

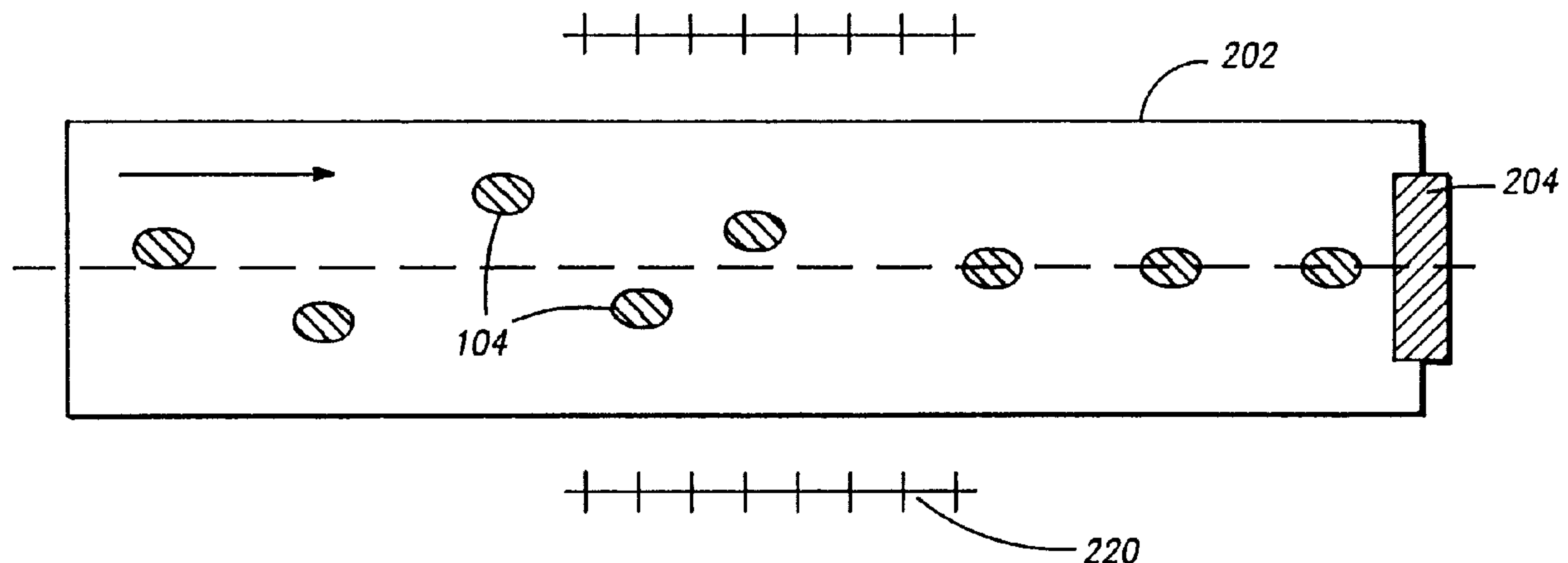


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(72) Inventeur/Inventor:  
GOIX, PHILIPPE, US  
(73) Propriétaire/Owner:  
GUAVA TECHNOLOGIES, INC., US  
(74) Agent: GOWLING LAFLEUR HENDERSON LLP

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(54) Title: METHOD AND APPARATUS FOR DETECTING MICROPARTICLES IN FLUID SAMPLES



(57) Abrégé/Abstract:

A method and apparatus for detecting a fluorescent substance tagged to a microparticle are described. The device comprises a single capillary flow carrier system for transporting the microparticle past a selected location, a source of electromagnetic radiation for irradiating the substance tagged to the microparticle, and a detection system for measuring fluorescent light emitted from the substance at the selected location. The method comprises transporting the microparticle to a selected location, irradiating a fluorescent substance tagged to the microparticle, and measuring the fluorescent light emitted from the fluorescent substance at the selected location.



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<b>(21) International Application Number:</b> PCT/US98/11958 <b>(22) International Filing Date:</b> 9 June 1998 (09.06.98) <b>(30) Priority Data:</b> 60/049,012 9 June 1997 (09.06.97) US <b>(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application</b> US 60/049,012 (CIP) Filed on 9 June 1997 (09.06.97) <b>(71) Applicant (for all designated States except US):</b> GUAVA TECHNOLOGIES, INC. [US/US]; Building 7, Alameda Point, 2500 Saratoga Street, Alameda, CA 94501-5012 (US). <b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only):</b> GOIX, Philippe [FR/US]; 5612 Florence Terrace, Oakland, CA 94611 (US). <b>(74) Agent:</b> SU, Jinntung; Wilson Sonsini Goodrich & Rosati, 650 Page Mill Road, Palo Alto, CA 94304-1050 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> METHOD AND APPARATUS FOR DETECTING MICROPARTICLES IN FLUID SAMPLES <b>(57) Abstract</b> <p>A method and apparatus for detecting a fluorescent substance tagged to a microparticle are described. The device comprises a single capillary flow carrier system for transporting the microparticle past a selected location, a source of electromagnetic radiation for irradiating the substance tagged to the microparticle, and a detection system for measuring fluorescent light emitted from the substance at the selected location. The method comprises transporting the microparticle to a selected location, irradiating a fluorescent substance tagged to the microparticle, and measuring the fluorescent light emitted from the fluorescent substance at the selected location.</p>		

**METHOD AND APPARATUS FOR  
DETECTING MICROPARTICLES IN FLUID SAMPLES**

5 **BACKGROUND OF THE INVENTION**

**Field of the Invention**

The present invention relates to a method and apparatus for detecting microparticles in fluid samples. More particularly, the present invention relates to a method and apparatus which uses a fluid delivery system and laser fluorescence  
10 detection system to detect fluorescently tagged microparticles in low concentrations in fluid samples.

**Description of Related Art**

Detection of microorganisms present at low concentration in fluids is critical  
15 to provide microbiological contamination answers faster to better treat patient diseases, to prevent deadly outbreaks, to better manage quality control processes in food, drink, and drug manufacturing plants, and to provide scientists with powerful and easy to use analytical research tools.

Testing methods for microorganisms such as *M. tuberculosis*, *Trichomonas vaginalis*, *Campylobacter*, *Salmonella*, *E. coli*, and *Cyclospora* include growth culture  
20 methods, PCR methods, fluorescently enhanced microscopic visualizations, ATP bioluminescence techniques, and bactometers. These methods are often slow and expensive, and have limited detection capabilities.

Testing devices include epifluorescent microscopes, fluorometers, and flow  
25 cytometers. Epifluorescent microscopes are coupled with cooled CCD high-resolution cameras to permit epifluorescent microscopic visualizations of microscopic particles. Fluorometers have limited detection capabilities, and is also not well suited when spectral differentiation in a large population of organisms is required. This is often the case when live versus dead organism differentiation is required. Flow



cytometers can be very accurate in detecting and differentiating immuno-fluorescently dead or live labeled particles. However, flow cytometers are expensive and require an experienced technician or scientist to operate it and interpret the data.

5 *Cryptosporidium* oocysts and *Giardia* cysts may be detected using an immunofluorescent assay (IFA) procedure. This method uses polyclonal antibodies to stain the cysts which then can be detected by epifluorescent microscopy. This method is extremely labor-intensive, considering the number of particles to be investigated under the epifluorescent microscope by an experienced technician. Flow cytometers may also be used, but they are very expensive and require an experienced and well-  
10 trained technician to operate. Furthermore, flow cytometers still require microscopy confirmation of oocyst identification.

Water quality monitoring is vital for managing supplies of unpolluted water for agriculture, industry, and human consumption. Water quality monitoring may be performed using test organisms as indicators of freshwater toxicity, for example, the  
15 fathead minnow *Pimephales promelas*, the cladoceran *Ceriodaphnia dubia*, and the green alga *Selenastrum capricornutum*. Test organisms are cultured under standard conditions, and exposed for a period of time to toxicants. Comparison of survival and reproduction rates of test organisms to control organisms provides an indication of water toxicity.

20 Bacteria enzyme activity may be used to assess water quality by using a specially designed enzyme substrate that becomes fluorescent when cleaved. This substrate is cleaved by enzymes in the bacteria and emits fluorescence light when exposed to light of the proper wavelength. The rate of enzyme activity can be measured using a fluorometer, and provides an indirect measurement of the level of  
25 toxicant stress on the bacteria.

Zooplankton feeding behavior may also be used to assess water quality. Extensive acute toxicity studies have been performed using plankton, in general, and various species of rotifer, in particular. Rotifer feeding and reproduction rates can be used as a rapid toxicity assessment tool. The effect of a wide range of chemicals  
30 including xylene, cadmium, copper, mercury, and diazanon on the feeding and reproduction rates of the rotifer *Brachionus calyciflorus* for fresh water and *Brachionus plicatilis* for marine waters has been extensively studied. In the feeding

rate method, the rotifers are exposed for several minutes to water containing a toxicant, and then allowed to feed on fluorescently labeled beads. The rotifers are then anesthetized, washed, transferred to a microscope slide, and individually examined using a fluorescent microscope. The feeding rate is estimated by  
5 quantifying the intensity of fluorescence of ingested beads in the digestive tract of individual rotifers using an imaging technique. This method requires a trained operator, a camera, and a fluorescent microscope, which makes it slow and expensive.

What is needed are methods and apparatus for detecting microparticles such as harmful microorganisms and assessing water quality which is rapid, sensitive,  
10 reproducible, substantially automatic, and cost-effective.

### SUMMARY OF THE INVENTION

The present invention is a device for detecting a fluorescent substance tagged to a microparticle. The device comprises a single capillary flow carrier system for  
15 transporting the microparticle past a selected location, a source of electromagnetic radiation for irradiating the substance tagged to the microparticle, and a detection system for measuring fluorescent light emitted from the substance at the selected location.

### BRIEF DESCRIPTION OF THE FIGURES

20 FIG. 1 shows a sample preparation system.

FIG. 2A shows a flow carrier system.

FIGS. 2B and 2C show detailed views of two embodiments of the capillary tube.

25 FIG. 3A shows one embodiment of a laser fluorescent measurement setup.

FIG. 3B shows another embodiment of a laser fluorescent measurement setup

FIG. 3C shows yet another embodiment of a laser fluorescent measurement setup

FIG. 4A shows a sample output of a digital processing unit.

30 FIG. 4B shows another sample output of a digital processing unit.

FIG. 5A shows the emission spectra for various fluorescent cyanide dyes used to tag microparticles.



FIG. 5B shows a flowchart of a signal processing strategy to detect a Cy5 tagged microparticle in the presence of other fluorescently labeled microparticles.

FIG. 6A shows a calibration curve obtained with water sample seeded with known amount of 2  $\mu\text{m}$  fluospheres using the device of the present invention.

5 FIG. 6B shows a graph of the dependence of the particle concentration on the particle arrival rate distributions (Poisson statistical model).

FIG. 6C shows a graph of the evolution of the integrated detected fluorescence signal versus the SYTO™ 60 *E. coli* concentration.

10 FIG. 7A shows normalized bead concentrations versus feeding time according to an analytical model for three different organism concentrations.

