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Hirschfeld

[11] **3,887,812**

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[54] **METHOD AND APPARATUS FOR DETECTING AND CLASSIFYING NUCLEIC ACID PARTICLES**

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Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 375,807, July 2, 1973.

[52] **U.S. Cl.** **250/458**; 250/461

[51] **Int. Cl.** **G01t 1/16**

[58] **Field of Search** 250/304, 361, 362, 363, 250/365, 369, 432, 435, 458, 461

[56] **References Cited**

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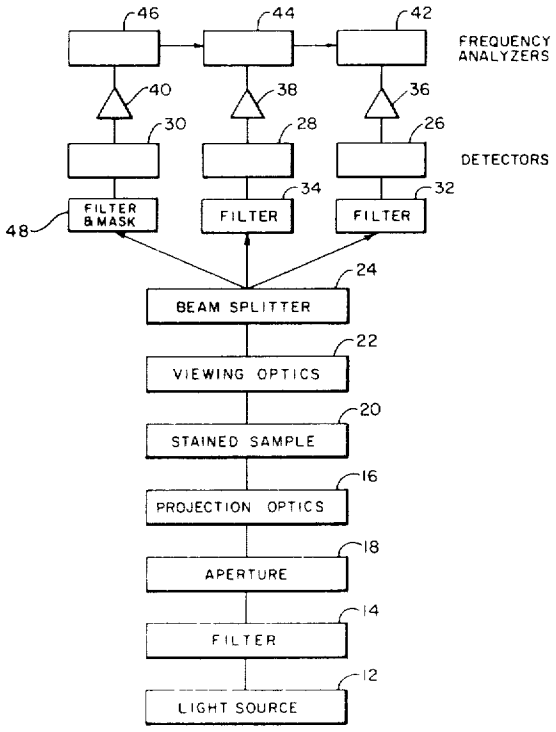
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Attorney, Agent, or Firm—Schiller & Pandiscio

[57] **ABSTRACT**

Free floating viruses are detected and sized by a method which combines fluorescent staining with the observation of a modulation of the fluorescence intensity by Brownian motion of the particles in combination with a particular spatial filter. Intensity modulation and fluorescence data provides a set of descriptors useful in distinguishing the type of virus involved, particularly if also combined with data regarding the scattering of light by the particles. The method and apparatus are also usable in connection with detection and classification of other biological particles having the same order of magnitude of size as viruses.

