THE VIROME PROJECT, A "MANHATTAN PROJECT' FOR BIOTERRORISM. II. TECHNIQUES FOR PHYSICAL VIRUS DETECTION USING MICROBANDING TUBES
Version 1.0

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Introduction

An essential first step in planning and organizing the Manhattan Project was an inventory of the technologies that might logically be required, followed by selection of those most promising, and then by intensive research and development to reduce them to practice with concurrent scale up as required. Under intense time pressures, many logically sequential projects were carried out in parallel.

For the Virome Project, a similar progression is required. Technology applicable to known viruses, from initial detection and diagnosis to vaccine production is relatively straightforward under "peacetime" conditions, however that will not be the case during a pandemic caused by a new lethal rapidly spreading pathogen.

In a major pandemic, hundreds of thousands could perish per day. If some curative measures turn out to be possible during such a disaster, every moment saved in developing them translates into lives saved at the end. The first requirement, therefore, is to rapidly discover whether virus infections exist in presenting patients *at all*. It is evident that the ability to answer this question has widespread implications, not the least of which is that prescribing antibiotics to febrile patients who turn out to have viral infections could be avoided.

A second challenge is to obtain a reliable estimate of viral load, to be able to quickly determine which drugs or therapies are effective in reducing it, and to identify those patients most in need of intensive care.

But it is the third research area, which includes the rapid acquisition of sequence data that presents the greatest challenge. Such data both provides positive identification, and facilitates early development of a variety of responses, including live, killed, subunit and DNA vaccines, and the exploration of RNA-based pharmaceuticals and antiviral drugs.

Planning the Virome Project

In long lead –time planning for military weapons systems it is routine to extrapolate from previous development history, whether increased aircraft speed or improvements in the strength of materials over time, and use such projections to define

future R&D expectations and targets. Given current rapid progress in the development of separations and sequencing techniques, I propose that it is reasonable to project as a target the isolation, quantitative analysis and genomic sequencing of virions recovered from a viremic patient ***in a single twenty-four hour period***. In this and succeeding papers we describe how this might eventually be done.

In planning a virome project we must address the question of how many different human viral pathogens may exist or could be made. If the set of all human viral pathogens is small, largely known, and evolving at a very slow rate, then the problem of characterizing and cataloging them is conceptually simpler than if quasi-astronomical numbers of viruses exist on earth, if they are rapidly evolving, if there are numerous different types or classes of viruses, and if new ones can be constructed in the laboratory.

Very early in the development of large scale centrifugal systems for vaccine purification (to be described in subsequent papers), the problem of obtaining large masses of particles to test these rotors arose. At that time crude vaccine preparations cost approximately one dollar per ml, and a single test batch of 100 liters would cost $100,000.

Before this problem was solved by using very small polystyrene latex suspensions as test particles, a search was made for more economical sources of virus. We discovered that the viral load of sea water was very high[1]. This work has since been extended and the best current estimates of this load range from ~3 x 10^6 virions/ml in the deep sea to ~ 10^8 virions/ml in productive coastal waters[2]. Astonishingly, these values are in the midrange of virus concentrations measured in blood samples from patients with various viral diseases.

The discovery of such massive quantities of marine viruses suggested that not only viral mass but viral diversity may be very large indeed. The world ocean volume[3] is estimated at ~1.32 x 10^{24} ml and using the deep sea concentration as the average, the oceans would contain ~ 4 x 10^{30} virions. Taking the average viral mass as 0.47 x 10^{-15} g, total global marine viral mass would be ~ 1.9 x 10^{15} g (assuming a density of 1) or ~ 1.9 x 10^9 metric tons. Assuming that this mass is ~28% nucleic acids, and that the average base pair mass is ~0.166 x 10^{-23} g, then the total number of base pairs present would be estimated at ~ 4.3 x 10^{41}. Assuming that one base pair substitution per 10^9 base pairs occurs per generation, and that the viruses turn over about once per day (as appears to be the case), the number of substitutions occurring per year would be ~1.6 x 10^{35}, or the gross equivalent of 5 x 10^{27} human genomes, but scattered at random throughout viral genomes. This is a large number.

Many interesting games can be played with these numbers, factoring in billions of years of evolution, to estimate how often a given sequence might appear. The conclusion is inescapable, however, that the oceans contain the major mutation engine of the planet and can and do generate enormous amounts of new genetic information. Estimates of the number of different viral types in the ocean are around 5,000[4].

The original Oak Ridge zonal centrifuge project started with the objective of finding human cancer viruses, based on animal models in which vertical transmission of oncogenic virus genomes had been demonstrated, and with the knowledge that viruses can pick up and transmit host genes quite widely. It was only a short jump from this knowledge to the conclusion that viruses generally may be important in evolution as I suggested in 1970[5]. This idea was not well received at a time when the prevailing view

was that genomes were only affected by calculable mutational processes, that these precious treasures had been passed down to us in an otherwise pristine condition, and that an eighth day of creation was about to occur in which man would do what nature was thought to be incapable of doing, namely moving genes across the entire spectrum of living organisms.

Gene transfer via virus infection is now known to occur widely[6], and the human genome has been shown to contain a vast graveyard of fragments of ancient viruses. Given that viruses can cross species and even phylum barriers, the virome constitutes a global FedEx system for moving genetic information about.

The relevance here is simply this: An enormous reservoir of rapidly turning over viruses is continuously producing new information that can ultimately be passed to nearly all species of animals and plants. In the process, new viruses can be expected to appear as long as life lasts on earth.

Note that marine virology has been explored by means that are almost unknown in medical virology. These means depend on physical separation and detection of virus particles without any prior knowledge of what they are or what they infect. Such techniques are now required if rapid detection, isolation and sequencing of new human viral pathogens is to be done quickly, especially for homeland defense and if the entire virome is to be surveyed.

Thus nature will increasingly provide new viral pandemics, especially as both world population and global travel increases. As more and more human viral pathogens are sequenced, it will be possible to use this sequence information to make new pathogens in the laboratory, either by design or accident. It therefore becomes increasingly urgent to be able to find and count new viruses rapidly by methods that are non-specific, are sensitive, and have an exceptionally wide dynamic range.

