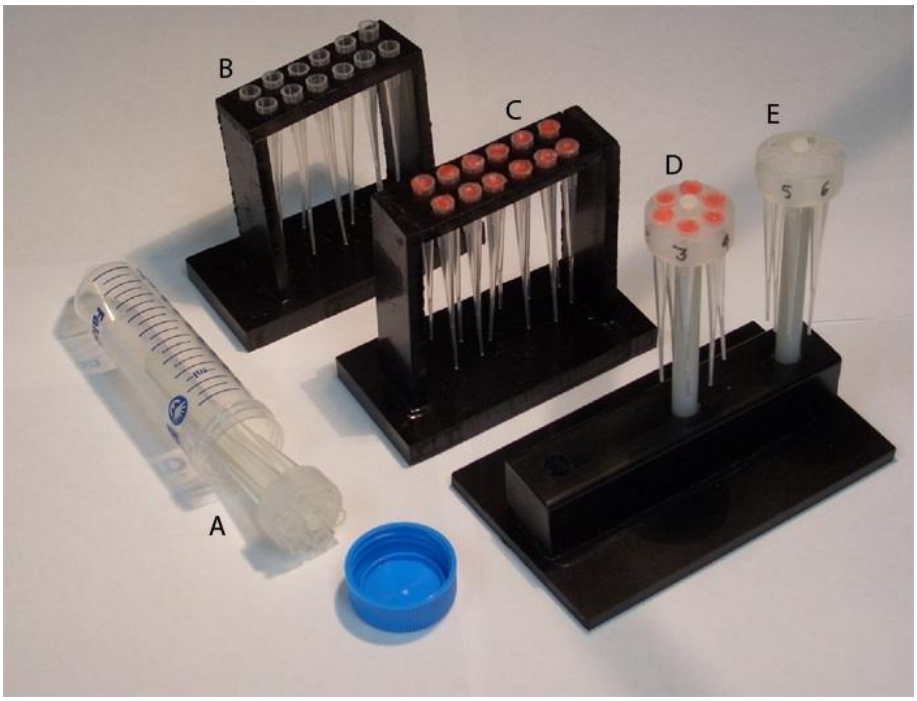

Figure 13. Accessories for Loading and Sealing Ultramicrobanding Tubes Made from Gel Loading Pipettes.

A. *Adapter for centrifuging ultramicrobanding tubes at low speed to remove air bubbles between added zones.* B. *Rack with sealed ends.* C. *Rack with tubes sealed with wax.* D. *Wax sealed tubes on adapter.* E. *Unsealed tube on adapter.*