FIG. 7B shows normalized fluosphere concentrations versus feeding time for a test sample containing 1 ppm of diazanon and a control sample, using a rotifer concentration of 500 rotifers/ml.

15 FIG. 7C shows normalized fluosphere concentrations versus feeding time for a test sample containing 1 ppm of diazanon and a control sample, using a rotifer concentration of 600 rotifers/ml.

FIG. 7D shows normalized fluosphere concentrations versus feeding time for a 600 rotifers/ml sample and an 1000 rotifers/ml sample.

DETAILED DESCRIPTION OF THE INVENTION

FIGURE 1 shows a sample preparation system 100. Sample preparation system 100 contains a fluid sample 102 suspected of containing microparticles 104. Fluid sample 102 may be run through a filter or trap to separate out any unwanted or macroscopic particulate matter. In order to prepare a sample to be tested, a fluorescent substance 106 is allowed to react with fluid sample 102 and microparticles 104. Fluorescent substance 106 may be one or more fluorescent stains, dyes, or reagents designed to stain, tag, or otherwise attach themselves to microparticles 104. A test sample 108 is obtained by filtering out any free remaining fluorescent substance 106 from fluid sample 102. Test sample 108 thus contains fluid sample 102 and fluorescent substance 106 attached to microparticles 104.

Fluid sample 102 may be a water sample, urine sample, blood sample, food product sample, or any other fluid to be tested. Fluid sample 102 may contain PCR-amplified samples for detection of viruses such as HIV. Microparticles 104 may be *M. tuberculosis*, *Trichomonas vaginalis*, *Campylobacter*, *Salmonella*, *E. coli*, *Cyclospora*, *Cryptosporidium* oocysts, *Giardia* cysts, or any other bacterium, virus, fungus, or microorganism that is capable of being tagged. Microparticles 104 may also be CD4 or CD8 cells for monitoring of AIDS patients. Different fluorescent substances 106 may be used with microparticles 104 to allow different types of microorganisms to be detected and distinguished from each other. For example, for bacteria, fluorescent substance 106 can be standard DNA or surface-label-reagent stains. For *Cryptosporidium* oocysts or *Giardia* cysts, fluorescent substance 106 can be a fluorescent dye conjugated with anti-*Cryptosporidium* or anti-*Giardia* antibodies, respectively. Fluorescent substance 106 may also be magnetically charged so that it can be affected by a magnetic field.

Microparticles 104 may also be microscopic beads containing a fluorescent substance 106. To measure the concentration of a toxicant in fluid sample 102, filter-feeder microorganisms such as rotifers or zooplankton may be added to fluid sample 102 in known quantities. Such filter-feeder microorganisms have a feeding rate which is a well-known function of toxicant concentration. After a known incubation period, microparticles 104 are added to fluid sample 102. Microparticles 104 may be fluospheres capable of being ingested by the filter-feeder organisms, such as latex



beads containing a fluorescent dye available from Molecular Probes, Inc., Eugene, Oregon. The fluospheres may have a uniform diameter of 2  $\mu\text{m}$  or have non-uniform sizes. They may have uniform spectro-photometric properties, with a maximum absorption wavelength of 624 nm, and a maximum emission wavelength of 645 nm, or have varying spectro-photometric properties. At known intervals of time, a test sample 108 is drawn from sample preparation system 100. Test sample 108 is obtained by filtering out any uningested microparticles 104 from fluid sample 102. Test sample 108 thus contains water sample 102 and organisms 104 with microparticles 104 in their digestive tracts.

FIGURE 2A shows a flow carrier system 200. Flow carrier system 200 is a fluid delivery system which introduces test sample 108 into a capillary tube 202. Capillary tube 202 may have very thin walls and excellent optical properties. Capillary tube 202 may have an internal diameter configured to admit microparticles 104 one at a time. A section of capillary tube 202 defines a test volume 204. Capillary tube 202 may be at least partially coupled to an optical table 206, which serves to hold capillary tube 202 in place. Multiple capillary tubes 202 may be arranged in parallel to obtain higher throughputs.

Flow carrier system 200 may include a pump system 216 coupled to capillary tube 202. Pump system 216 may be a syringe 208 which contains test sample 108, and injects test sample 108 through capillary tube 202 and test volume 204. After passing through capillary tube 202, sample 108 may pass into a dump 212. In this manner, microparticles 104 in test sample 102 may be passed one at a time through capillary tube 202. Pump system 216 may further include a syringe pump 214 coupled to syringe 208. Syringe pump 214 is configured for precise control of flow of test sample 108 through capillary tube 202. Pump system 216 may also be a peristaltic pump.

FIGURES 2B and 2C show detailed views of two embodiments of capillary tube 202. FIGURE 2B shows microparticles 104 flowing through capillary tube 202 towards test volume 204. FIGURE 2C shows capillary tube 202 with a magnetic element 220 positioned in a concentric fashion around capillary tube 202. Magnetic element 220 may be a continuous ring, or be comprised of one or more separate elements. Magnetic element 220 may be used in conjunction with microparticles 104



and fluorescent substance 106 which are magnetically charged. This configuration may assist in substantially focusing microparticles 104 tagged with fluorescent substance 106 to the center of capillary tube 202 as they flow through test volume 204, thus improving detection of microparticles 104.

5           FIGURE 3A shows a laser fluorescent measurement setup 300. A laser 302 generates a laser beam 304. Laser beam 304 may be focused through one or more lenses 306 onto test volume 204. The wavelength and beam size of laser 302 is selected according to the absorption wavelength of fluorescent substance 106 and the size of capillary tube 202.

10           When test sample 108 is passed through test volume 204, any fluorescent substance 106 present in test sample 108 is exposed to laser beam 302. Alternatively, a standing test sample 108 in capillary tube 202 may be moved relative to laser beam 302 to expose test sample 108. A collecting lens 310 collects and images fluorescence light emitted by fluorescent substance 106 onto a photo-multiplier 312. A set of interference filters 314 may be placed in front of photo-multiplier 312 to filter  
15 out the resonant light from the fluorescence light. A photodiode 316 may be placed on the opposite side of test chamber 308 to collect the resonant light. Output from photo-multiplier 312 may be sent to a first digital processing unit 318 to analyze fluorescence peaks. Output from photodiode 316 may be sent to a second digital  
20 processing unit 320 to analyze Mie scattering peaks.

          FIGURE 3B shows another laser fluorescent measurement setup 300. In this case, the fluorescence emissions pass through a diffraction grating 313 and are imaged onto a multiple detector array 315. The focal length and aperture of collecting lens 310, the dispersion characteristics of grating 313, and the size and separation of the  
25 multiple detectors in array 315 are optimized to detect at least two or three fluorescent emission bands specific to the emission spectrum of fluorescent substance 106 as well as resonant light. A set of interference filters 317 may be used to single out the fluorescence emission of fluorescent substance 106 used to tag microparticles 104. By reading the fluorescence emission at multiple spectral locations using multiple  
30 interferential filters 317 with specific transmission characteristics, the particular fluorescent substance 106 used can be detected and distinguished. The contribution of the total fluorescence signal to each detector will provide the data needed to



differentiate the particular fluorescent substance 106 from the fluorescence emissions of non-tagged particles. Output from multiple detector array 315 may be fed to a digital processing unit 318, which processes and digitizes the multiple signals delivered by multiple detector array 315.

5           FIGURE 3C shows another embodiment of a laser fluorescent measurement setup 300. A plurality of lasers 302 generates a plurality of laser beams 304. Laser beams 304 may be focused through one or more lenses 306 onto test volume 204. The size of laser beam 304 may be matched to the size of capillary tube 202. The wavelengths of lasers 302 are tuned to specific absorption bands of fluorescent  
10           substance 106. This multiple laser and detection system may assist in reducing false positive and negative results associated with a single laser system.

          FIGURE 4A shows a sample output from digital processing unit 318. The voltage signal coming out of photo-multiplier 312 is digitized and transferred to a computer where it can be manipulated and analyzed. The voltage signal may be  
15           digitized at a frequency of up to 3000 Hz with 8-bit precision.

          Every time fluorescent substance 106 passes through test volume 204, a fluorescence peak 402 is created. A threshold value 404 may be selected according to the baseline signal level and its variance. The number of fluorescence peaks 402 detected above threshold value 404, along with the size of voltage spikes, give a  
20           measurement of the amount of fluorescent substance 106. In the case of toxicant concentration, comparing this data with the data for an uncontaminated control sample permits determination of the toxicant concentration in water sample 102.

          FIGURE 4B shows another sample output from digital processing unit 318. When an microparticle 104 which has been tagged by fluorescent substance 106  
25           passes through test volume 204, the it generates a burst of fluorescence light with a time signature 406 and spectral signature 408. The time signature 406 and spectral signature 408 is then processed by the digital signal processing unit 318 and compared with the expected time and spectral signatures of microparticle 104 and fluorescent substance 106 to be detected.

30           Because a wide range of particles and organisms naturally fluoresce at a wide range of wavelengths, it is crucial to spectrally differentiate an microparticle to reliably detect it. Multiple laser sources and detectors may be used in close



wavelength proximity to spectrally pinpoint the fluorescence pattern of the dye attached to the microparticle targeted for detection. The electronic signal analysis techniques can be tailored to the understanding of the pre-defined shape and spectral properties of the target microparticle prior to detection.

5           The use of multiple signals provides normalization and improved selectivity. Measurements at more than one fluorescence emission wavelength and/or at more than one excitation wavelength give spectral selectivity which can distinguish different dye sources. Because the dyes used for immunofluorescence commonly have relatively narrow emission peaks compared to background fluorescence sources,  
10 ratios of on-peak to off-peak signals may reliably distinguish dye-labeled particles from background events of similar absolute fluorescence.

Background particle signals are rejected through the use of electronic filtering, thereby allowing a sustained and very high sampling data rate. Electronic filtering involves the use of several detectors and is made possible by the uniqueness of a  
15 particle's light scattering signature and the presence of at least two fluorescent wavelength signatures. Based on the differential signal analysis of each of the detected log amplified signals, the capability of singling out the tagged microparticle at a data frequency rate of up to 50 kHz can be achieved.

FIGURE 5A shows emission spectra of various fluorescent cyanide dyes  
20 which may be used to tag microparticles 104: Cy5, Cy5.5, and Cy7, with maximum absorption peaks of 650 nm, 675 nm, and 743 nm, respectively. Multiple detector array 318 may be used to first record the fluorescent spectrum of the particular dye. The fluorescent spectra emitted by the tagged microparticles 104 in the sample is then compared to the recorded fluorescent spectrum of the dye. In this fashion, tagged  
25 microparticles 104 can be identified and distinguished from other fluorescently tagged microparticles 104. In addition, because the flow rate is controlled, the width of the trace signal can be considered proportional to the diameter of microparticle 104 crossing test volume 204.

FIGURE 5B shows a flowchart of a signal processing strategy to detect a Cy5  
30 tagged microparticle 104 in the presence of other fluorescently labeled microparticles. Four detectors are used. Detector D1 is centered on the resonant laser excitation, in

this case 635 nm. Detectors D2, D3, and D4 are centered at 650 nm, 670 nm, and 690 nm, corresponding to features of the Cy5 fluorescence emission.