However, any detection and counting method will, in many if not most applications, required some form of purification, and concentration. Extensive studies will also be required to determine whether any single detection and counting methods works for a wide variety of different samples types and with different classes of virions.

In this and several subsequent papers we explore a variety of different approaches to this problem, and defer to later studies their application to specific sets of samples.

Techniques and Parameters

Viruses range in mass from ~ 6 x 10^{-15} to ~5.4 x 10^{-17} grams, have sedimentation coefficients in water at 20^0C ranging from ~ 90-1,000 S, and isopycnic banding densities, depending on the medium used, ranging from ~ 1.12 to ~ 1.5 g/ml. The ratio between the mass of an average cell and the mass of an average virus is approximately 10 million.

Viruses are the only known natural particles in their size range that have nucleic acid cores surrounded by relatively impermeable protein coats.

An additional unique property is that their isopycnic banding densities can be experimentally changed. For example, viruses band at different densities in gradients made with nonionic solutes as compared with gradients made with salts such as cesium chloride. Polysaccharides generally have banding densities greater than those of viruses or nucleic acids, and heavily glycosylated particles of viral dimensions may be initially present. A major example, mucus, is composed of subunits which fortunately

disaggregate over time with dilution, and in the presence of mild reducing agents or suitable enzymes.

Hence the separations armamentarium available includes means for differential manipulation of banding densities, and treatment with enzymes and reagents that differentially affect viruses and contaminating particles. Viruses have been banded in a variety of materials including cesium and rubidium salts, in sucrose, and in iodinated gradient materials such as iodixanol[7]. In the latter the banding densities of many particles are reversed from those observed in CsCl, and nucleic acids band above proteins[8]. Thus there is the possibility of using sequential banding in two different gradient materials to resolve complex mixtures.

Viruses have also been isolated from contaminants using precipitation techniques[9], and extraction with solvents including freons[10,11,12] Further viruses have been shown to be differentially sensitive to a range of detergents and enzymes[13,14]. For viruses that traverse the gut, resistance to digestive enzymes, exposure to changes in pH and deoxycholate would be expected, and a classification of virus bases on deoxycholate sensitivity has been proposed[15]. Thus methods can be developed to exploit the differential sensitivity of virions and sub cellular fragments to enzymes, detergents, and solvents, and to chemically dissect cells and tissues to liberate them[16]. While there have been many isolated observations, there have been no systematic studies in which sensitivity to biochemical reagents has been exploited for virus isolation, identification and classification using many different types of viruses.

Viruses have been non-specifically detected by light scatter, or by UV absorbance or by a resistive pulse technique[17]. Using several types of Corona viruses and T2 bacteriophage there was an unavoidable lower limit near 5 x 10^7 virions/mL. Mayor et[18] al first showed that nucleic acids in some types of viruses could be stained with acridine orange, and that single and double-stranded nucleic acids fluoresced at different wavelengths. With the development of a variety of stains whose fluorescence increases very markedly after attachment to nucleic acids[19], detection sensitivity has increases, and individual virions may now be detected and counted in the epifluorescence microscope[20,21,22]. Thus by combining fluorescent staining with s-ρ separations described in a previous paper[23], it appears feasible to develop sensitive biophysical methods for detecting and isolating virus particles from biological sources, and for counting them.

Initially it was thought that some viruses were impermeable to stains[24]. Gradually stains were developed which were more selective for nucleic acids, and which increased in fluorescence when so bound. These included stains which bind to phosphate groups, incalated between bases, or are selective for regions rich in AT or GC. To date there has been no systematic study of the use of different fluorescent stains to distinguish between viruses with single or double stranded DNA and RNA, although the possibility exists that such distinctions could be rapidly made and would assist in virus identification. In section or in homogenates, methyl green stains DNA selectively, and pyronin Y similarly stains RNA, and is slightly flourescent. Therefore these dyes may selectively block binding of fluorescent dyes in a manner that would allow the type of nucleic acid present in individual virions to be detected by epifluorescence microscopy.

We conclude that virus generally constitute a unique class of particles in that all have nucleic acids shielded by protein or lipoprotein coats, fall in a size range separate from the size range of most sub cellular particles, have isopycnic banding densities different

from nearly all other sub cellular particles thus defining the "virion window", and can be stained with fluorescent dyes relatively specific for nucleic acids, facilitating their detection during isolation.

Natural waters may be easily freed of particles larger than viruses by filtration and then the viruses readily concentrated on aluminum oxide filters, stained and counted[25], however this method has not thus far been successfully applied to clinical samples. Hence some other approach is required that includes the elements of an S-rho separation, and transient exposure to reagents that sediment much more slowly than viruses.

Production and Use of Gradients

A common element in the centrifugal separations described is the preparation and use of liquid density gradients, and every molecular biologist is familiar with them, usually in a simplified form. The K-II gradients used for vaccine production (reviewed in a subsequent paper) are either simple step gradients or are self-generating, as are many of the gradients used in the banding region of the micro-banding tubes describe below. With zonal centrifuges, as also described in subsequent papers, complex gradients are often accurately generated.

However, for most other applications, methods for making precision gradients, especially on small scale, have been lacking. Not only should the shape of gradients be closely defined, but the composition of the gradients must be carefully and reproducibly controlled if reagents are to be inserted into them at defined levels.

For large gradients in the liter range, the Angelique second dimension polyacrylamide electrophoresis gel caster previously developed can be used. This was a computerized system that allows computer control of gradient shape and composition in gradients that may be many liters in volume, depending on how they are programmed.