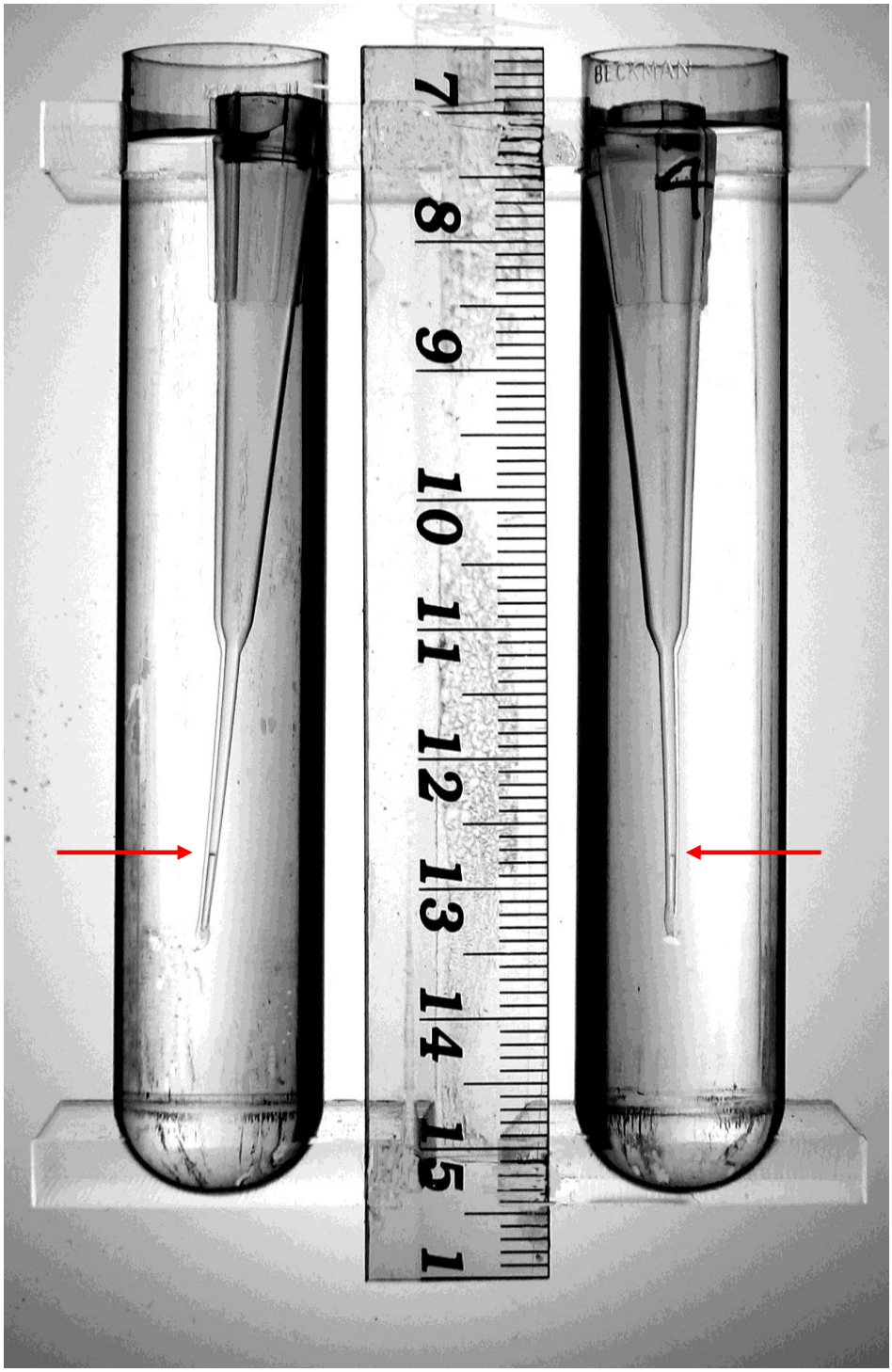

Figure 14. Lambda Phage has been Sedimented on to PF 5050 Fluorocarbon.

Ultramicrobanding tubes floating in water. Phage are at the level of 12.8 cm as indicated by arrows, and are barely visible in each tube. Scale is radius from the axis or rotation. Tubes were floating in water in a Beckman SW 41 Ti rotor during centrifugation.

The virus sedimented against fluorocarbon in Figure 14 is shown at much higher magnification using a modified epifluorescence microscope in Figure 15. A 90^0 prism was inserted in the optical path to allow vertical microtubes to be observed and photographed.

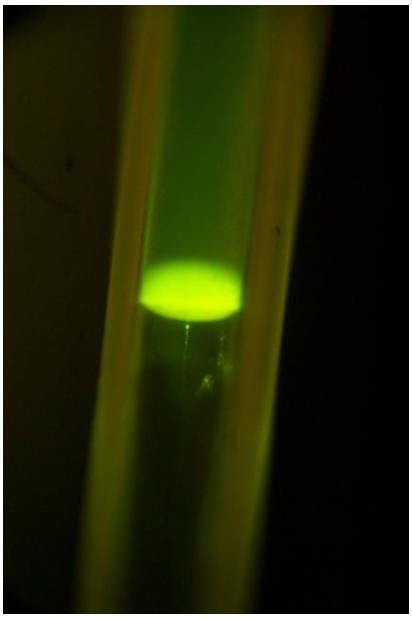

Figure 15. Epifluorescence Microscope Image of Virus Band in Figure 14.

Lambda phage was stained with Sybr gold, and is sedimented against fluorocarbon PF 5050.

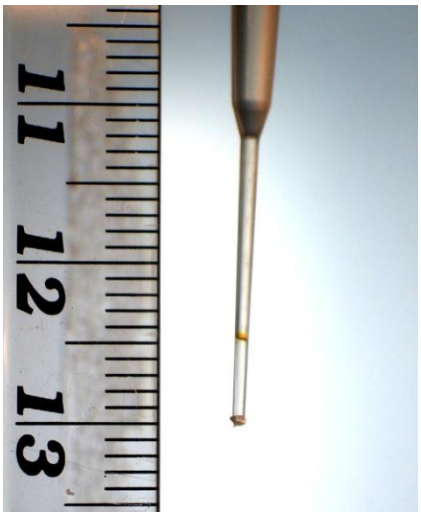

Figure 16. Ferritin Sedimented Against Fluorocarbon PF 5050

Centrifuged for 4 hours at 41,000 rpm, 288,000 x g_{max} in SW 41 Ti rotor.