4 Claims, 6 Drawing Figures



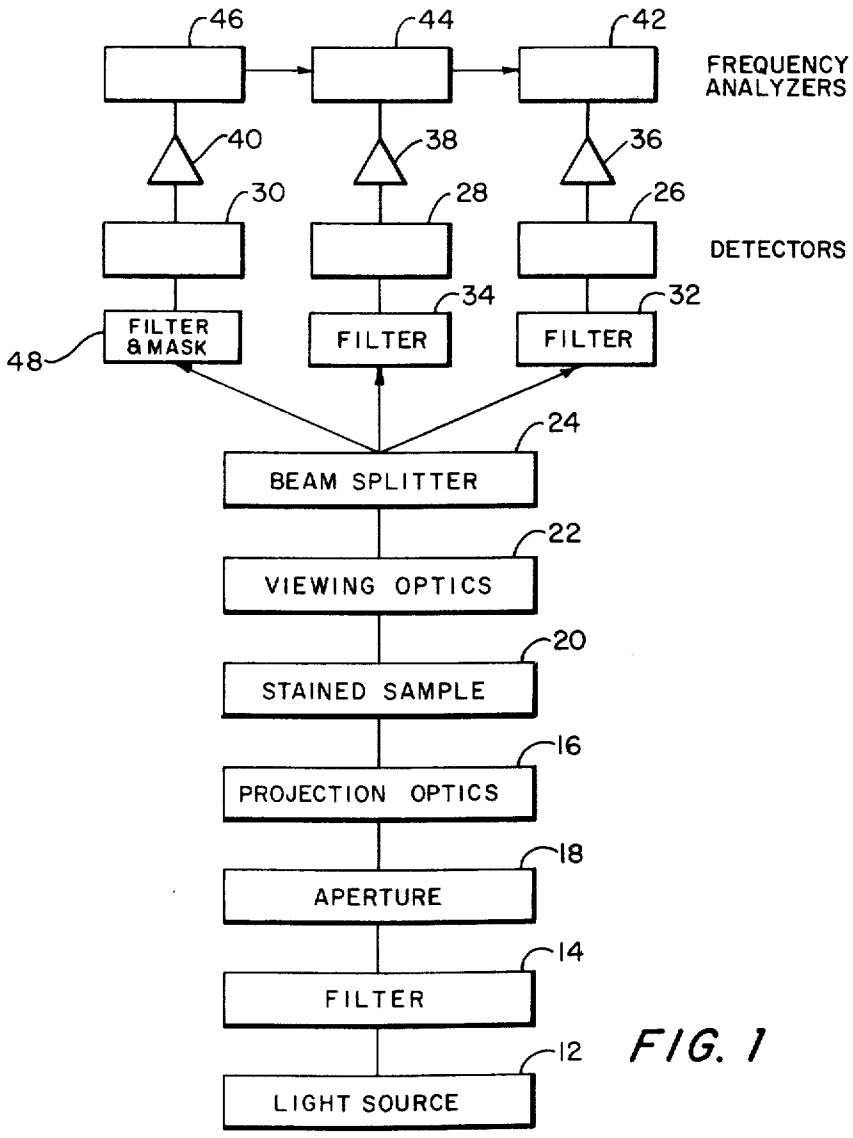

46
44
42
FREQUENCY ANALYZERS
40
38
36
30
28
26
DETECTORS
34
32
48
FILTER & MASK
FILTER
FILTER
24
BEAM SPLITTER
22
VIEWING OPTICS
20
STAINED SAMPLE
16
PROJECTION OPTICS
18
APERTURE
14
FILTER
12
LIGHT SOURCE