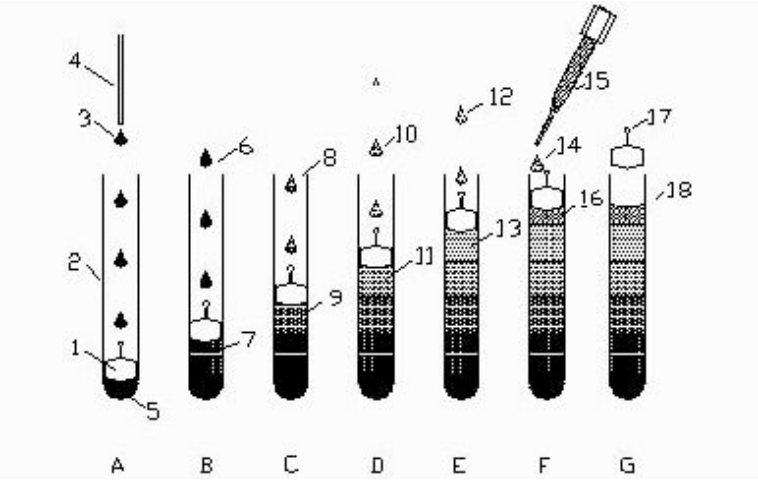

Figure 1. Preparation of Liquid Step Gradients Using Floats.

In A, float 1 is placed in centrifuge tube 2, and individual liquid aliquots of decreasing density 3 are introduced using pipette 4. In B through E this process is repeated to produce succeeding less dense bands. In F the biological sample 15 is layered on top, and in G the float 17 is removed. The tube is then carefully placed in a swinging bucket rotor and centrifuges at high speed.

For small gradients, use is made of specially designed floats that allow gradients composed of many small steps to be formed as shown diagrammatically in Figure 1. The steps are simply pipetted sequentially into the tops of the tubes, and flow and mixing are controlled by the floats[26]. Figure 2 shows a 20 step gradient prepared in an SW 28 centrifuge tube using Iodixanol as the gradient solute, and with cobalamine in every other layer to produce color. Using floats, very small zones containing enzymes, including nucleases or low concentrations of detergents, may be prepared and viruses centrifuged through them. In this manner viruses may be transiently treated with non- or slowly-sedimenting reagents as they sediment. Figure 3 shows floats for several different size centrifuge tubes.

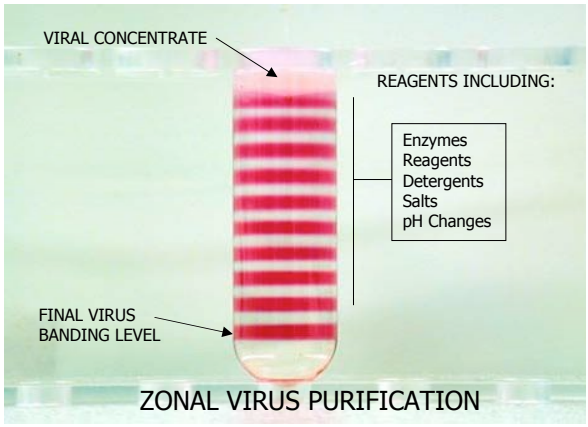

Figure 2. Twenty-Step gradient in SW-28 Centrifuge tube prepared using floats.

Gradient was prepared using Iodixanol for the gradient solute, and cobalamine in every other step to visualize bands.

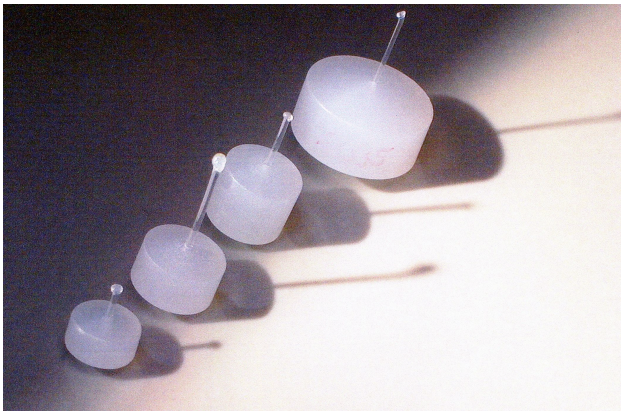

Figure 3. Polypropylene Gradient Floats

Floats are tapered top and bottom and have polycarbonate whiskers to allow easy insertion and removal. Clearance between the float and the centrifuge tube is determined by the viscosity of the gradients used. Performance can be improved by making the surface of the float hydrophilic, for example by exposure to a plasma arc.

Systems for Intermediate Volume Zonal Purification

The zonal systems described in subsequent articles allow for initial rapid fractionation of kilogram quantities of tissue and hundreds of liters of crude vaccines. There is need, however, for intermediate scale systems that will further both purify and concentrate the products of the large scale systems to very small volumes. Since infectious materials will ultimately be involved, containment is an important issue.

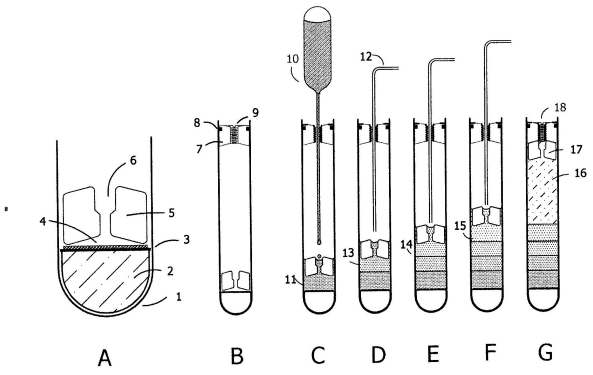

Figure 4. Schematic Diagram of Closed Gradient Processing System for Virus Purification

For the isolation of virions from small infected samples a closed sterile system, such as is shown diagrammatically in Figure 4, is required in which the infectious particles can be sedimented through step gradient zones that can be conveniently set up, with the particles collected either as a pellet, in zones or covalently attached to a flat transparent surface such as a cover slip.

As shown in Figure 4A, a flat-topped Delrin® (acetyl homopolymer) insert 2 is placed in bottom of clear plastic centrifuge tube 1 after small amount of the first and densest gradient liquid has been introduced. Delrin has a density of 1.424 g/cm^3 which is above the highest density of most gradients used for sedimentation-rate separations. If denser liquids are required, Teflon® (density 2.2 g/cm^3) may be used instead. A thin flat polished sapphire disc 3 is placed on top of the insert to support a circular cover slip 4 treated with 3-aminopropyltriethoxysilane (and optionally glutaraldehyde). Without the sapphire disc, glass cover slips often break in a high centrifugal field. The cover slips are numbered with a resistant ink along one edge, and both inserted and subsequently recover using a vacuum "pick and place" device with a small diameter suction cup. A special polypropylene float 5 with a restricted center hole 6 is then inserted to control gradient formation. As shown in B the polypropylene cap 7 with sealing O rings 8 and center nylon screw 9 closes the tube.