When a microparticle 104 is detected in test volume 204, first, the time trace of the signal detected by detector D1 is analyzed (block 502) and the signal intensity S1 and the pulse width W1 are compared with the expected time trace (Sc, Wc) generated by the passage of the particular microparticle in the test volume (block 504). If the detected signal does not meet this criteria, then the data is rejected (block 506). If the detected signal passes this first test, then the fluorescence intensity ratio of the detectors D2, D3, and D4 are analyzed (block 508). The fluorescent ratio of S2/S3 and S2/S4 are compared with the expected fluorescence ratios corresponding to the Cy5 fluorescence spectra Sca and Scb, respectively (block 510). If these two tests are positive, a microparticle is counted (block 512); if the tests are negative the data is rejected (block 514).

#### EXAMPLE 1

Flow carrier system 100 was calibrated using water samples with known fluosphere concentrations. A reference solution of  $3 \times 10^9$  beads/ml was diluted 1000 times. Then water samples containing 0, 3, 500, 7000, 14000 and 28000 beads/ml were prepared with a 10% confidence interval using a 20  $\mu$ l micropipet. These water samples were passed through the device. FIGURE 6A shows detected fluorescence peak counts versus expected counts for the calibration samples. An excellent correlation was consistently obtained.

A reference sample of  $10^7$  *E. coli* SYTO™ 60 DNA-stained was prepared by first killing the bacteria using a 70% isopropanol exposure for one hour and then following with three sterile washes. The *E. coli* bacteria population was then stained with a 5  $\mu$ mol concentration SYTO™ 60 dye. The spectral characteristics of the SYTO™ 60 dye (Abs = 650 nm, Em = 678 nm) are very well suited for the laser-based system of the present invention.

Five graded concentration samples from  $10^7$  to 0 *E. coli* per ml stained were prepared using a 20  $\mu$ l micropipette and 2  $\mu$ m filtered de-ionized water. A 100  $\mu$ l solution of each sample was drawn using a 1 ml syringe. The syringe was placed onto a syringe pump, and a 10  $\mu$ l/min flow rate of the solution was injected into a 70  $\mu$ m



single capillary towards the test volume. The fluorescent test volume was defined by a 20  $\mu\text{m}$  focused laser beam using a 635 nm, 5 mW laser diode and a single 10 mm focal lens. The test volume was imaged onto a 3 mm x 3 mm slit using a 40x objective microscope. The photodetector signal was digitized at 3000 Hz and 8 byte dynamic range. The digitized signal was transmitted through a single serial cable onto a laptop computer. The signal was displayed on-line on a window screen using proprietary software. A time series corresponding to an injection of each sample at 10  $\mu\text{l/min}$  flow rate during 30 sec was recorded. A triplicate experiment was performed for each sample, which corresponds to a 90 sec injection. By controlling the flow rate, the injection time, and the expected concentration, an expected fluorescent peak count was calculated and compared with the actual measured count. For each sample, the average peak residence time, peak intensity, and peak power (peak integral) were also computed.

The arrival process of the particle across the test volume was assumed to follow a random arrival process and therefore follow a Poisson process. The expected count number was corrected accordingly, to take into account the probability of having more than one particle arriving into the test volume during a time window equivalent to the particle transit time across the laser beam, taking into account the fact that a single detected count could be attributed to more than one particle.

FIGURE 6B represents the particle arrival rate distribution at the test volume for the organism concentration investigated. For concentrations greater than  $10^5$  p/ml at a 10  $\mu\text{l/min}$  flow rate, a Poisson statistical correction is necessary. In particular, at a concentration of  $10^7$  *E. coli* per ml, there is more than one *E. coli* crossing the test volume 80% of the time.

FIGURE 6C represents a correlation between the expected concentration and the integrated detected fluorescence signal corresponding to the passage of individual stained *E. coli* through the laser test volume. The integrated signal was computed as the product of the number of detected peaks corrected by Poisson statistics, and the average peak power (V/ms). The correlation is excellent, with a 98% slope.

However, when there was no *E. coli* present in the sample, an integrated background noise of 4 peaks every 30 seconds was detected. These background peaks were attributed to bubbles deflecting the beam reflection into the photo-detector slit or to

naturally fluorescent particles. The use of multiple wavelength detection arrays and a light scattering detector may eliminate these false positive counts.

### EXAMPLE 2

5 Water quality monitoring using rotifers was performed using a capillary tube with an internal diameter of 70  $\mu\text{m}$ , a narrow band, 635 nm, 3 mW diode laser with a beam diameter of 40  $\mu\text{m}$  was used. Interference filters were selected to transmit 12% at 670 nm (20 nm FWM), and  $10^{-6}$  at all other wavelengths.

#### 10 Method

1. A live *B. plicatilis* rotifer culture was obtained from Aqua-Farms, Florida. These rotifers were chosen because they are easy to raise, and the influence of toxic samples on their feeding, reproduction, and death rates have been studied extensively. The average concentration of rotifers in a 100 ml vial was  
15 counted using five 20  $\mu\text{l}$  samples examined under a 50x microscope. An average count of 10 rotifers per 20  $\mu\text{l}$  sample was measured, or about 500 rotifers/ml.
2. Two samples of 8 ml each were used to make the feeding rate measurements, a reference sample and test sample. These two vials were filled with the 500  
20 rotifers/ml reference solution.
3. A 2000 ppm diazanon solution was prepared using the rotifer medium solution, so as to maintain water quality parameters such as pH,  $\text{O}_2$ , alkalinity, salinity, and temperature as constant as possible. A 20  $\mu\text{l}$  amount of the diazanon solution was added to test sample.
- 25 4. After a 5 minute incubation, 20  $\mu\text{l}$  of a  $40 \times 10^6$  beads/ml solution of crimson fluospheres was added to both the reference and test samples. The time was noted as  $t = 0$ .
5. Using two identical syringes connected with a luer union to a 100  $\mu\text{m}$  piece of nylon tubing terminated with a 20  $\mu\text{m}$  plankton filter, a 50  $\mu\text{l}$  sample was  
30 extracted from the reference and test samples. In both cases, the organisms were filtered out from the bead solution.



6. The reference and test samples were successively passed through the test chamber using the syringe pump at a flow rate of 15  $\mu\text{l}/\text{min}$ . The data from data acquisition were stored for later analysis.
7. Steps 5 and 6 were repeated at  $t = 5, 10, 15,$  and 20 minutes.

5

### Data Analysis

Each data file was retrieved, using TOXANA™, a time series data analysis software program. This program allows visualization of the digitized photo-multiplier trace signal on a 0-5 V scale for each data file. An assessment of the signal baseline mean and variance values (where no peak is detected) was made for each file. From this measurement, a peak detection threshold,  $T_d$ , was computed as follows:

10

$$T_d = \text{mean} + 2\sqrt{\text{variance}}$$

The number of peaks with an intensity above  $T_d$  were calculated for each file, as well as the average peak intensity, the average peak width, and the average peak area.

15

### Analytical Model

The clearance volume  $V_{cl}$  for an organism with an average motility  $\Omega$  and clearance diameter  $d$  can be assumed to be:

$$\text{(Eq. 1)} \quad V_{cl} = \Omega \cdot \pi \cdot \frac{d^2}{4}$$

20

The number of beads present in the clearance volume per unit of time is equal to:

$$\text{(Eq. 2)} \quad \frac{dN_b}{dt} = C_b(t) \cdot V_{cl}$$

Assume that a volume  $V$  contains organisms with an average motility  $\Omega$ . In this volume the concentration of food particles or beads is  $C_b(t)$ .

The change in bead concentration varies per unit of time:

$$(Eq. 3) \quad \frac{dC_b(t)}{dt} = -\frac{dN_b(t)}{dt} \cdot \frac{N_0}{V}$$

By substituting (1) and (2) into (3), the rate at which the bead concentration varies with time is governed by the differential equation:

$$(Eq. 4) \quad \frac{d(C_b(t))}{dt} = -C_b(t) \cdot \pi \cdot \frac{d^2}{4} \cdot \Omega \cdot \frac{N_0}{4}$$

Define the constant K as:

$$(Eq. 5) \quad K = \pi \cdot \frac{d^2}{4} \cdot \Omega \cdot \frac{N_0}{V}$$

Then 1/K is a time constant which reflects the rate at which the bead concentration decreases. Then (5) becomes:

$$(Eq. 6) \quad \frac{d(C_b(t))}{dt} = -K \cdot C_b(t)$$

Integrating (6) gives:

$$(Eq. 7) \quad C_b(t) = \lambda \cdot e^{-Kt}$$

When  $t = 0$  and  $C_N(t = 0) = C_{B0}$ , (7) becomes:

$$(Eq. 8) \quad C_b(t) = C_{B0} \cdot e^{-Kt}$$

The feeding rate is defined as the number of beads ingested per organism and per unit of time. It can be expressed by:

$$(Eq. 9) \quad FR = \frac{dN_b}{dt} \cdot \frac{1}{N_0} = \frac{d(C_b)}{dt} \cdot \frac{V}{N_0}$$

Combining (8) and (9), F can be expressed as follows:

$$(Eq. 10) \quad FR = K \cdot C_b(t) \cdot \frac{V}{N_0}$$



Assuming that  $d(C_b)/dt$  is small compared with  $C_b(t)$ ,  $F$  becomes a constant which can be expressed as:

$$(Eq. 11) \quad FR = K \cdot C_b \cdot \frac{V}{N_0}$$

FIGURE 7A shows normalized bead concentrations versus feeding time according to the model given in Eq. 8 for three different organism concentrations: 100 rotifers/ml, 250 rotifers/ml, and 1000 rotifers/ml. The analytical value used to model the feeding rate (FR) in this case is 2.7 beads/min.

This value was computed based on the following assumptions: the organism is a rotifer and its clearance rate is proportional to a 150  $\mu$ m diameter section with a motility of 15 cm/s. It is important to note that the sensitivity of the technique strongly depends strongly on the organism concentration.

### Results

FIGURE 7B shows normalized fluosphere concentrations versus feeding time for a test sample containing 1 ppm of diazanon and a control sample. To compare the experimental results to the model, a concentration of 500 rotifers/ml was used.

For the control sample the correlation between model and experiment is excellent for the first 600 seconds. In this region, the average feeding rate is  $29 \times 10^{-3}$  beads/sec. The departure of experiment from the model after 900 seconds can be attributed to two factors. First, the model assumes that the variation of  $C_b$  remains small compared to  $C_b$  and therefore the feeding rate is a constant. In fact, the feeding rate depends on the food concentration, which after 900 seconds has dropped by 50%. This variation cannot be neglected. Second, the rotifers have an average digestion transit time of 1200 seconds. Since the fluospheres are not metabolized by the rotifers, they are ejected back into the sample by the rotifer after 1000 seconds, which may contribute to an increase in bead concentration.

For the test sample the concentration of fluospheres decreases slightly with a slope corresponding to an ingestion rate per organism of  $10^{-3}$  beads/sec. Here, the feeding rate was reduced by a factor of 30 from exposure to 1 ppm of diazanon.