FIG. 1

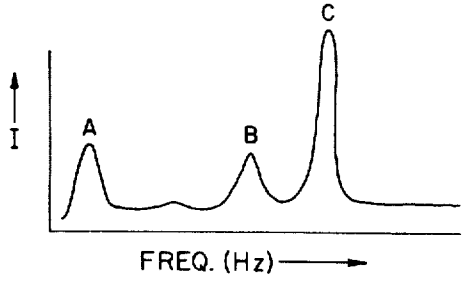


FIG. 2

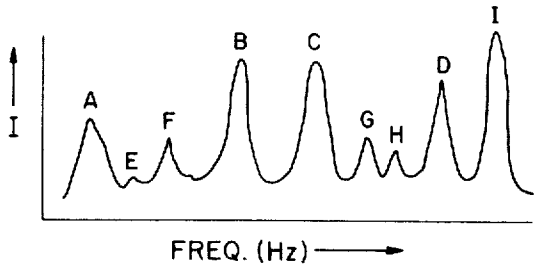


FIG. 4

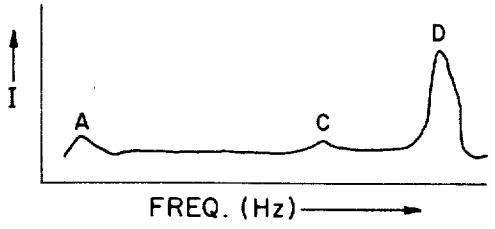


FIG. 3

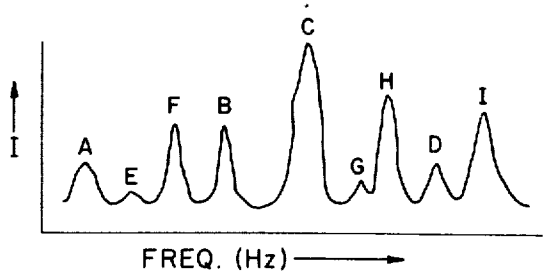


FIG. 5

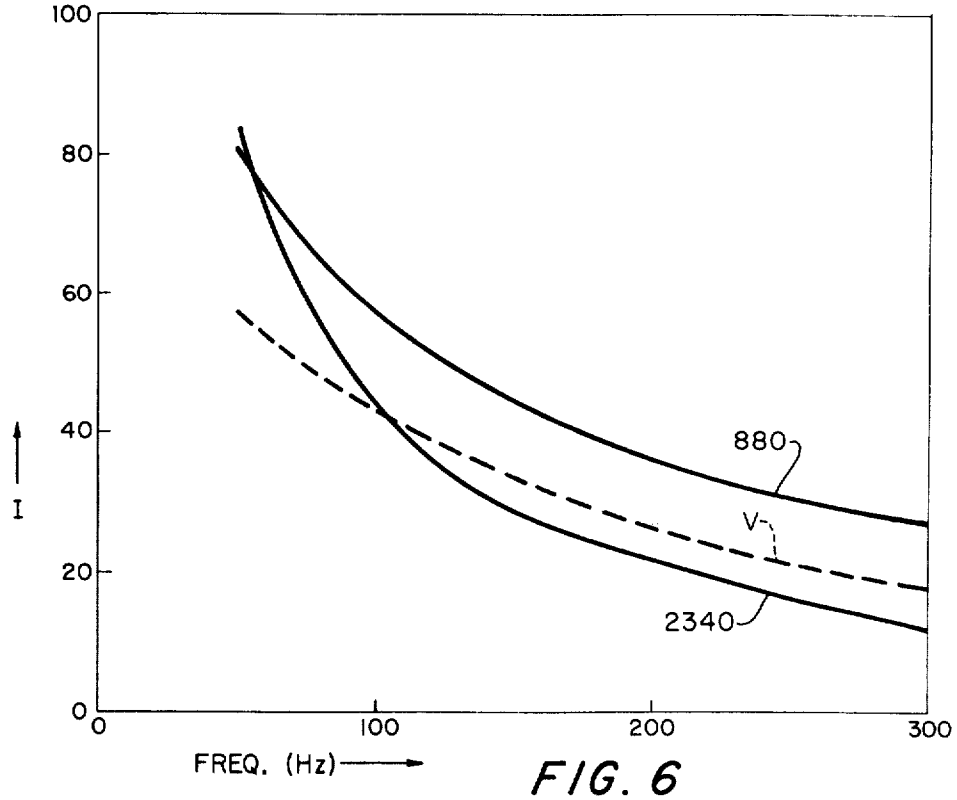


FIG. 6

METHOD AND APPARATUS FOR DETECTING AND CLASSIFYING NUCLEIC ACID PARTICLES

This application is a continuation-in-part of copending application Ser. No. 375,807, filed July 2, 1973.

The present invention relates to clinical laboratory methods and apparatus and more particularly to detection and classification of virus particles.

Virus particles, responsible for a wide variety of plant and animal pathology, comprise exceedingly small particles sized on the order of a wavelength of visible light or smaller (3500 to 200 Angstroms) and consist essentially of either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) surrounded by a protein shell. The ratio of nucleic acid content to protein content varies from 1:100 to 1:2.

Viruses are usually studied in animal, bacterial or plant hosts or in cell cultures derived from tissues of the hosts. Virus presence if inferred by observing the development of infectious symptoms in a patient due to viral infection. The long time involved and the danger of spread of the infection limit the desirability of this technique.

Virus-suspect specimens may be subjected to a variety of chemical reactions using known techniques of immunochemistry to detect and classify viral species, if any, therein. The reactions involved are highly specific and a large number of specific antibodies are necessarily involved. At present however, only a limited number of such antibodies are known. Culturing suspect specimens and examination in the electron microscope to observe differences is limited in its effectiveness by the long time involved in culturing and sample preparation. It has also been known in the prior art to produce a suspension of a suspect specimen in liquid, illuminate the suspension with coherent light and observe backscattered light by heterodyne spectometry to obtain data on Brownian motion of suspended particles correlatable with size of the particles. In other words, the method measures particle velocity by observation of the Doppler shift. The use of coherent and essentially monochromatic radiation permits measurement even of the comparatively slow Brownian velocities. However, inadequate ability to descriminate among viral species and between viral particles and similarly sized cell debris in the specimen limit the utility of this technique.

It is therefore an important object of the present invention to provide method and/or apparatus for virus detection avoiding the above cited problems of the prior art.

It is a further object of the invention to provide classification among viral species present in a suspect-sample consistent with the preceding object.

It is a further object of the invention to provide a short time of speciment treatment and data extraction consistent with one or more of the preceding objects.

It is a further object of the invention to provide inexpensive and simple apparatus and/or operating steps consistent with one or more of the preceding objects.

According to the invention, virus-suspect specimens are treated by the following process:

a. Taking from the patient a body fluid sample containing a suspension to be examined for virus, and treating the sample with one or more fluorescent dyes specific to a nucleic acid;

b. Illuminating the sample with light in an absorption band of the dye or dyes and observing fluorescence, if any, from the stained sample at spectral peaks associated with deoxyribonucleic or ribonucleic acid, and substantially simultaneously spatially filtering either or both the excitation illumination and the emitted fluorescence, whereby the observed fluorescent intensities of dyed particles is modulated by the Brownian motion of such dyed particles.