The reason for using float 5 in Figure 4 in place of the type shown in Figure 3 is that the former can be assembled in one sterilized closed system, and left in place until the device is disassembled under contained conditions to recover the cover slip. Otherwise the removable floats give superior results.

In use, the cap screw is removed, and the liquid for the remainder of the bottom gradient step 11 inserted optionally using plastic bulb 10. These bulbs can serve to conveniently store and insert liquids for different gradient steps, and can be recapped with silicone rubber caps. As shown in D-F, additional gradient steps may be added optionally using a stainless steel needle 12 attached to an automatic pipetting device. As illustrated in G, a virus-containing sample layer 16 is then added, the float is left in place, and the cap sealed with screw 18. Note that the top screw may be designed to include an upper section which can be grasped by a hemostat-like device to make insertion and withdrawal of the screws easier to do manually or with an automated device.

After centrifugation, the top screw is removed and the contents aspirated using a hollow needle passing through the float. This withdrawal needle may be automatically moved down at a constant rate, and, if attached to a small peristaltic pump will, with the proper adjustment of needle movement downward and pump speed, recover the gradient as a stream of liquid segments interspersed with air bubbles as was done the Technicon autoanalyzer. Note the shape of the float serves to move gradient zones to the center and out through the aspirating needle.

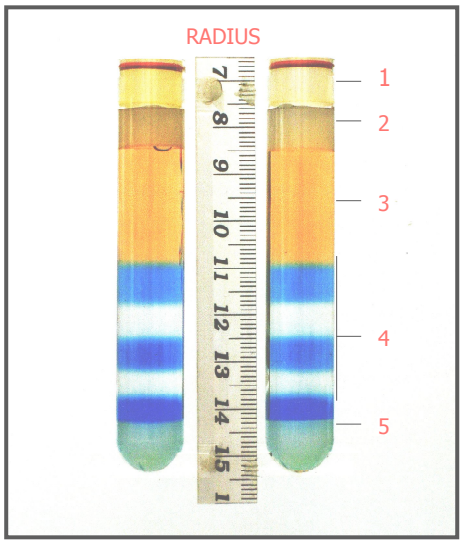

Figure 5. System for Gradient Purification of Infectious Agents.

Components are polypropylene caps 1 with sealing O rings, polypropylene gradient floats 2, sample layers 3, five step gradient with alternate layers stained blue to enhance visibility, and Delrin bottom plugs 5. Center scale gives radius from a zone to the axis of rotation of the rotor.

Cap 7 is designed to float at a level such that the virus-containing sample does not reach the O ring even at high speed. However, standard clear plastic centrifuges tubes, for example the Beckman Ultraclear tubes for the SW 41 Ti swinging bucket rotor, have a smaller external diameter than the inside diameter of the buckets, and hence expand at speed, lowering the level of the floating cap. This occurs when here is no liquid between

the plastic centrifuge tube and the titanium bucket. During deceleration, the tube shrinks back to its original diameter, and liquid may be forced up over the cap.

This problem can be avoided by insuring the space between plastic tube and the bucket is filled with a fluid having the same average density as the filled tube. This liquid can also contain virocidal agents. Note that the filled tube can be externally decontaminated before and after centrifugation. During manipulation involving the cap, the tubes are held in a vice-like device to prevent the caps from moving vertically.

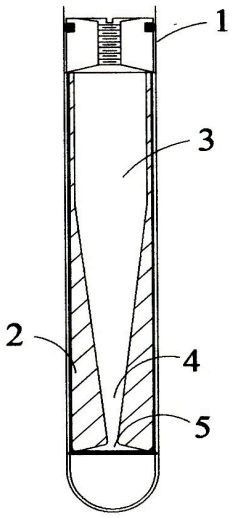

Figure 6. Use of Funnel Insert to Concentrate Viruses for Counting

Funnel insert 2 funnels sedimenting virions to center 4, and focuses them in center area 5 of cover slip. Closure 1 seals tube during use.

The sensitivity of the counting system of Figure 5 may be increased by the introduction of a tapered insert 2 shown in Figure 6. This insert concentrates sedimenting virions in area 3 into funnel 4 and onto a narrow circle on coverslip 5.

When particles are to be sedimented to the bottom, the Delrin plug is omitted, and the infectious particles either recovered as a pellet, or from bands in the lower portion of the gradient.

An S-rho separation on a small scale may be accomplished by making an S-rate separation in one tube, recovering a virus zone, and then doing an isopycnic separation on it in a second tube with a denser gradient. Virus detection may be based on fluorescence for a pellet, or either fluorescence or scatter in the case of either rate-zonal or isopycnic banding density separations.

When infectious particles are to be sedimented against glass cover slips for counting, the cover slip may be initially treated with a 3-aminopropyltriethoxysilane, and the second liquid zone above the cover slip may contain glutaraldehye. The sedimenting particles are thus reacted initially with glutaraldehyde during sedimentation, and then react with the amino groups on the glass to immobilize them. They are further cross linked with glutaraldehyde as it diffuses down to the level of the glass during and after centrifugation. An example of attachment of a mixture of lambda phage and bacteria stained with Sybr gold® to a glass cover slip is shown in Figure 7. The number of virions

in a sample may be counted by reference to killed, fixed and stained bacteria add to viral suspensions.