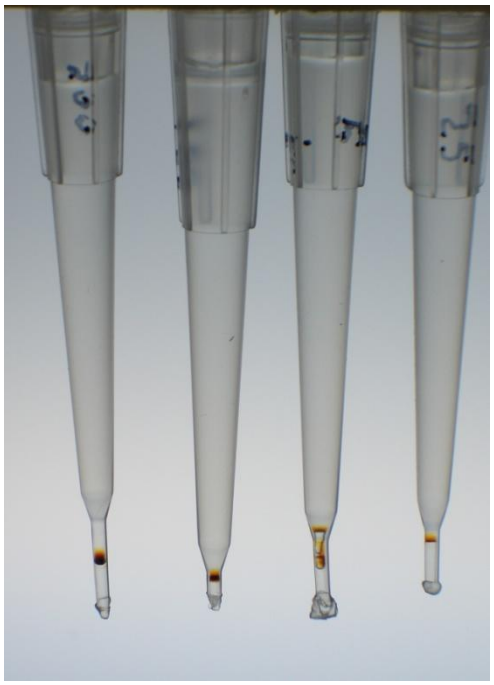

Figure 17. Ferritin Sedimented Against PF 5050

Centrifuged four hours at 55,000 rpm, and 368,000 x g_{max} in SW 55 Ti rotor. Ferritin load was 200, 100, 50, and 25 mg from the left to right.

It is of interest to know whether this concentration method would work with proteins to provide a small concentrated sample for MALDI or electrospray analysis. To examine this possibility, ferritin was sedimented against PF 5050 as shown in Figure 16 to give a tight pellet. To see whether this would work at the highest g forces available, we spun ferritin at 55,000x g as shown in Figure 17. The short length of the rotor buckets necessitated using shorter ultramicrobanding tubes.

With wax sealed tubes, the narrow capillary section at the tip end may be cut off without any of the contents flowing out, and the narrow bore capillary section recovered and itself sectioned from its top using the "Virotome" shown in Figure 18.

In this instrument a glass plate is attached to two vertical steel posts, and the unsectioned tip end is attached with adhesive tape at its bottom. A single edge razor blade is mounted on an optical X-Y-Z movement to push the razor blade slowly but firmly against the tip as shown in Figure 18. One can follow this by looking though the glass plate. After each section, the blade is moved back, leaving the small section

on the blade held by a very thin film of silicone grease. The blade is then moved down ~1 mm, and then moved forward to make another cut. Cutting is done against a transparent adhesive tape attached to the glass to counteract the effect of small differences in the flatness of the glass and the straightness of the edge.

Additional views of the sectioned tips are shown in Figure 19-21.