Scattering behavior and/or polarized light response may also be observed.

Spatial filtering of observed fluorescence intensities is achieved in the present invention in one or two basic ways. In the first method, the radiation used to illuminate the particles is spatially filtered: For example, a regular grid or a simple aperture is placed between the illumination source or optics and the suspension of particles, thereby providing one or more zones of high and low illumination. In the second method the fluorescence radiation itself is spatially filtered, for example, by placing a grid between the suspension of particles and the viewing optics, thereby providing zones within which fluorescence can or cannot be observed. In either case, as the particles move from zone to zone by Brownian motion, the fluorescent emission will be modulated to produce a repetition rate spectrum which is a function of the velocities of the fluorescing particles. The particle velocities in turn are a function of the particle masses. This system can be readily distinguished from conventional scanning fluorometry in which either the object or illuminating beam are moved or scanned in a fixed scanning pattern which creates a signal indicating a contour of the fluorescent intensity distribution across the scanned object. The present system depends instead on the statistical random or Brownian motion of a particle past an edge to modulate the fluorescent intensity as a function of particle velocity. By wavelength filtering, one can readily identify the wavelength of the fluorescent emission and thereby determine the particular nucleic acid to which the fluorochrome is bound. This, together with simultaneous observation of the intensity modulation of the fluorescing particles serves to distinguish viral-like particles from background and provides useful viral classification descriptors. The number of useful descriptors can be enhanced through correlation of simultaneous observations of two-color fluorescent modulation of instensity by Brownian motion and further enhanced by correlation of polarized light response and scattering data with the measurements of intensity modulation by Brownian motion. The method and apparatus of the invention are also usable in connection with detection and classification of other biological particles having the same order of magnitude of size as viruses, i.e. submicron size.

A conventional laboratory microscope may be modified by the addition of aperture, filters and a fluid-well specimen holder in accordance with the present invention to carry out the above described process. Preferably, the microscope is provided with multiple detector channels for simultaneous readout of intensity modulated wavelengths corresponding to each of DNA and RNA. However, a single channel may also be utilized with changes of filters for sequential observation for the two colors to be detected. Scattering data is taken separately or together with the fluorescence data. Readout is preferably done automatically by photodetectors coupled to frequency analyzers for each read-

out channel. The frequency analysis output for each channel is a graphical or ditigal representation of intensities of signal at various frequencies. Whether a signal is generated at all is determined by coincidence of the color selective properties of the detection system with the fluorescent emission wavelength of the fluorochrome attached to the viral particle, which is dependent on the selection of fluorochrome and whether it is linked to a DNA or RNA-containing particle.

These and other objects, features and advantages of the invention will be apparent from the following detailed description with reference therein to the accompanying drawing in which:

FIG. **1** is a block diagram of apparatus for detection of viral species and classification by composition of a particular nucleic acid; and

FIGS. **2–6** are graphical representations of the relationship of light intensity to intensity modulation frequency in operation of the invention, all graphs having the same coordinate increments in abscissa and ordinate.

According to a preferred embodiment of the invention, the known techniques of tissue culture preparation are used to obtain a virus-suspect sample. The known techniques are modified to the extent of being carried out in a time too short to produce the growth areas of cell destruction associated with plague preparation. The sample so obtained is suspended in a liquid. Alternatively, a natural body fluid suspension of virus particles (e.g., plasma or spinal fluid) may be directly utilized. The suspension is mixed with fluorochrome dye stains which are soluble in the fluid and specific to the viral or other small nucleic-acid-containing particle to be detected.

The apparatus of a preferred embodiment of the invention comprises a modified microscope with a laser or other high intensity light source **12** for projecting light through a wavelength filter **14,** a spatial filter **18,** and conventional projection optics **16.** Filter **14** restricts the illumination to an excitation band of the fluorochrome dye. Spatial filter **18** may be a single aperture or a multiaperture filter such as a grid or the like as hereinafter described. The spatial filter is imaged in the plane of the sample which is held in a transparent fluid well in sample holder slide **20.** It will be understood the spatial filter may precede the slide or the slide may precede the spatial filter in the light path. In the latter case, the sample is imaged onto the spatial filter by viewing optics **22.**