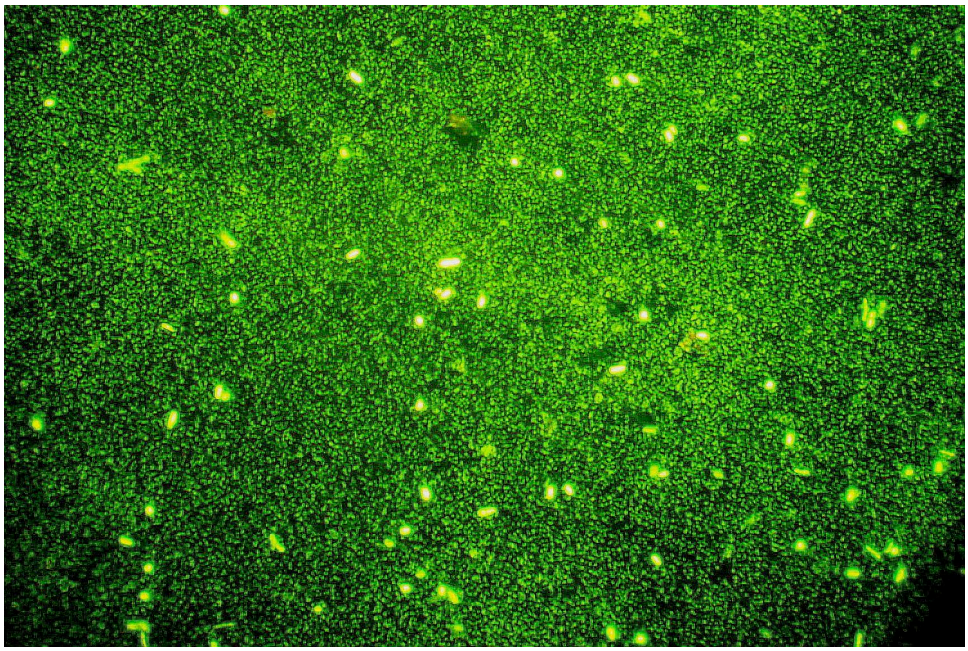

Figure 7. Fluorescently Stained Virions and Bacteria Covalently Attached to Glass Cover Slip

Lambda phage and bacteria stained with Cybr gold and attached covalently to a glass cover slip. Phage particles (small dots) are beyond the limits of resolution of the light microscope, and the images are those produced by point fluorescent sources. Their diameters are a function of the circle of confusion of the lenses used and the number of pixels in the electronic camera. Image is more clearly reproduced in black and white. 40x objective.

Details of studies using this system for computerized virus counting using a series of different viruses and stains are described in a subsequent paper.

Centrifugal Microbanding and Zone Purification

In virus isolation, methods for effectively sedimenting viruses through very small gradients containing reagents zones, and for microbanding them in microliter gradients have been missing, as have been methods for rapidly removing gradient solutes before concentrating viruses for mass spectrometric or other types of analysis. To fill this void we have developed the polycarbonate microbanding tubes as shown in Figure 8. These tubes serve several important functions which are to concentrate viruses from ~ 4 ml volumes to a few microliters or a small pellet, to remove contaminants by sedimenting viruses through zones of non-sedimenting reagents, to isopycnically band viruses in small volume gradients, and to remove the gradient solutes from banded viruses by dilution followed by sedimentation.

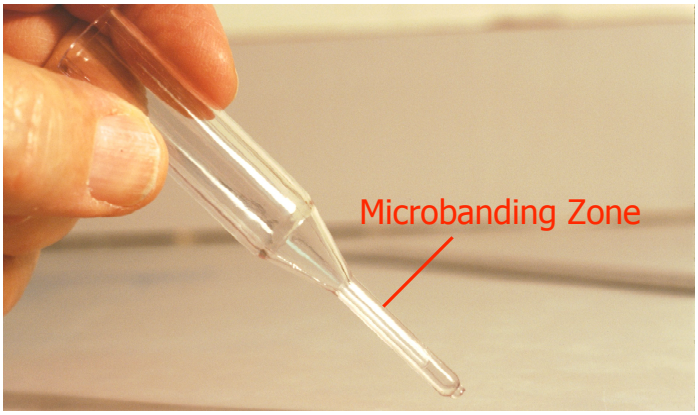

Figure 8. Polycarbonate Microbanding Tube
Banding zone volume is ~ 40 uL.

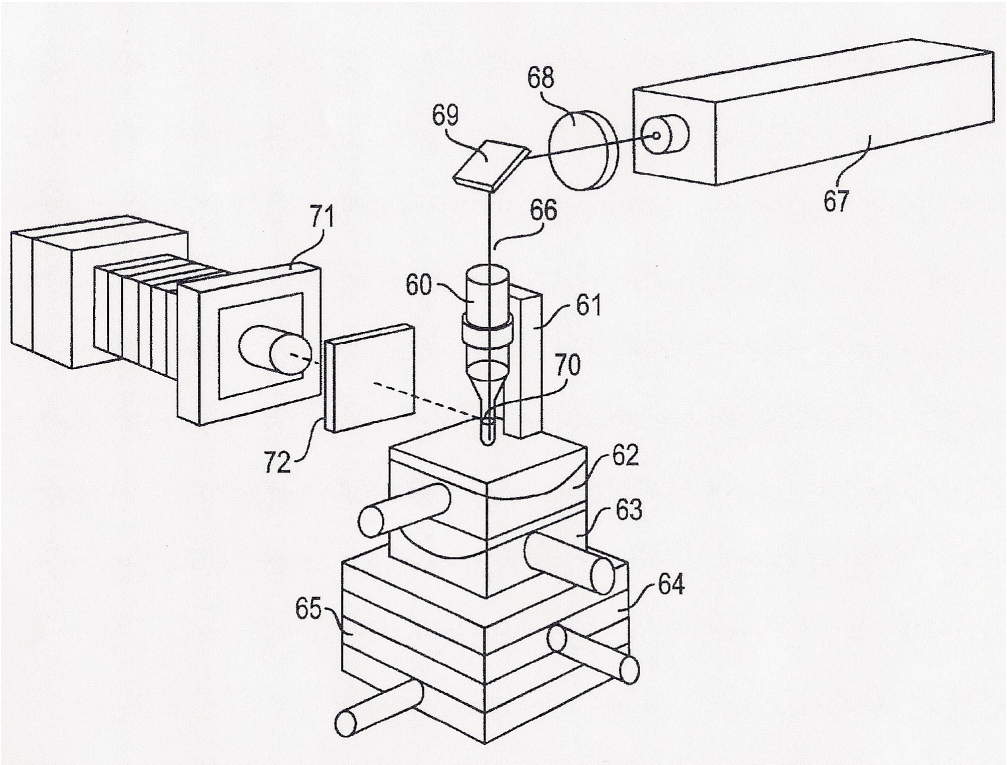

Figure 9. Schematic Diagram of Virus Microbanding System
Microbanding tube 60 is aligned in a vertical position by post 61, alignment is done by goniometers 62 and 63, while horizontal movements are done by 64 and 65. Laser beam 66 from laser 67 passes filter 68 and right angle mirror 69 to illuminate virus band 70. Emitted fluorescent light or scatter is detected by CCD camera 71 after passing filter 72.