FIGURE 7C shows normalized fluosphere concentrations versus feeding time for a test sample containing 1 ppm of diazanon and a control sample. The concentration of organisms is now 600 rotifers/ml.

Again, for the control sample, the agreement between model and experiment is excellent for the first 600 seconds. The feeding rate is now 3 beads/min per organism, compared well to the 1.7 beads/min per organism value obtained earlier. For the test sample, the concentration of beads remains almost unchanged with time and indicates a feeding rate of less than 0.05 beads/min. This measurement is consistent with previous experiments.

FIGURE 7D shows normalized fluosphere concentrations versus feeding time for a 600 rotifers/ml sample and an 1000 rotifers/ml sample. The two samples are exposed to concentrated 2  $\mu$ m fluospheres for 1700 seconds. The fluosphere concentrations are monitored continuously and the normalized concentrations are reported and compared to the clearance rate model described earlier. The agreement between model and experiment is optimum for an average feeding rate per organism equal to 4.8 beads/min.

To express feeding rate in terms of mass, the following equation may be used:

(Eq. 12) 
$$\dot{M} = FR \cdot \rho \cdot V_{flu}$$

where FR,  $\rho$ , and  $V_{flu}$  are the average feeding rate per individual rotifer, the fluosphere density, and the individual fluosphere volume, respectively. Here, for a feeding rate of 4.8 beads/min and spherical fluospheres with a density of 1.055 g/ml and a diameter of 2  $\mu$ m,  $\dot{M} = 21 \times 10^{-12}$  g/min per organism.

\* \* \* \*

The foregoing description of the invention has been presented for purposes of illustration and description. It is not intended to be exhaustive or to limit the invention to the precise forms disclosed. Obviously, many modifications and variations will be apparent to practitioners skilled in this art. It is intended that the scope of the invention be defined by the following claims and their equivalents.

What is claimed is:



## CLAIMS

1. A device for detecting a microparticle in a fluid, the microparticle being tagged with a fluorescent substance, the fluorescent substance emitting fluorescent light when exposed to electromagnetic radiation, the device comprising:
  - a sample tube;
  - a fluid delivery system coupled to the sample tube, the fluid delivery system capable of causing the microparticle and the fluid to flow through the sample tube;
  - a source of electromagnetic radiation for projecting a beam of electromagnetic radiation through sample tube to expose the fluorescent substance to electromagnetic radiation in a volume partially defined by the beam size; and
  - a detection device configured to detect fluorescent light emitted from the fluorescent substance when the microparticle flows through the volume.
2. The device of claim 1, wherein the microparticle is a microorganism, and the sample tube, the fluid delivery system, the source of electromagnetic radiation, and the detection device are adapted to detect the microorganism.
3. The device of claim 1, wherein the microparticle is a bacterium, virus, or parasite, and the sample tube, the fluid delivery system, the source of electromagnetic radiation, and the detection device are adapted to detect the bacterium, virus, or parasite.
4. The device of claim 1, wherein the microparticle is a CD4 cell, and the sample tube, the fluid delivery system, the source of electromagnetic radiation, and the detection device are adapted to detect the CD4 cell.
5. The device of claim 1, wherein the microparticle is a fluosphere, and the sample tube, the fluid delivery system, the source of electromagnetic radiation, and the detection device are adapted to detect the fluosphere.
6. The device of claim 5, wherein the fluosphere has been ingested by a filter-feeder.

7. The device of claim 6, wherein the filtro-feeder has a feeding rate sensitive to a toxicant level in the fluid sample.
8. The device of claim 1, wherein the fluorescent substance is a dye-conjugated antibody.
9. The device of claim 1, wherein the fluorescent substance is a DNA stain.
10. The device of claim 1, wherein the fluorescent substance has a magnetic charge.
11. The device of claim 10, further comprising:  
a magnetic element positioned in a surrounding relationship to the capillary, the magnetic element having a magnetic charge which repels the fluorescent substance.
12. The device of claim 1, wherein the fluid delivery system is a syringe coupled to a syringe pump.
13. The device of claim 1, wherein the fluid delivery system is a peristaltic pump.
14. The device of claim 1, wherein the source of electromagnetic radiation is at least one laser.
15. The device of claim 1, wherein the detection device is an array of detectors.
16. A device for detecting a fluorescent substance tagged to a microparticle, comprising:  
a single capillary flow carrier system for transporting the microparticle past a selected location;  
a source of electromagnetic radiation for irradiating the substance tagged to the microparticle; and  
a detection system for measuring fluorescent light emitted from the substance at the selected location.



17. The device of claim 16, wherein the source of electromagnetic radiation comprises a source of light.
18. The device of claim 17, wherein the source of light comprises a laser.
19. The device of claim 16, wherein a plurality of microparticles are individually transported past the selected location at a substantially uniform velocity.
20. The device of claim 16, wherein the microparticle is an organism, and the sample tube, the fluid delivery system, the source of electromagnetic radiation, and the detection device are adapted to detect the organism.
21. The device of claim 16, wherein the microparticle is a fluosphere, and the sample tube, the fluid delivery system, the source of electromagnetic radiation, and the detection device are adapted to detect the fluosphere.
22. The device of claim 21, wherein the fluosphere is ingested by a filtro-feeder.
23. The device of claim 22, further comprising a device for exposing the filtro-feeder to a toxic substance.
24. The device of claim 23, wherein the fluorescent substance is ingested by the filtro-feeder, and exposure of the filtro-feeder to the toxic substance affects the rate of ingestion of the fluorescent substance by the filtro-feeder.
25. The device of claim 24, further comprising means for calculating the ingestion rate as a function of the amount of fluorescent light emitted from the fluorescent substance at the selected location.
26. The device of claim 16, wherein the fluorescent substance has a magnetic charge.
27. The device of claim 26, further comprising:
  - a magnetic element positioned in a surrounding relationship adjacent to the selected location, the magnetic element having a magnetic charge which repels the fluorescent substance.

28. A method for detecting a microparticle tagged with a fluorescent substance, comprising:
- transporting the microparticle to a selected location in a sample tube;
  - irradiating the fluorescent substance tagged to the microparticle; and
  - measuring fluorescent light emitted from the fluorescent substance at the selected location.
29. The method of claim 28, wherein the microparticle is a bacterium, and the sample tube, the fluid delivery system, the source of electromagnetic radiation, and the detection device are adapted to detect the bacterium.
30. The method of claim 28, wherein the microparticle is a virus, and the sample tube, the fluid delivery system, the source of electromagnetic radiation, and the detection device are adapted to detect the virus.
31. The method of claim 28, wherein the microparticle is a parasitic cyst, and the sample tube, the fluid delivery system, the source of electromagnetic radiation, and the detection device are adapted to detect the parasitic cyst.
32. The method of claim 28, wherein the microparticle is a CD4 cell, and the sample tube, the fluid delivery system, the source of electromagnetic radiation, and the detection device are adapted to detect the CD4 cell.
33. The method of claim 28, wherein the microparticle is a fluosphere, and the sample tube, the fluid delivery system, the source of electromagnetic radiation, and the detection device are adapted to detect the fluosphere.
34. The method of claim 33, wherein the fluosphere has been ingested by a filter-feeder.
35. The method of claim 34, wherein the filter-feeder has a feeding rate sensitive to a toxicant level in the fluid sample.
36. The method of claim 28, wherein the fluorescent substance is a dye-conjugated antibody.



37. The method of claim 28, wherein the fluorescent substance has a magnetic charge.

38. A method for detecting microparticles in a fluid tagged with a fluorescent substance, comprising:

causing the microparticles to flow through a test volume in a sample tube substantially one at a time;

irradiating the fluorescent substance tagged to the microparticles passing through the test volume; and

detecting fluorescent light emitted from the fluorescent substance in the test volume.

39. The method of claim 38 wherein the microparticles are caused to flow through the test volume substantially one at a time by controlling a concentration of the microparticles in the fluid.

40. A device for detecting microparticles in a fluid, the microparticles being tagged with a fluorescent substance that emits fluorescent light when exposed to electromagnetic radiation, the device comprising:

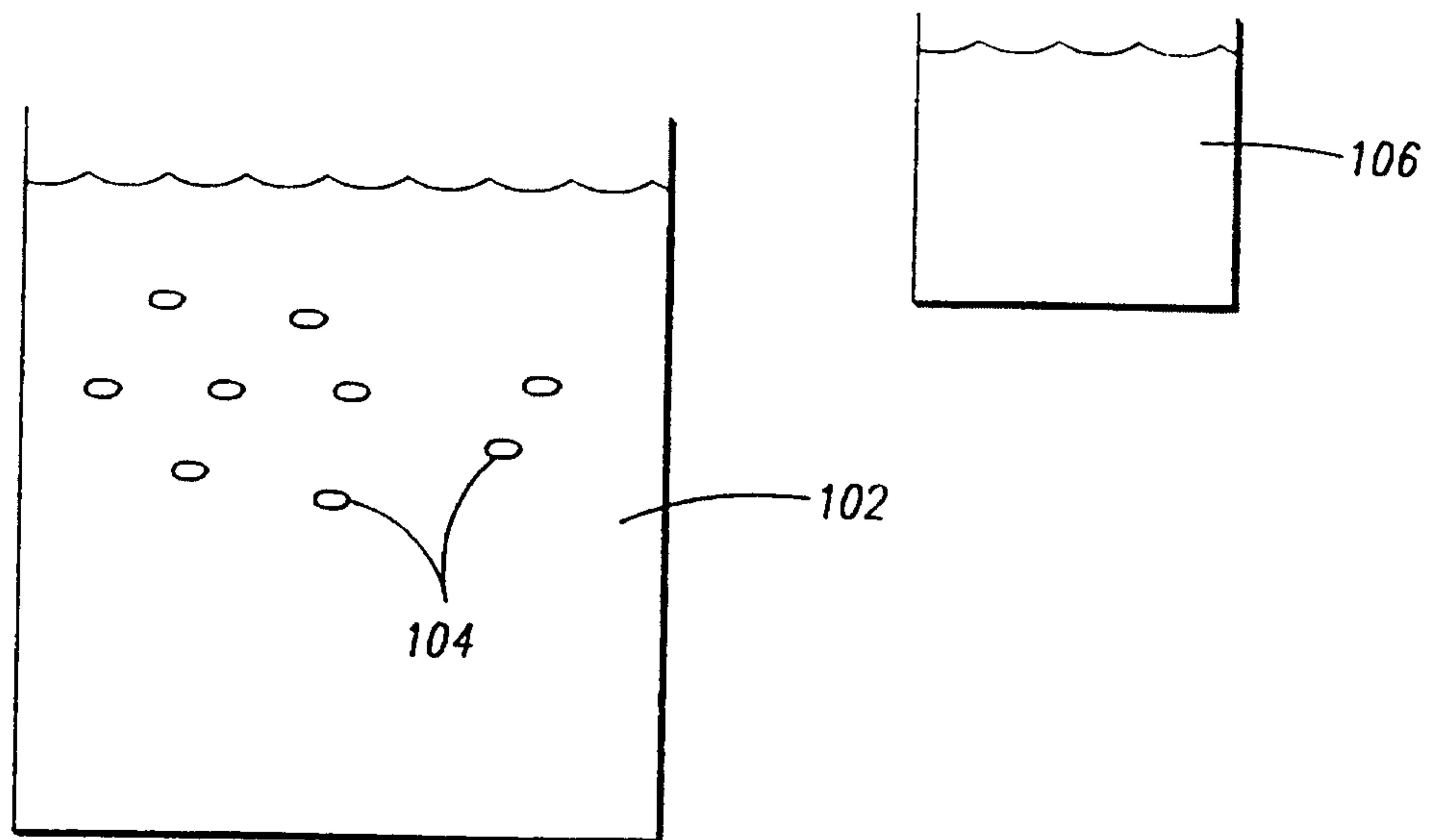
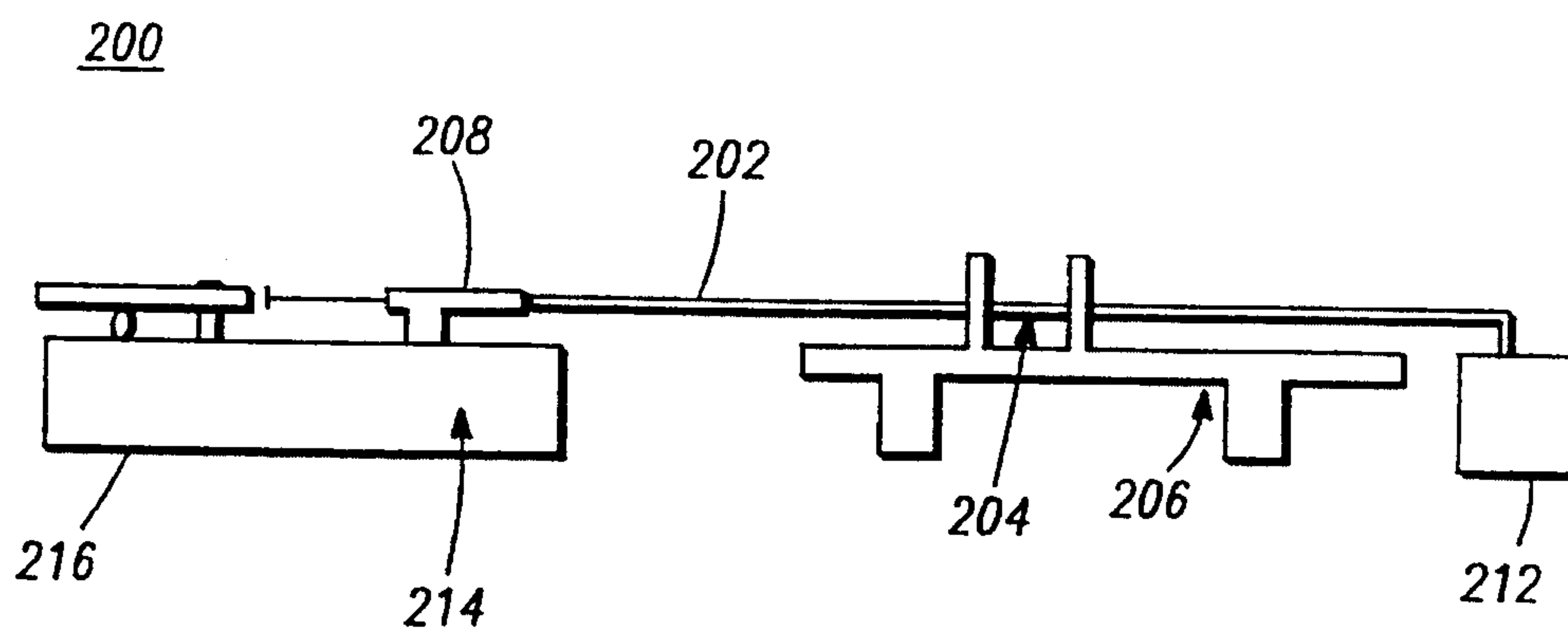
a sample tube;

a source of electromagnetic radiation for projecting a beam of electromagnetic radiation to the sample tube to form a test volume partially defined by the beam size;

a fluid delivery system coupled to the sample tube causing the microparticles to flow through the test volume substantially one at a time; and

a detection device configured to detect fluorescent light emitted from the fluorescent substance when the microparticles are exposed to the electromagnetic radiation in the test volume.

41. The device of claim 40 wherein the fluid delivery system comprises at least a pump for pumping the fluid and microparticles.