Spatial filters generally are well known to those skilled in the optical arts and may take the form of gratings, grids, annuli, and the like. Each defines one or more zones or edges between a relatively light-transmitting element and a relatively non-transmitting element. It will be apparent that as a particle, moved by Brownian forces, crosses the edge or zone of a filter to a light-transmitting area, the detector will see that particle somewhat as a light burst or scintillation. As the particle crosses the edge into a non-transmitting area, the burst is extinguished. Thus, assuming that a particle travels in a straight line across a grid, the observed intensity of emission from the particle will fluctuate between maximum and minimum values at a frequency depending on the grid spacing and the particle velocity. Theoretically, for optimum modulation and duty cycle, the grid spacing should, at least in order of magnitude, match the particle size. For particles of submicron size, such matching is a difficult task at least. However, spatial filters approaching diffraction-limited apertures for the wavelengths employed, although having aperture sizes far greater than viral dimensions, will nevertheless provide quite useful results. It should be noted that the present invention is intended to provide viral classification descriptors which are derived by frequency analysis of a viral population of statistically valid magnitude. A typical sample can be expected to contain viral particles in excess of about $1 \times 10^6/ml^3$ which thus provides an adequate population for the proposed analysis. However, if one assumes that this population of particles is viewed in a volume 1μ thick (e.g. the depth of field of the viewing optics is 1μ) through a spatial filter which is simply a diffraction-limited circular aperture, the observation time to obtain adequate data for analysis would be intolerably long because of the very few scintillations per minute that would occur. Simply increasing the aperture area would improve the situation by providing a longer edge across which more particles would be expected to move, but the mean dwell time of the particles in the aperture would also increase thereby impairing the signal-to-background ratio, so little gain would be made toward providing an adequate observational sample in a reasonable time period.

The criterion used in the present invention to select an adequate spatial filter is that the ratio of viewed volume to the root mean square distance across the aperture of the filter, be greater than 1000/1. The root mean square distance (also known as the mean crossing distance) is defined as the mathematical root mean square distance to the edges of the aperture (or the several apertures of a multiple aperture) from the mean of all points in the aperture (or in each of the apertures in the multiple aperture situation). In other words, to obtain a statistically large number of scintillations per unit time it is desirable to increase the viewed volume (i.e. the product of the aperture times depth of field) but the dwell time of the individual particles in the viewed volume is minimized to reduce background.

For example, an aperture in the form of an elongated slit 1μ wide (assuming a depth of field of 1μ deep) should then be more than 1000μ long to achieve adequate signals. Such slit need not be linear but can be in the form of a spiral. Closely related filters formed of multiple slits are gratings (e.g. an array of parallel linear slits), a group of concentric ring segments (e.g. an array of curved slits having a common center of curvature) and the like. Similarly, one can employ a grid or a plurality of small (rectangular or otherwise) openings to form the desired filter. All of the latter are of course multiple volume apertures that can be employed in the present invention provided that they meet the criterion hereinbefore set forth.

The light passing through the sample and/or emitted by the sample is viewed through conventional viewing optics **22,** a beam splitter **24,** and photoelectric detectors **26, 28, 30.** Detectors **26** and **28** may have wavelength filters **32** and **34** associated therewith to limit their effective spectral ranges to different fluorescence bands or may themselves have limited spectral ranges. Detector **30** may also be provided with a wavelength filter and known means **48** to mask out direct radiation from light source **12** so that detector **30** sees only scattered light.

The electrical output signals from the detectors are amplified by preamplifiers **36, 38** and **40** and applied

to known frequency analyzers **42, 44, 46** which are synchronized by mechanical or electronic means such as a common clock circuit associated therewith. The outputs of the frequency analyzers are applied to data reduction apparatus which may be a strip chart recorder and/or a computer.

To obtain desired specificity of nucleic acid staining, solubility, and the property of fluorescence at desired wavelengths under excitation by light of desired wavelenghts, one may use the cationic dyes: acridine orange or yellow GR, quinacrine mustard, ethydium bromide, pyronine B, aurophosphine, euchrysine 2GNX and 3R, vesuvin, rhodamine S, B and 6G, coriphosphine 0, civanol, acrifalvine, atabrine, phosphine, benzoflavine, rheonine A, thioflavine T and berberine. Many other dyes can be used with appropriate adaptation of sample chemistry or the light source to suit such dyes.