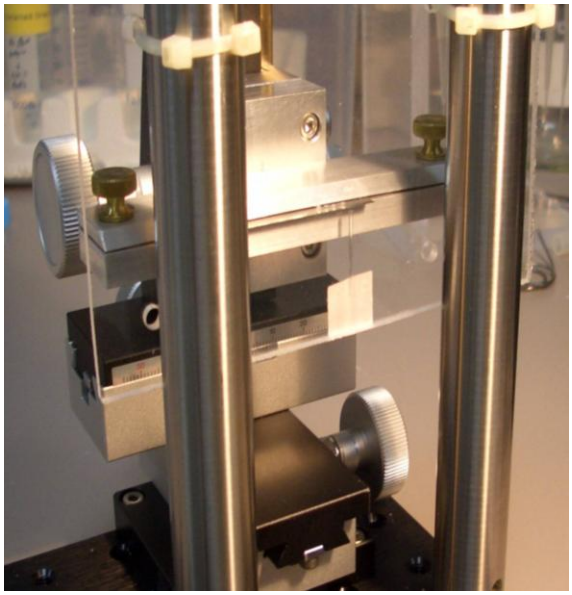

Figure 18. Microtome (Viratome) for Sectioning Microbanding Tubes

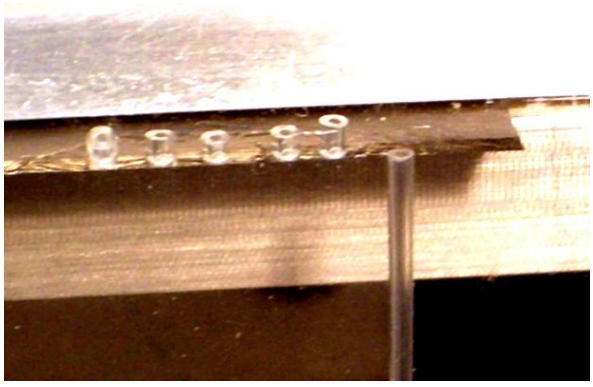

Figure 19. Sections of Ultramicrobanding Tubes Attached to a Single Edge Razor Blade.

Tube being sectioned shown at right. Sections are held on the blade by a thin layer of silicone grease.

Figure 20. Ultramicrobanding Sections Still Attaches to Blade

The Z movement was controlled by hand, accounting for the differences in length of the tube sections recovered.

Figure 21. Indication of the Scale of the Collected Ultramicrobanding Sections.

One millimeter sections of these tips hold from ~300 to 900 nanoliters depending on where in the tapered portion they are taken.

It was observed that the liquid in 1 mm sections evaporated very rapidly, and it was further discovered that the rate of drop of a meniscus is indeed much faster than if the same liquid is in a larger tube. A very small amount of oil may be sprayed on the top of these sections to prevent evaporation, should that turn out to be a problem.

Micro Gradient Recovery by Extrusion

Since the pellets obtained using a fluorocarbon cushion are very small indeed, some way of extruding them was sought. This will be useful for MALDI anaysis and for dotting on nitrocellulose or other filters, for extrusion into ultramicrotiter plates, and for ultra-microsequencing studies. It is desirable to be able to: (A). Determine + or – whether a viral load is present above a certain experimental range. (B). Determine the packed volume of the virus. C). Extrude the virus onto a slide for counting, (D). Recover enough virus for sequencing. (D). Simultaneously identify the virus, if it is a known one, by immunological means or PCR-based methods. (E). Store the viruses on the slide for future use.

For this it is essential to have fine control over the virus recovery process. This requires incorporation of means for eliminate air in the system, for have the system completely filled with liquid, and to have included cheap disposable, and very precise ultramicrosyringes included in each and every ultramicrobanding tube.

A simple solution to this very difficult problem was to seal the loaded tubes with the wax previously mentioned which will flow in high centrifugal fields to form a tight closure at the top of the ultramicrobanding tubes as shown in Figure 22. During centrifugation any air present is forced out and the closure becomes flat. The end of the tip can then be cut off without any fluid coming out because the wax is stiff enough to allow a needle to be pushed through it without being itself deformed. The procedure is described in the legend of Figure 13, and involves loading a step gradient sequentially to the top of the tube, and then centrifuging each step down a low speed using the adapters shown in Figure 13.

A device for moving the needle smoothly and accurately is shown in Figure 23. This may be used to produce ul to <100 nl droplets that may be collected as shown on a Teflon coated slide in Figures 23 and 25.