100*FIG. - 1**FIG. - 2A*



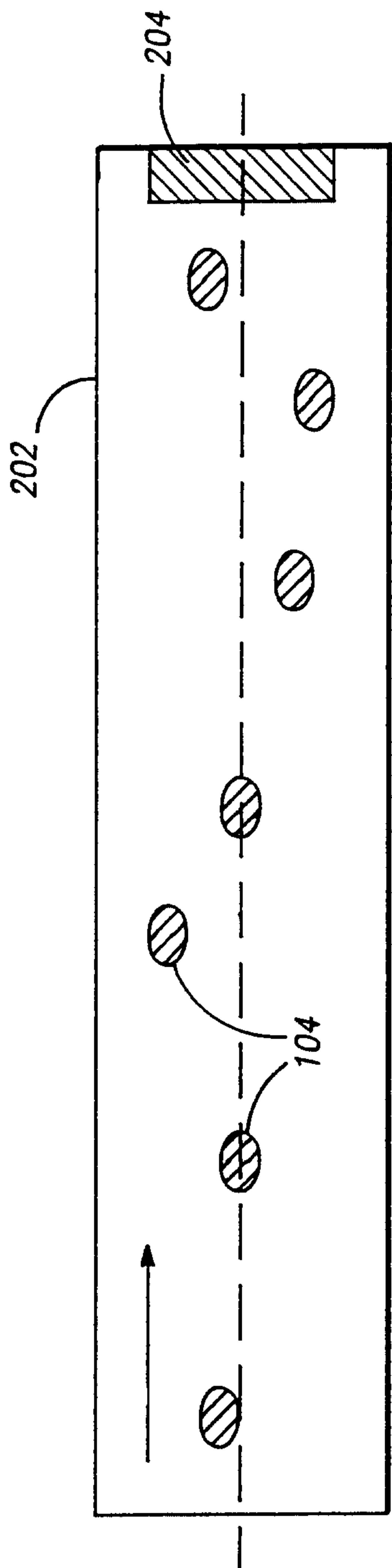


FIG. -2B

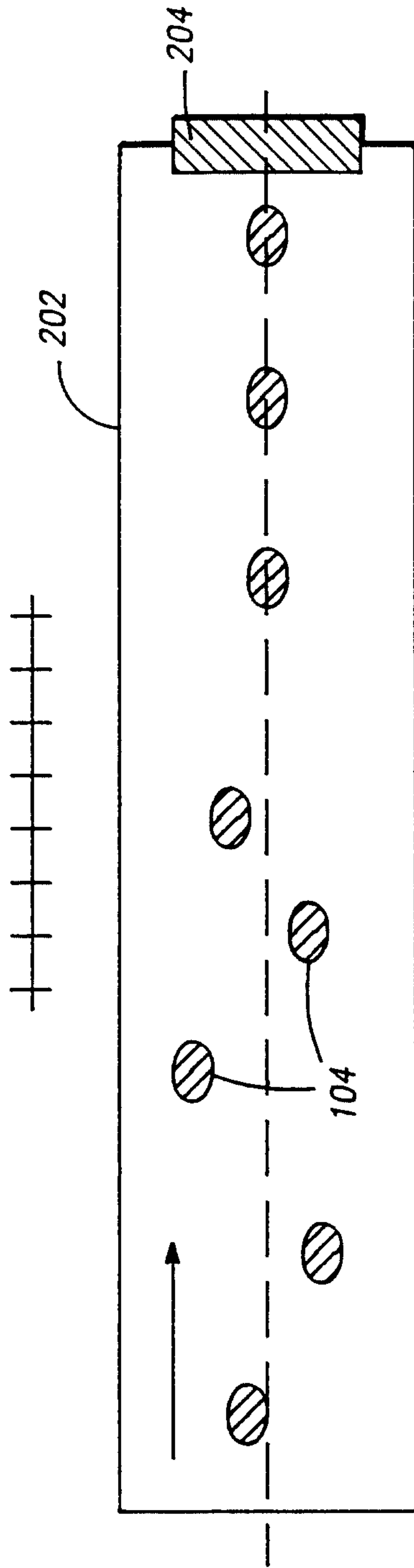


FIG. -2C

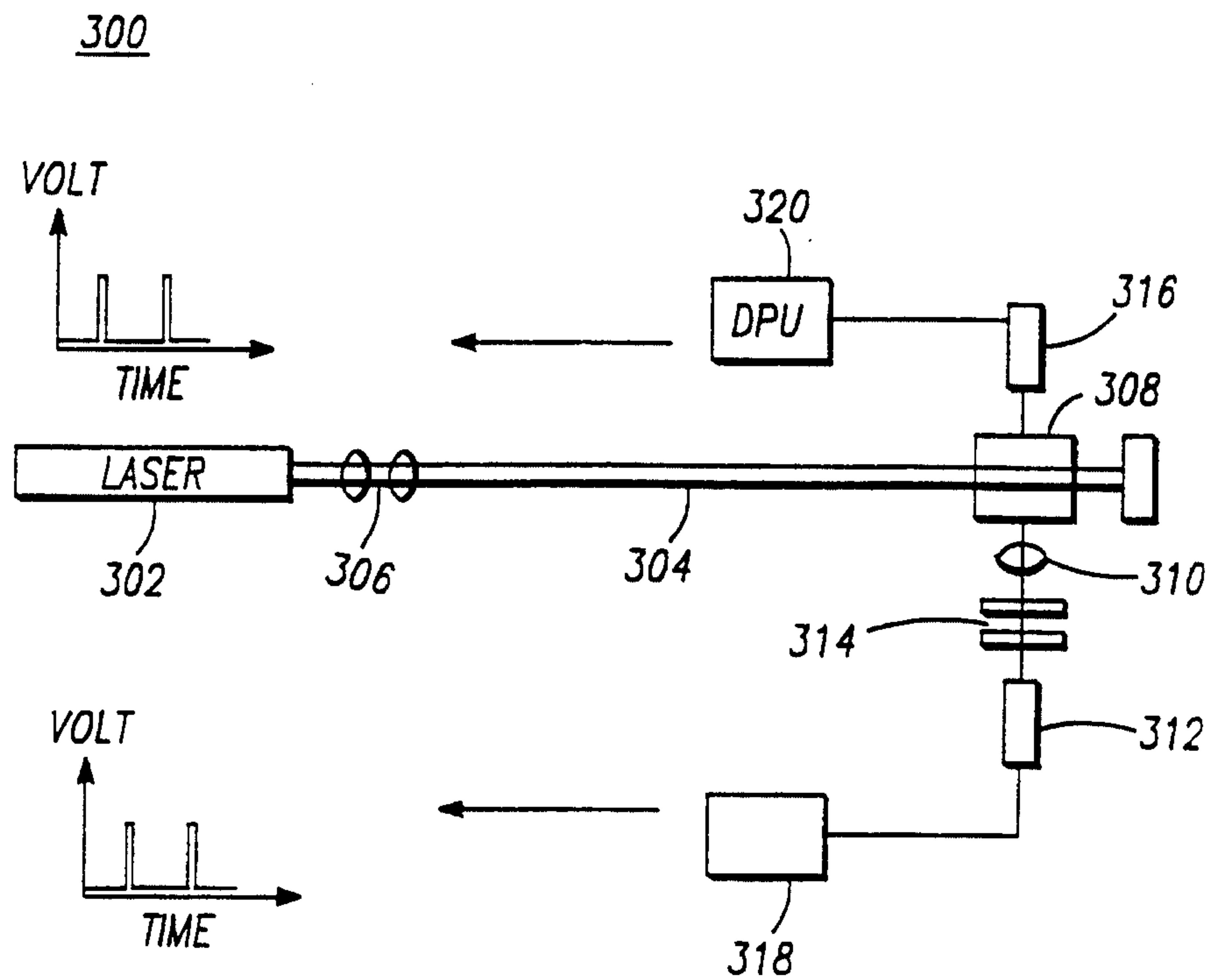


FIG. - 3A

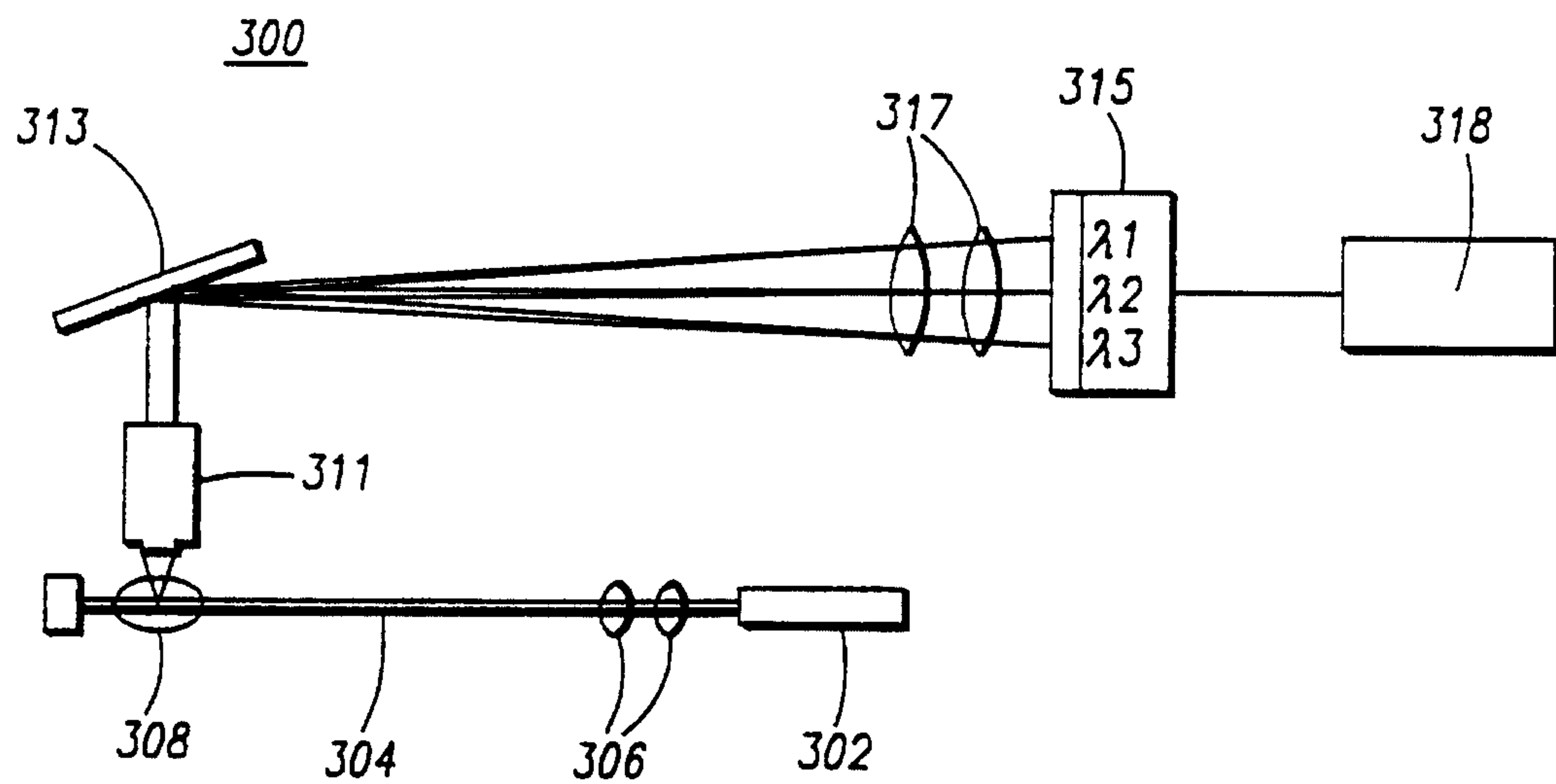
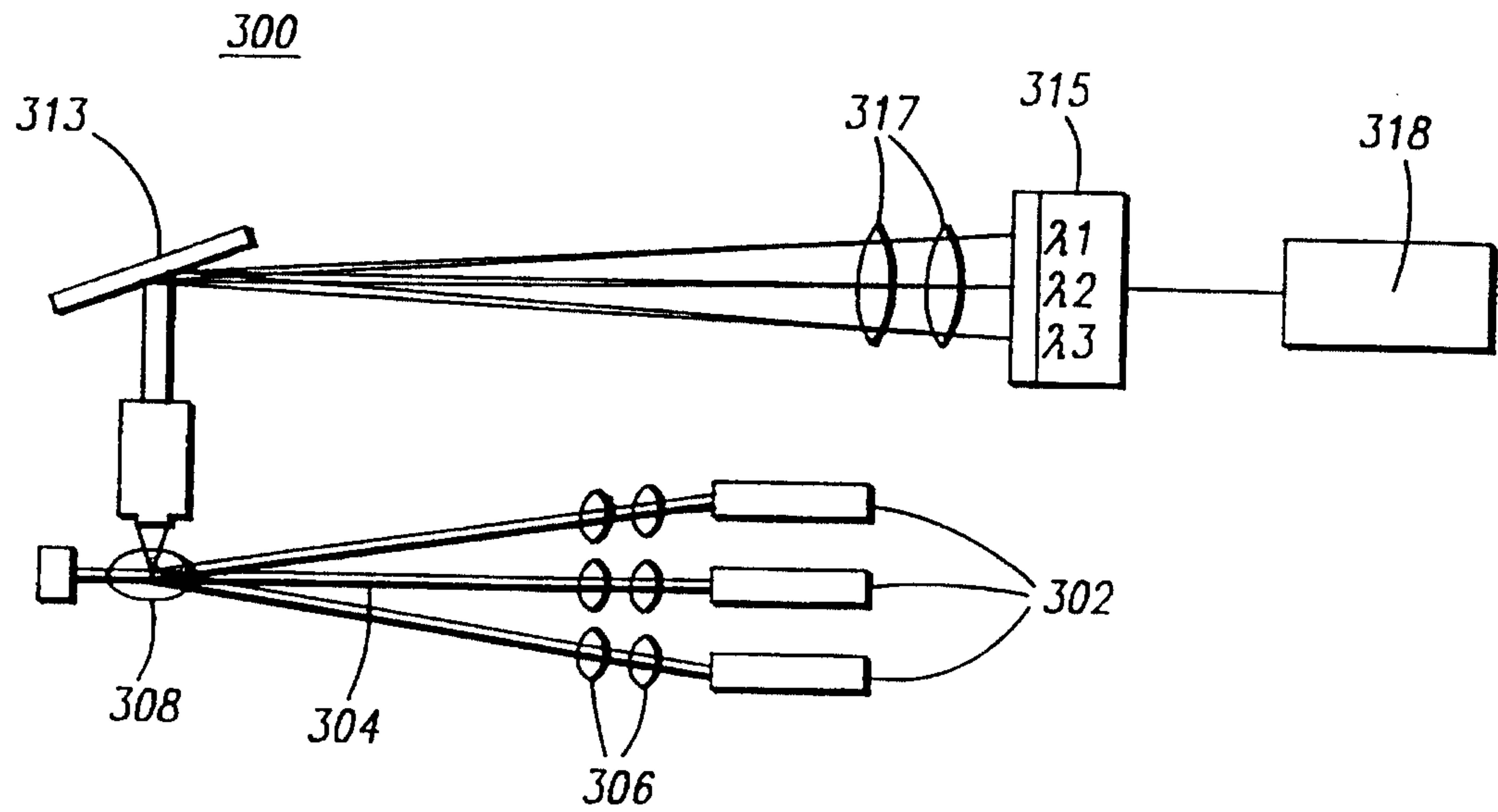
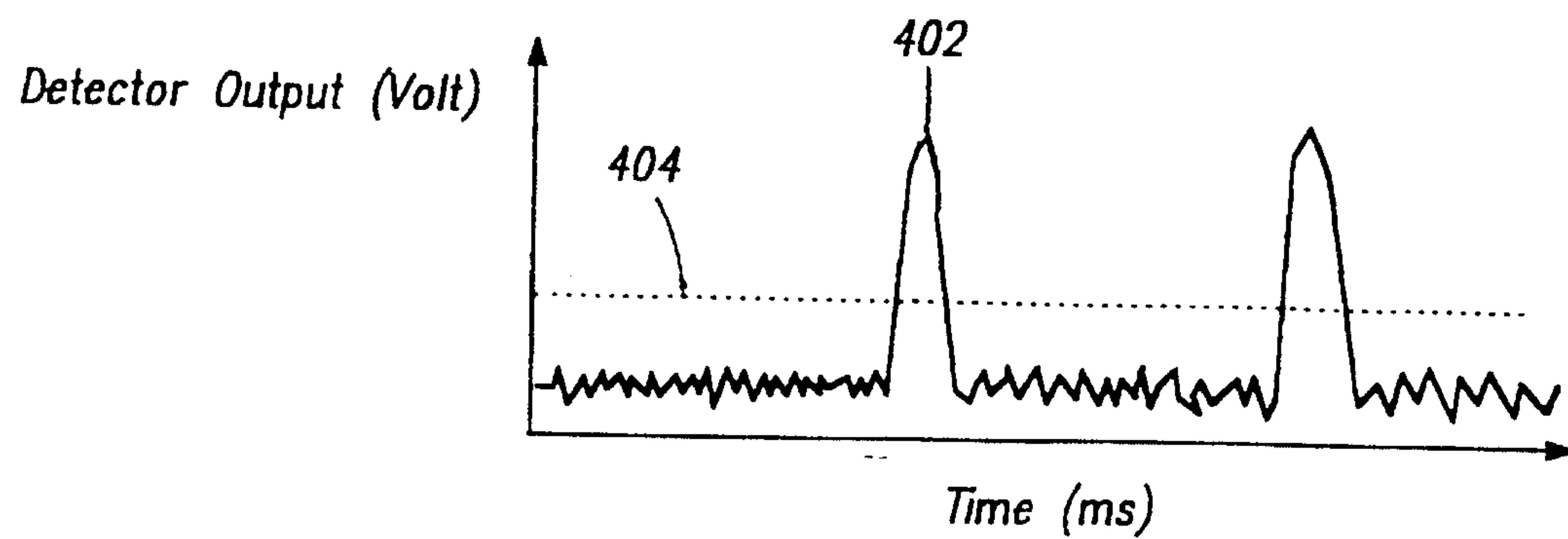