With an appropriate spatial filter, the apparatus of FIG. **1** can be operated with a 0.05 cc. sample having a particle concentration of about 10^8 particles (of approximately 200 to 3500 Angstrom size) per cubic mm., to make classification measurements in one minute or less. Sample handling can be accomplished at a rate of five minutes or less per sample. Accuracy of size classification can be within ± 3 percent.

The outputs of the frequency analyzer for a single sample are shown schematically in FIGS. **2–5** which are intensity vs. modulation frequency graphs (in Hertz). The sample was studied with acridine orange and its fluorescence was measured through different channels or in a single channel with changes of detection filtering between observations.

FIG. **2** is a graph taken with a filter passing an appropriate wavelength (5500 A in this example) to detect the presence of DNA particles. The location of each of peaks A, B, C on the resulting curve are proportional to the equivalent diffusional diameters of various DNA-containing particles. Peak heights and areas are proportional to quantities of the DNA-containing particles of the sizes indicated by peak location.

The curve in the graph of FIG. **3** is created by the frequency analyzer and its printout device when using a wavelength filter (5500 A in this case) chosen to detect RNA-containing particles.

Clearly peak B of the curve in the graph of FIG. **2** corresponds to a DNA-containing virus because there is no corresponding peak in FIG. **3**. Similarly peak D of FIG. **3** corresponds to an RNA-containing virus. As viruses have to belong in one of these types, the continued presence of peaks A and C indicates a slightly shifted DNA spectral behavior which indicates a structural change in the DNA'S arrangement. These fluctuations in the overlap between both channels have recognition value.

FIG. **4** shows the result of a repeat run with polarized filter specific for scattering due to the total particle mass. The illuminating source is an argon laser. This filter is chosen to correspond to the illumination, and is 4880 Angstroms in this case.

It is clear from comparison of FIG. **4** with FIGS. **2** and **3** that the peaks E, F, G, H, and I of FIG. **4** are due to the presence of particles that do not contain nucleic acid and may be disregarded.

FIG. **5** shows the result of a repeat run with the 4880 Angstrom polarized filter with a different orientation.

The ratio between the readings of FIGS. **4** and **5** describes the polarization in the particle scattering and thus the particle's elongation.

Tha average of the curves of FIGS. **4** and **5** gives the total particle mass, whose ratio to the FIG. **2** or **3** curves gives the nucleic acid/total weight ratio in the particle.

FIG. **6** is a similar plot, for apparatus calibration purposes, of intensity vs. modulation frequency, using the apparatus of FIG. **1** with a single tiny aperture as a spatial filter to detect suspended particles of 880 Angstrom polystyrene spheres, a known phage virus, and 2340 Angstrom polystyrene spheres. The polystyrene spheres were observed in scattered light.

The results are shown, respectively, on the curves marked 880, V, and 2340. The half-width of the half-heights of the respective curves occur at 34.5, 13.2 and 27.5 Hertz indicate the frequencies relatable to known diameters of the particles.

It is evident that those skilled in the art, once given the benefit of the foregoing disclosure, may now make numerous other uses and modifications of, and departures from the specific embodiments described herein without departing from the inventive concepts. Consequently, the invention is to be construed as embracing each and every novel feature and novel combination of features present in, or possessed by, the apparatus and techniques herein disclosed and limited solely by the scope and spirit of the appended claims.

What is claimed is:

1. Apparatus for detecting and classifying, in a fluid specimen, submicron-dimensioned particles containing a nucleic acid and stained with a fluorescent dye specific to said nucleic acid, said apparatus comprising,

a radiation source for providing illumination within an excitation band of said dye,

means for directing said illumination into said specimen,

means for viewing fluorescence emission produced from said dye responsively to said illumination, and

means for spatially filtering at least one of said illumination and said fluorescence emission, said means for spatially filtering comprising aperture means providing, in conjunction with said means for viewing, a ratio of viewed volume of said specimen to the root mean square distance across said aperture means of greater than 1000/1.

2. Apparatus as defined in claim **1** wherein said means for spatial filtering is disposed in the optical path between said source and said specimen.

3. Apparatus as defined in claim **1** wherein said means for spatial filtering is disposed in the optical path between said means for viewing, and said specimen.

4. Apparatus as defined in claim **1** wherein said means for spatial filtering includes one or more light transmissive portions, substantially all of which are dimensioned to be as closely matched to the average dimension of said particles as the wavelengths of said illumination permits.

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