The tubes are of molded polycarbonate designed to withstand high centrifugal forces (up to 288,000 x g). Molds for several tube designs have been constructed, and additional ones may be needed as the design is further refined. Floats and tube caps similar to those shown in Figure 5 have also been designed and tested. Thus zone purification may be done in the body of the tube, and further purification and concentration by isopycnic density banding can be done in the microbanding region. Microbanding gradients typically have a volume of 40 microliters, and virus bands may be recovered in as little as one microliter using long gel loading pipette tips.

The tubes are supported by Delrin® adapters and centrifuged at 41,000 rpm in a Beckman SW Ti rotor. A specially designed system for detecting viruses in microbands and for determining their banding densities is shown diagrammatically in Figure 9, and is shown with a computer and printer in Figure 10. A 488 nm beam is shown illuminating a virus band in Figure 11.

A photograph taken of the microbanding region of a tube scribed at 1 mm intervals is shown in Figure 12 and it is used to calibrate pixel positions in the camera relative to positions in the tubes. Note that the image of the tube has been rotated +90° so that the tube is seen to lie on its side with the top to the right.

Figure 10. Operational Virus Microbanding System.

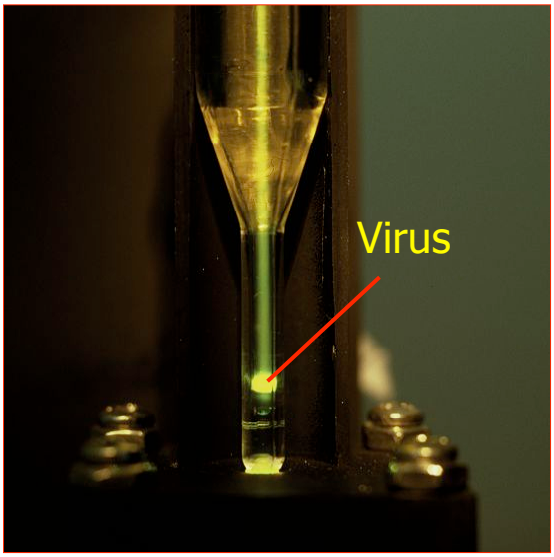

Figure 11.

Microbanded lambda phage in a cesium chloride gradient.

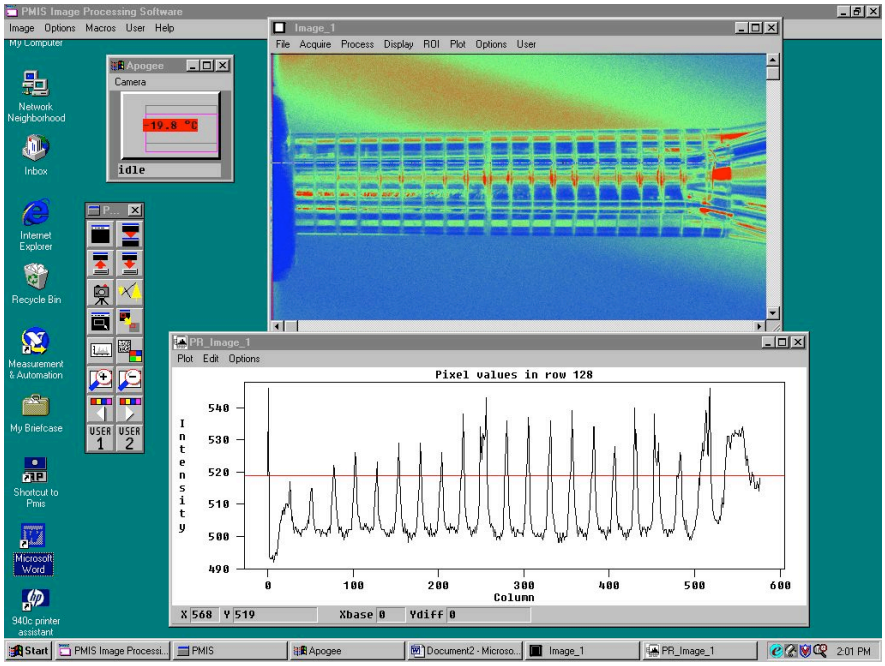

Figure 12. Calibration Marks at 1 mm Intervals In Microbanding Section Of Tubes.

Upper Panel: Pseudocolor image of scored microbanding tube. Note that bottom of microbanding tube is to the left. Lower Panel: Intensity across upper image indicating positions of score lines for calibration against camera pixels.

The argon-ion laser provides lines at 458, 488, and 514 nm, and excitation filters are provided to select only one line. The emission filters include 488, 510, 520, 535, 550, 580, 600 nm, all with half maximum bandwidth of 10 nm. The image in this system is captured by an Apogee CCD camera and displayed using the PMIS program.

Since one of the fundamental concepts of the present project is to both separate and identify viruses on the basis of their isopycnic banding densities, it is essential to be able to measure density at short intervals along the microgradient with high accuracy. It is infeasible to recover the gradient and measure density along it by conventional methods. We had invented density beads during the Oak Ridge Project[27]; however, they are too large for present work and were not fluorescent. Hence small homogeneous beads, generally a micron or smaller in diameter were synthesized for us under contract using different ratios of styrene and 3-bromostyrene to control density. These latex particles have densities of 1.073, 1.114, 1.188, 1.373 and 1.53 g/ml, and both unstained and fluorescently stained batches were prepared. Banding of these beads is illustrated in Figure 12. This allows the gradient to be calibrated, and the density of banding viruses determined by interpolation. Since both size and density of polystyrene latex beads may be controlled, a set of beads may be made to calibrate both S and rho separations.