FIG. - 3B



*FIG. - 3C**FIG. - 4A*

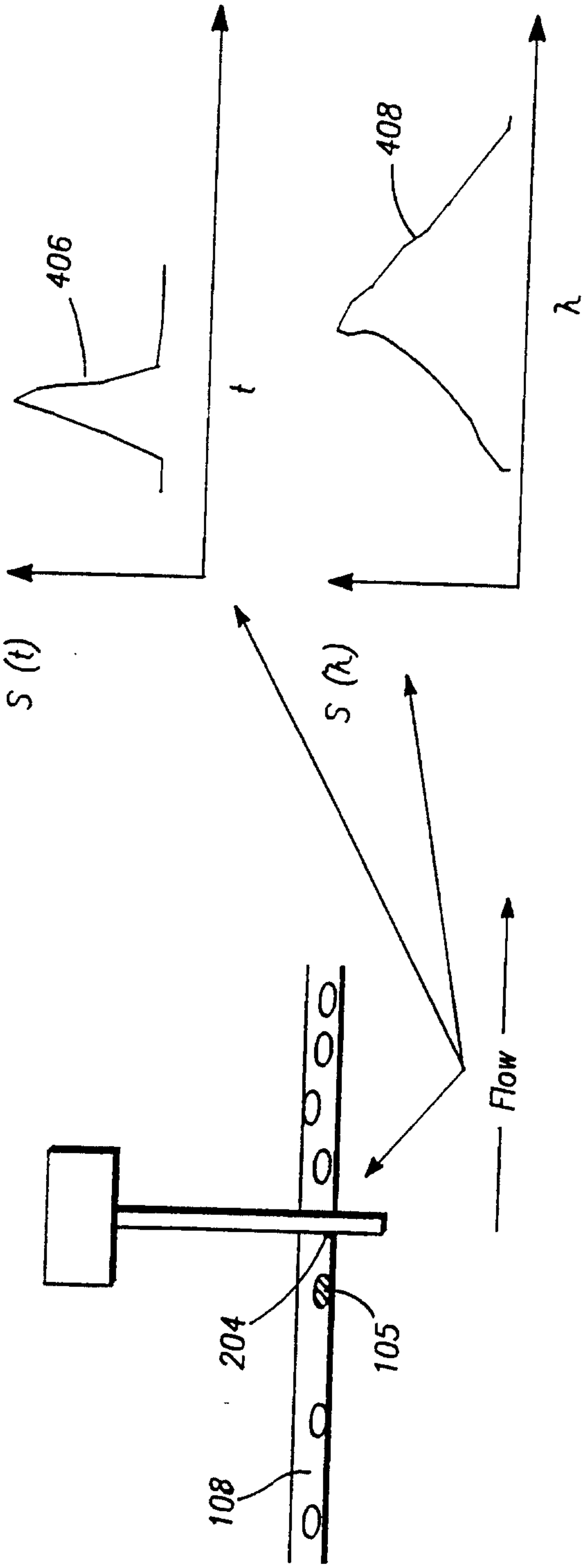
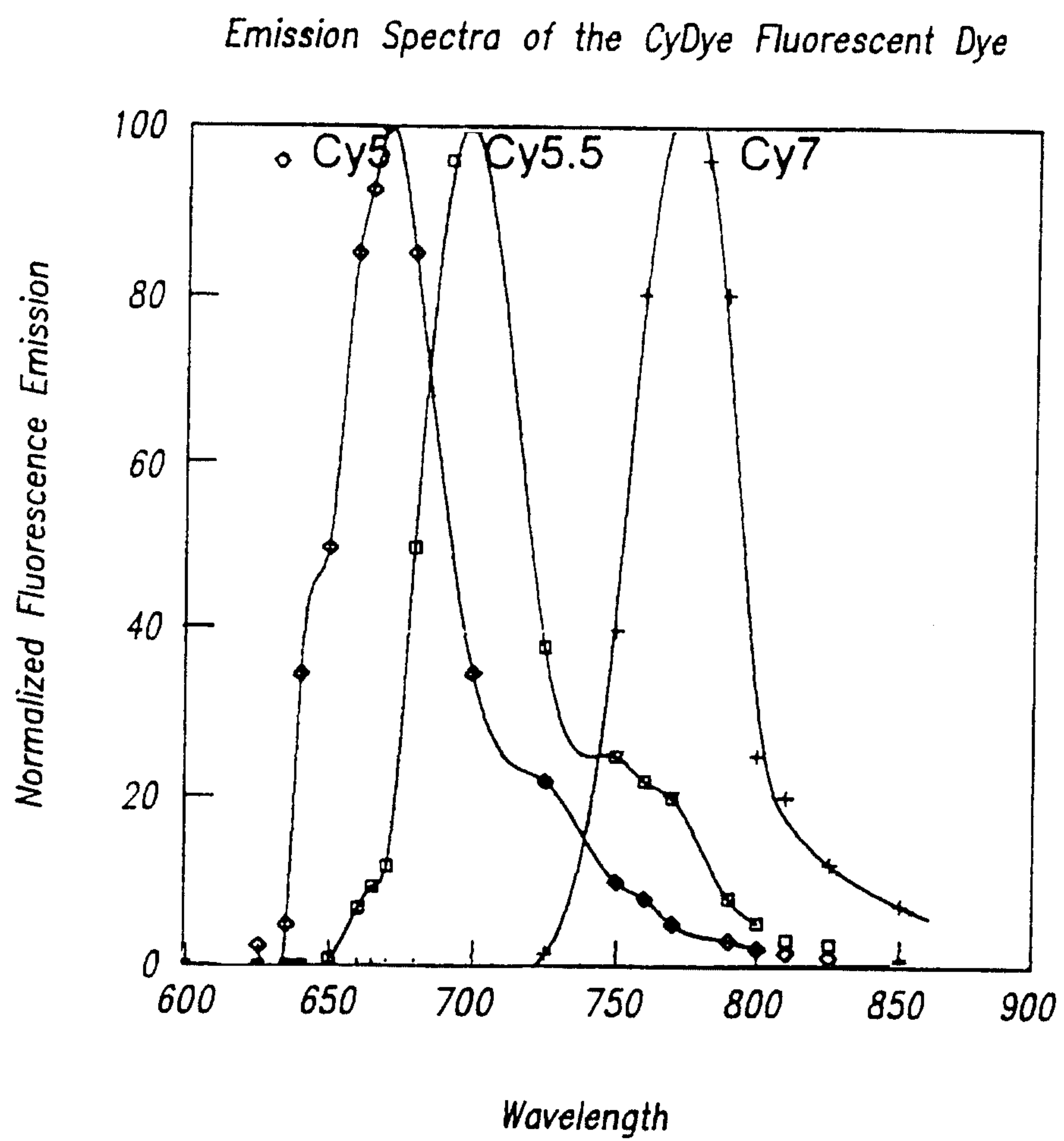