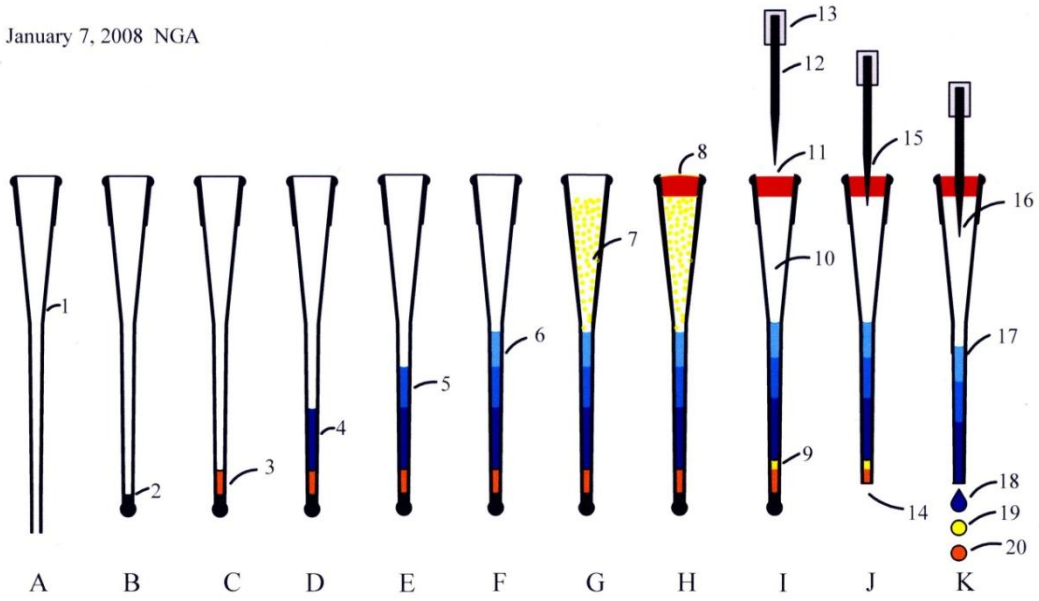

Figure 22. Procedure for Loading, Centrifuging and Recovering Virus Bands from Microbanding Tubes.

A1. Original gel loading pipet. B2. Tip heat sealed. C3.Fluorocarbon is introduced to the bottom of the tip, and centrifuged down into position, or allowed to evaporate to that volume. D4. Densest step in step gradient introduced and centrifuged down at low speed. E5 and E6. Next steps in gradient introduced and spun down. G7. Sample, ~200 ul, introduced and spun to remove air bubbles if necessary. H8. Soft seal was put in place. I. Microbanding tube ultracentrifuged and virus. I9 clearing virus from sample volume 10, a flat wax plug 11 with no air bubbles under it, and width needle 12 attached to movement 13 poised to be inserted into wax. K15. After cutting off tip 14, needle is slowly inserted into wax cap, beginning to extrude the fluorocarbon. Step gradient 17 is gradually moved down, extruding droplets 18-20, which may be recovered in order on a glass slide, or a Teflon surface. If the virions have been stained, these droplets may be examined for them, and/or recovered for sequencing.

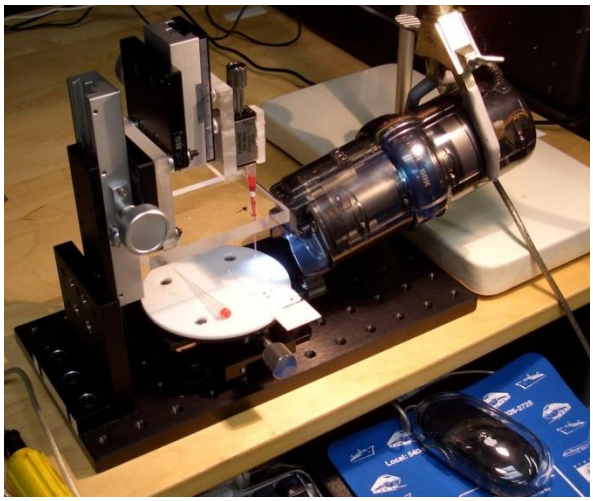

Figure 23. Spot Fraction Collection Using Teflonized Slides

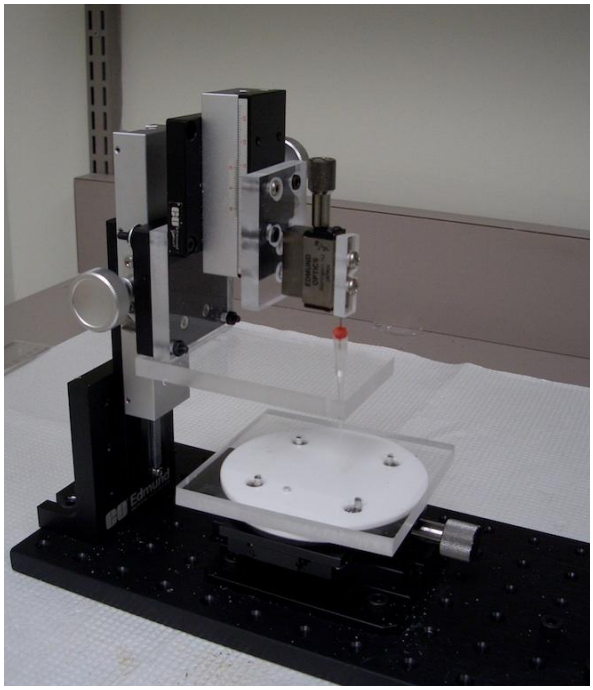

Figure 24. Apparatus for Recovering Microgradient by Needle Extrusion.