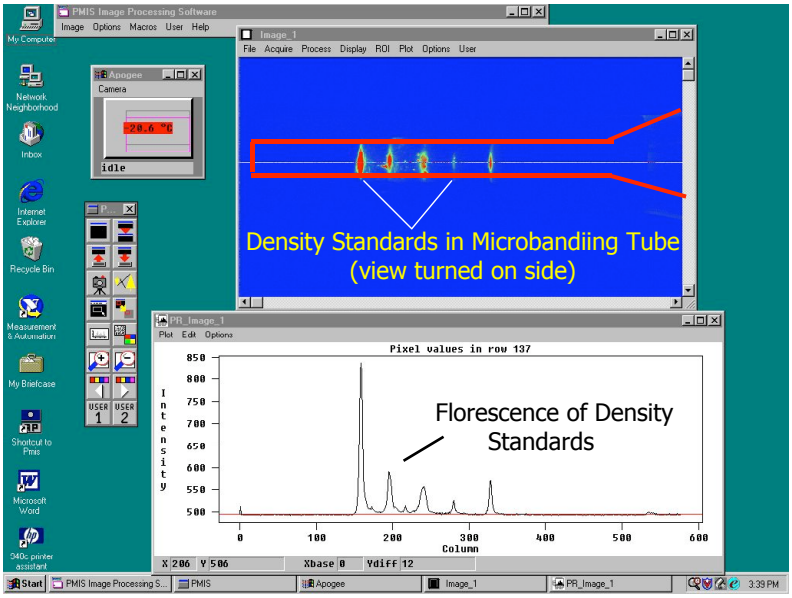

Figure 13. Banding of Density Indicating Beads in Iodoxanol-CsCl Gradient.

Beads have densities of 1.073, 1.188, 1.373, and 1.53 g/cc. Upper Image: Microbanding tube image seen on its side with top to right. Densities of beads are: 1.073, 1.114, 1.188, 1.373, and 1.53 g/mL. Lower Image: Light intensity along scan line shown in top image, indicating positions of beads.

Light Scattering vs Fluorescence

In the experiments to date comparing fluorescently stained and unstained viruses, scatter provides more sensitivity than does fluorescence. Scatter is proportional to the

reciprocal of the 4th power of the wavelength, hence scatter experiments were done at the lowest wavelength available (456 nm) and no emission filter was used.

Early on it was noticed that banded unstained viruses were easily visible when one looked at the tubes from one direction, and were not visible at a right angle to that direction. This is explained by the fact that argon ion lasers emit polarized light, and the scattered light is also polarized. In order to scan the tubes from two directions at right angles to each other at the same time, a mirror was placed next to the microbanding tube, and the two views of the tube 90^0 apart are displayed one above the other in the upper panel of Figures 14 and 15. The results in Figures 14 and 15 show the same tube containing banded lambda phage. A and B indicate the scan line for these two views. In the upper plane A the view is at a right angle to the plane of the electrical vector of that light, and the lower scan line B is in that plane.

In Figure 14 the data presented in the bottom panel is for scan line A, and shows the scatter peak. Figure 15 is identical to Figure 14, except that the lower panel shows the results for scan line B, and no scattered light is detected.

This bears on the question of whether the density beads or the virus or both should be fluorescently labeled. In all of our studies scatter at this angle has been very much more intense than fluorescence. Hence it may be better to use scatter to detect viruses, and fluorescence to detect the positions of density beads. However this loses the advantage of using specific staining to demonstrate that the particles of interest do indeed contain nucleic acids. A solution to this problem is to make larger density beads that fluoresce at a very much different wavelength than viruses stained with currently used dyes, thus minimizing density bead scatter, and maintaining the option of using both scatter and fluorescence to detect viruses and measure their concentration, possibly at the same time.

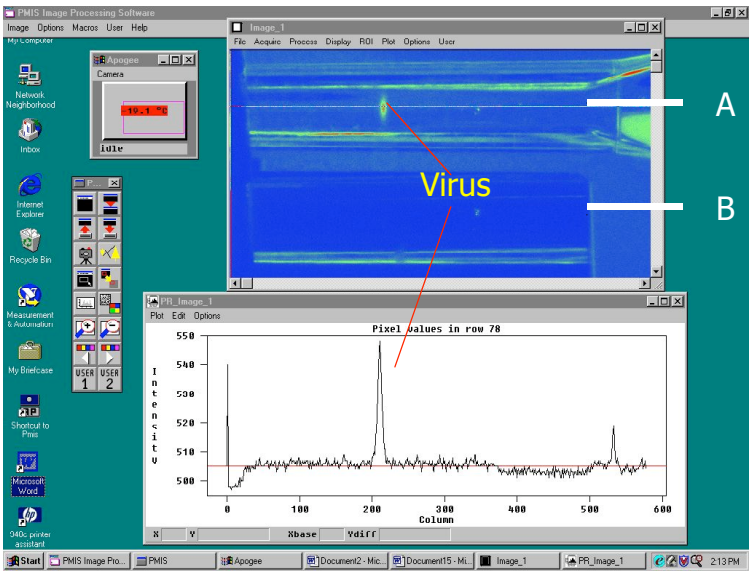

Figure 14. Isopycnic banding of Lambda Phage in Iodixanol-CsCl Gradient in a Microbanding Tube as detected by scattered light at 456 nm.

Upper Image: Two views of the microbanding tube are incorporated into the same image during the same exposure to indicate the effect of polarization on scatter. Microbanding tube shown in A is positioned normal to the plane of the electrical vector of the laser illumination, and in B, normal to plane of the magnetic vector of the polarized laser beam. Lower Image: Intensity of scattered light along the center of tube image in A.

In future work the relationship between scattering intensity and titer will be determined in the hope of making micro-banding measurements quantitative. The reason for pursuing scatter in preference to fluorescence is that fluorescence depends on virus permeability, dye concentration, length of exposure to exciting light, length of staining, and possibly on salt concentration in a gradient. However scattering is extremely sensitive to the presence of dust, hence in practice one would like to measure both scatter and nucleic-acid-binding dye concentration.

A micrometer-controlled band recovery device has been built that can recover bands into sections of very small polyethylene tubing. The volume of the recovered band is determined by measuring the length of tubing occupied by the droplet, and the ends, separated by an air space from the droplet, are heat sealed and stored cold until analyzed.

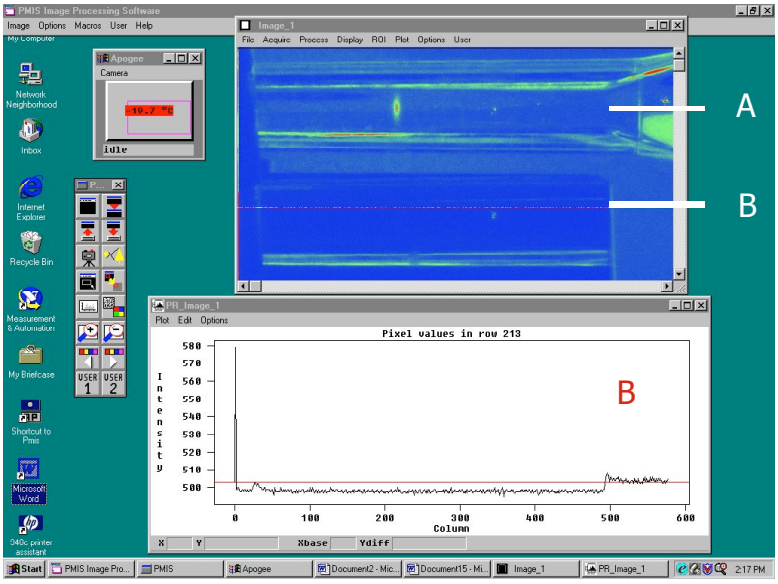

Figure 15. Isopycnic banding of virus in Iodixanol-CsCl Gradient in a microbanding tube.

Upper image is a duplicate of that shown Figure 27. Lower image: Amazing total lack of any scatter from microbanding tube viewed along scan line B above, in which the image is viewed normal to the magnetic vector of the laser illumination.

Bands recovered from dense gradients contain high concentrations of gradient solutes that must be removed in preparation for mass spectrometric analysis. Dialysis of such small volumes is infeasible; therefore the microdropet is diluted by a factor of 100 or more in a very dilute buffer and sedimented in a microbanding tube onto the surface of

~10 uL of a dense fluorocarbon. All but a microliter or two of the fluid over the virus is removed, and the tiny pellet resuspended and recovered in a polyethylene tube as described. Small volumes may also be recovered using long gel loading micropipettes. For analysis these small volumes are expelled directly onto a MALDI target.

Discussion

The overall objective of the Virome Project is to isolate new infectious agents quickly, and prepare vaccines against them as fast as technically possible. Methods that depend on agent cultivation for biological amplification are luck dependent, and there is no way to know in advance what will work for a new agent. Biophysical methods, as is being shown in this series, can be rapid but depends on having sufficient agent concentration at the beginning for whatever analytical means (ideally sequencing) are adopted at the end. To insure that success is achieved it is essential therefore to scale up the amount of sample at the start, and to microminiaturize analytical processes at the end.

With biophysical processes such as are under investigation here, it is essential to develop a series of techniques spanning a very wide dynamic range, and, ideally, to keep the infectious particles in suspension from start to the final steps. It is also essential that all procedures be adapted for remote safe contained operation, and for rapid start up.

In this paper we describe a system for gradient purification at an intermediate scale using high speed swinging bucket rotors, for adapting this system for virus counting by epifluorescence microscopy of target cover slips, for banding at near the bottom of the gradients, or for recovering purified viruses as pellets. Also described is a hybrid system in which infectious particles may be purified by sedimentation through zones in liquid gradient, and then funneled into a microbanding region where viruses are banded isopycnically.

Polycarbonate microbanding tubes are difficult to fabricate because of the high viscosity of the molten plastic. The pin which defines the microbanding region (1.1 mm in diameter) always bent, even when high strength steel was used for the mold. This problem was partially solved by using a mold in which the distal end of pin was held in place during the first injection step by a mold component that was then withdrawn for the final injection. Unfortunately small irregularities in the internal mold surface also caught sedimenting virion, thus reducing the amount of virus recovered in the microgradient bands.

Attempts have been made to remove small surface irregularities that might trap viruses from the internal surfaces of polycarbonate tubes by methylene chloride surface vapor polishing. However this process requires special equipment and a hazardous reagent.

For these reasons, further work in his direction will center on finding other more suitable plastics, and on ways to greatly decrease the bore of the microbanding region. For this purpose glass centrifuge tubes will also be explored.

Density beads provide a convenient means for determining the portion of a gradient that corresponds to the virion window, and deserve further development.

When different viral loadings were used to test the sensitivity of the microbanding-fluorescence system the lower limit of detection was about 10^8 virions total. This is in contrast to the limit of 10^5 virions for counting on electron microscope grids[28]. Thus we

have not, at this point, reached either the dynamic range or sensitivity goals desired. A combination of a microbanding approach with epifluorescence detection on a flat transparent surface may offer the best chance to reach these goals.

Northrop discovered in 1926 that living organisms generally are resistant to proteolytic enzymes[29]. Gradient zone digestion of cytoplasmic constituents and dead bacteria can therefore also be used to recover live bacteria from infected tissue homogenates. As shown here both bacteria and viruses can be sedimented onto and covalently bound to a glass surface. This opens the possibility of a general method for isolated all types of infectious agents from infected tissues, for distinguishing between bacterial and viral infections, and for very rapidly recovering sufficient material from either for direct sequencing. In addition, once infectious particles are immobilized on a transparent surface, immunochemical fluorescence techniques may be applied to their identification.

We stress that this paper concerns technology development. Much further research and development will be required to adapt these systems to a wide variety of clinical samples, to discover what conditions and dyes or optimal for different types of viruses, and to further develop and modify the systems described to make them routinely useful in the clinic. This will involve collaborative studies on a wide scale.

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