Waxed-sealed microtube shown on left, Teflon coated microplate shown in center, and tip of GLP shown in upper right.

One of the problems with use of gradients is that the material recovered contains a large amount gradient solutes such as sucrose, CsCl, or iodixanol. It is essential to remove these before analysis by mass spectroscopy. The fractions are too small for gel filtration.

A fraction, recovered in the ul to nl range can be diluted to ~200 ul, layered over D_2O,

Figure 25. Extrusion of Nanoliter Volume Fractions onto a Teflon Coated Slide

which has a density close to that of 20% sucrose, and sedimenting the virus through D_2O onto a fluorocarbon, thus eliminating all of the gradient solutes, and yieding a virus pellet suitable for direct mass spectrometric analysis.

Estimating virus concentration by fluorometric analysis of virions packed against fluorocarbons, as shown in Figures 6 and 15, and extruded as shown in Figure 25 can be done on images obtained with an epifluorescence microscope.

Note that the slide can be metal or metalized, and that the extrusion needle can serve as an electrode. This allows contact between the protruding liquid in the tip of the capillary section of the ultramicrobanding tube and the slide to be determined electronically, and the movement stopped without actually contacting the slide. When the tip is withdrawn, a very small measured droplet is left behind on the slide.

Gradient Recovery from Conventional Ultracentrifuge Tubes

The wax closure-needle system may also be used to collect gradients. The wax seals the tube completely so that if the bottom is pierced no liquid flows out. If the needle at the top is advanced until just visible, and then withdrawn slightly, individual droplets flow out under gravity, and are expelled at a controlled rate, making the wax-needle combination an inexpensive and exquisite microvalve. The system is shown in Figure 26.

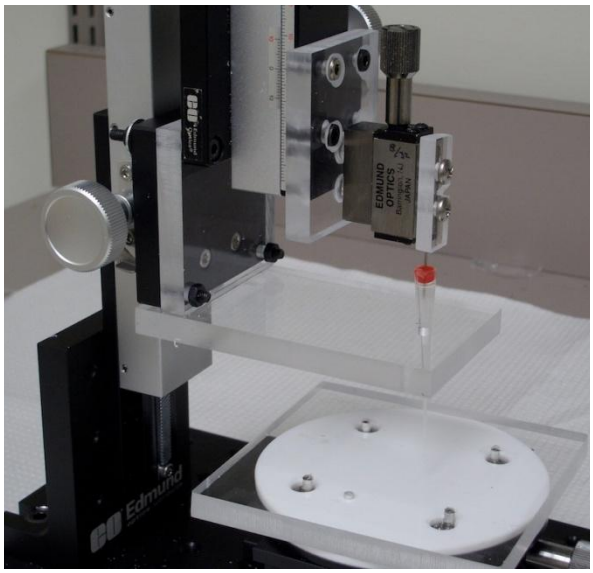

Figure 26. Apparatus for Extruding Pellets and Gradients from Pipette Tips.

This simple approach uses a completely filled system, does not require an expensive microsyringe, and is all disposable, and can yield samples in the nanoliter range.

Figure 27. Demonstration of Effectiveness of Wax-Needle Valve

Droplet maintained in position shown for over a minute

Discussion

In this paper we have explored and demonstrated means for concentrating viruses from suspensions through density gradients in a funnel-shaped region of micro- and ultra-microbanding centrifuge tubes. where the virions may be either pelleted or isopycnically banded. These tubes float on a liquid of suitable density which allows them to be supported at very high speeds. The tubes are made of a soft plastic such as polypropylene and may be sectioned and recovered in ul to nl volumes.

Methods for using specially prepared wax which will flow in high centrifugal fields to make tube closures are described. When these tubes are cut off the tip, a needle may be inserted through the wax at the top to act as a microsyringe to expel and recover micro-gradients, or the wax-needle combination may be used as a valve to allow controlled flow of droplets in a microfraction collector. These devices allow viruses to be recovered in a high state of purity from experimental mixtures.

Means for sectioning tubes to recover viruses pelleted agains dense fluorocarbons and also described.

These systems also provide sufficient viral mass, in many instances, for single molecule sequencing, and for epifluorescent virus counting.

We believe this work provides the basis for solving the first problem in viral diagnostics and viroterrorism, which is rapid definitive identification of viruses from individual patient samples. We propose that one-day virus isolation and sequencing is now in sight.

Contact: normananderson@viraldefense.org

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