FIG. -4B



*FIG. -5A*

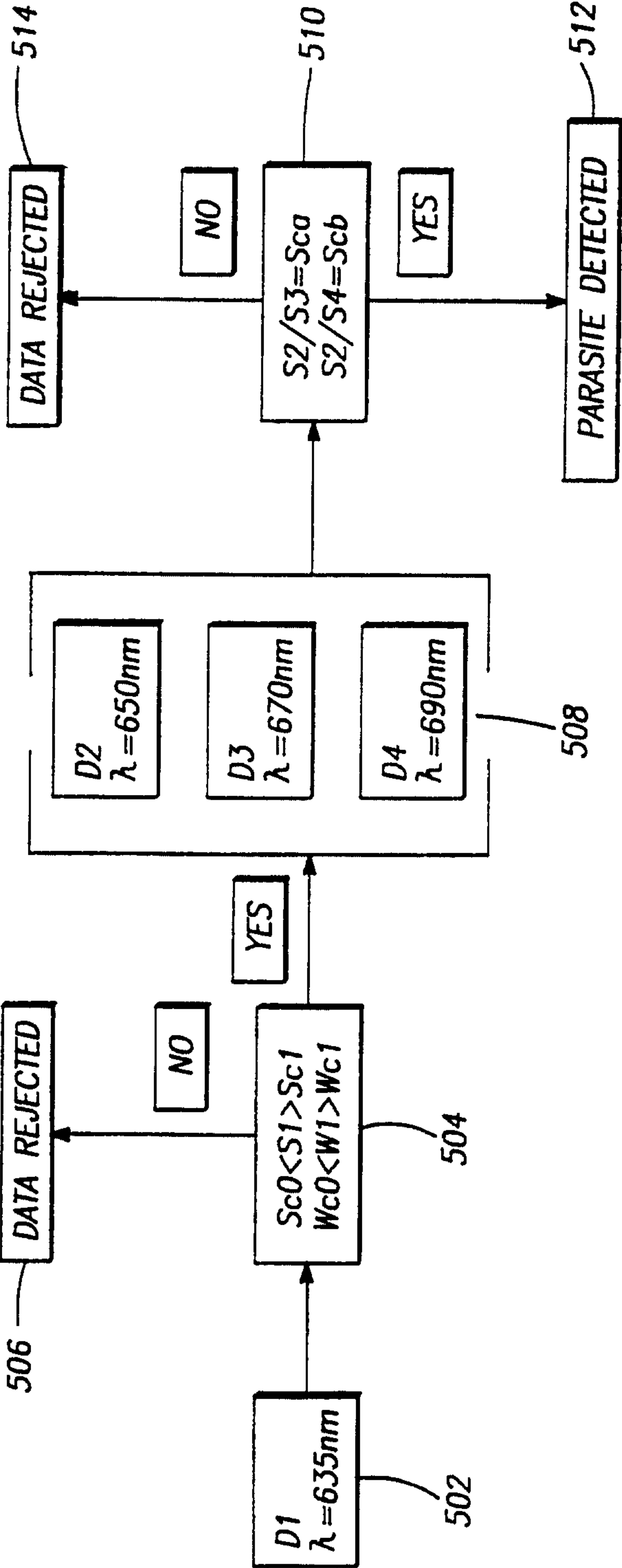
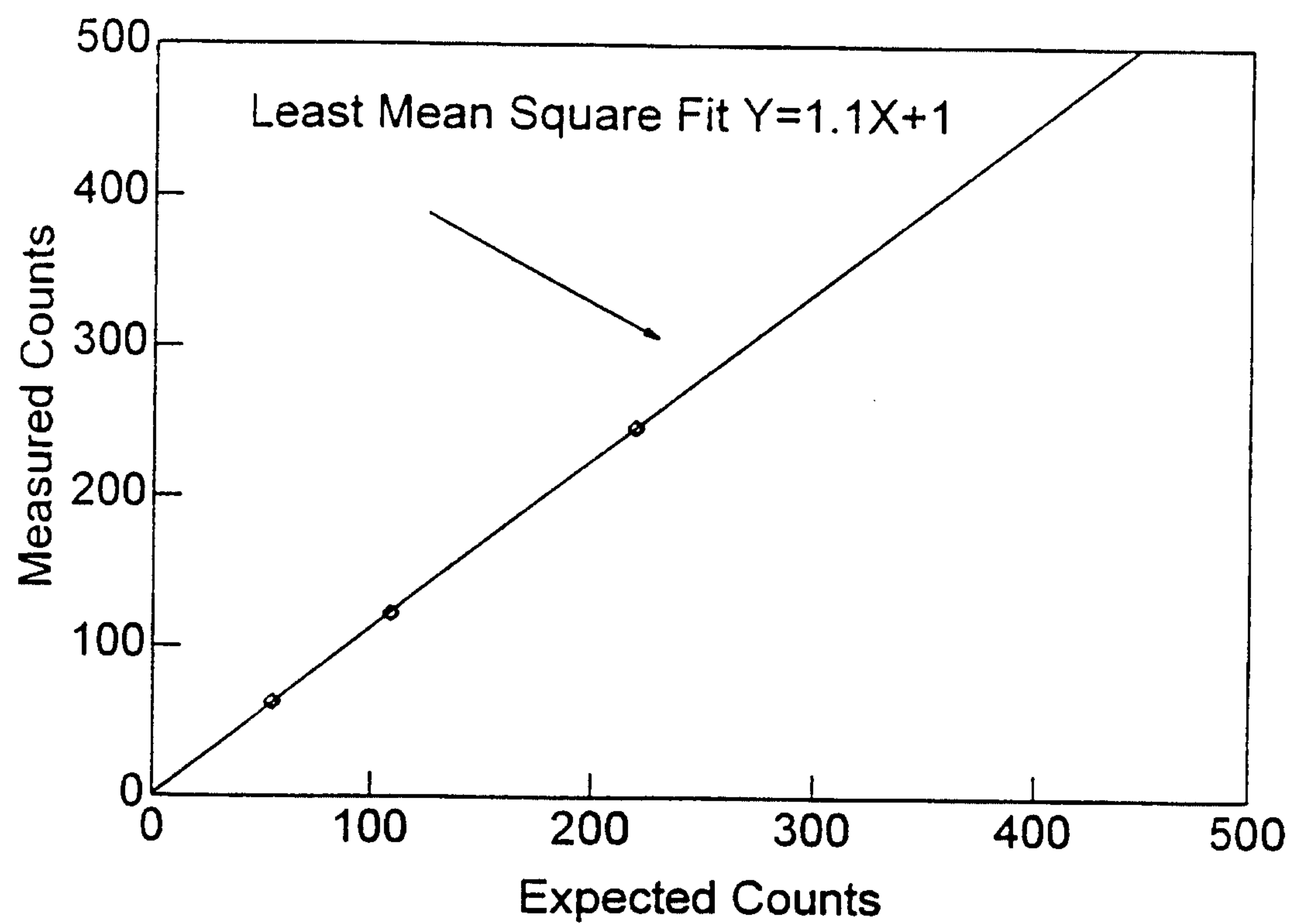
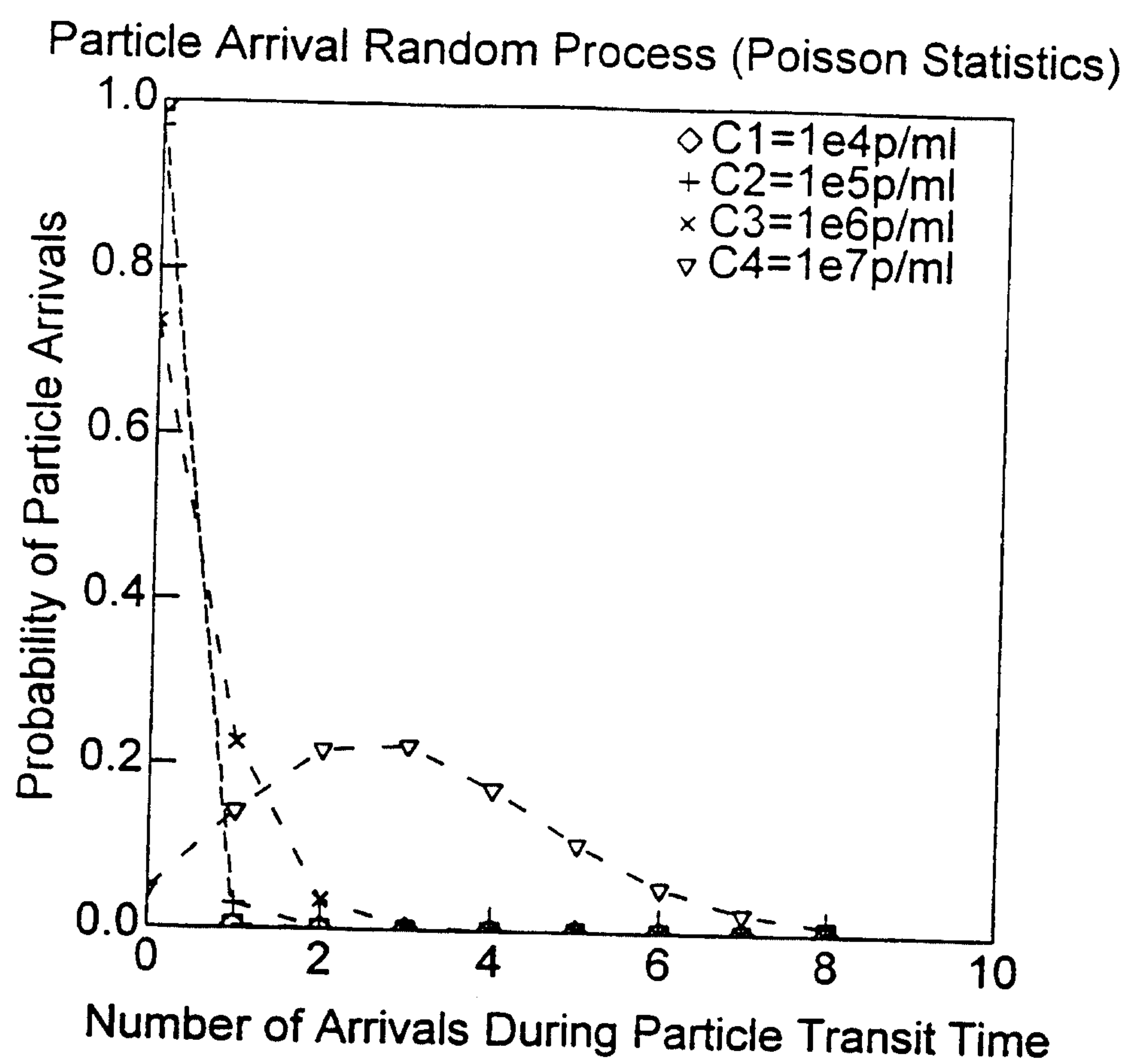


FIG. 5B



## Calibration Experiment: 2 micron crimson beads

*FIG. - 6A*

*FIG. -6B*



SYTO60 Stained Ecoli calibration curve (Guava Device)  
Detection Time: 30 seconds

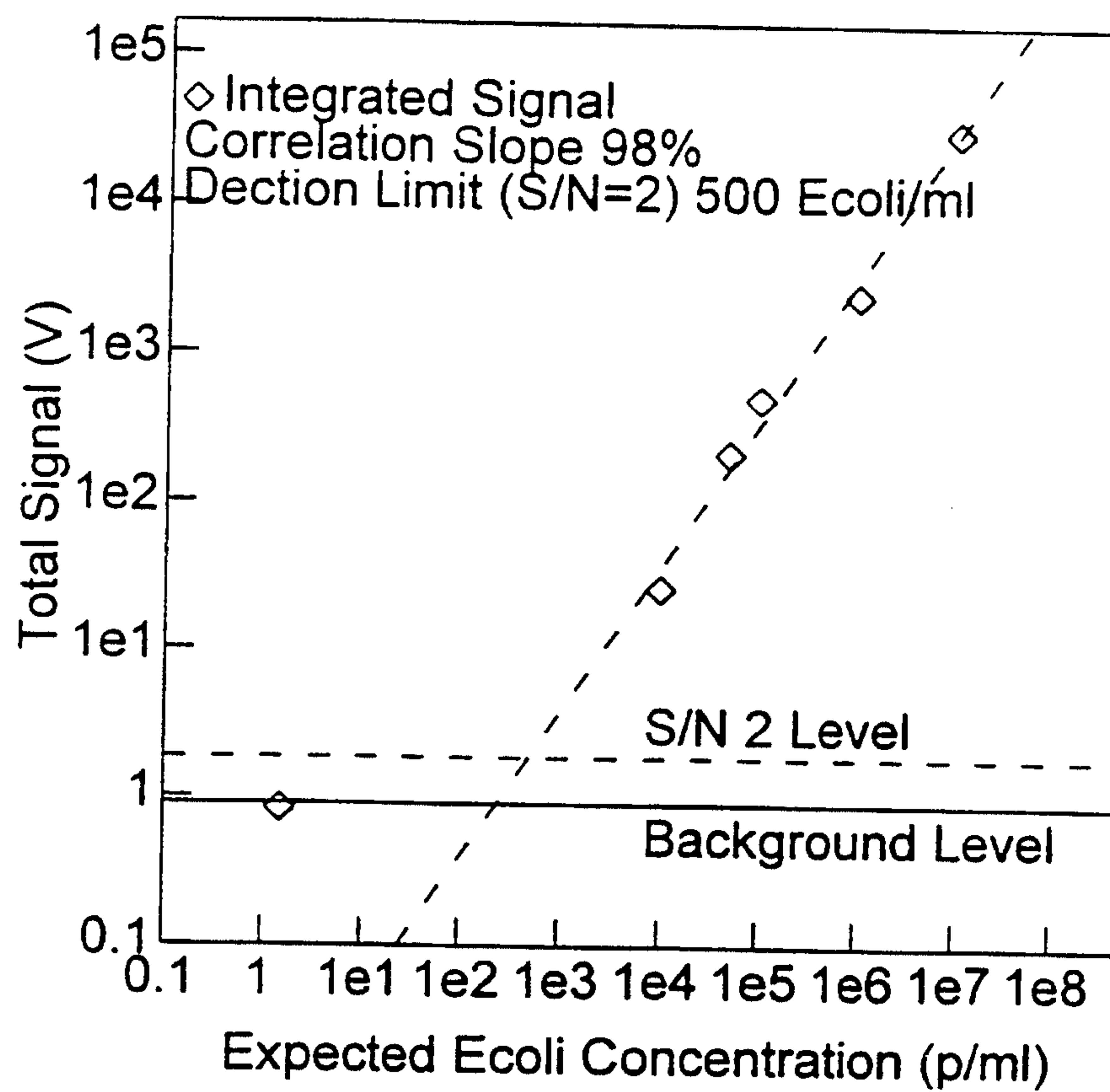
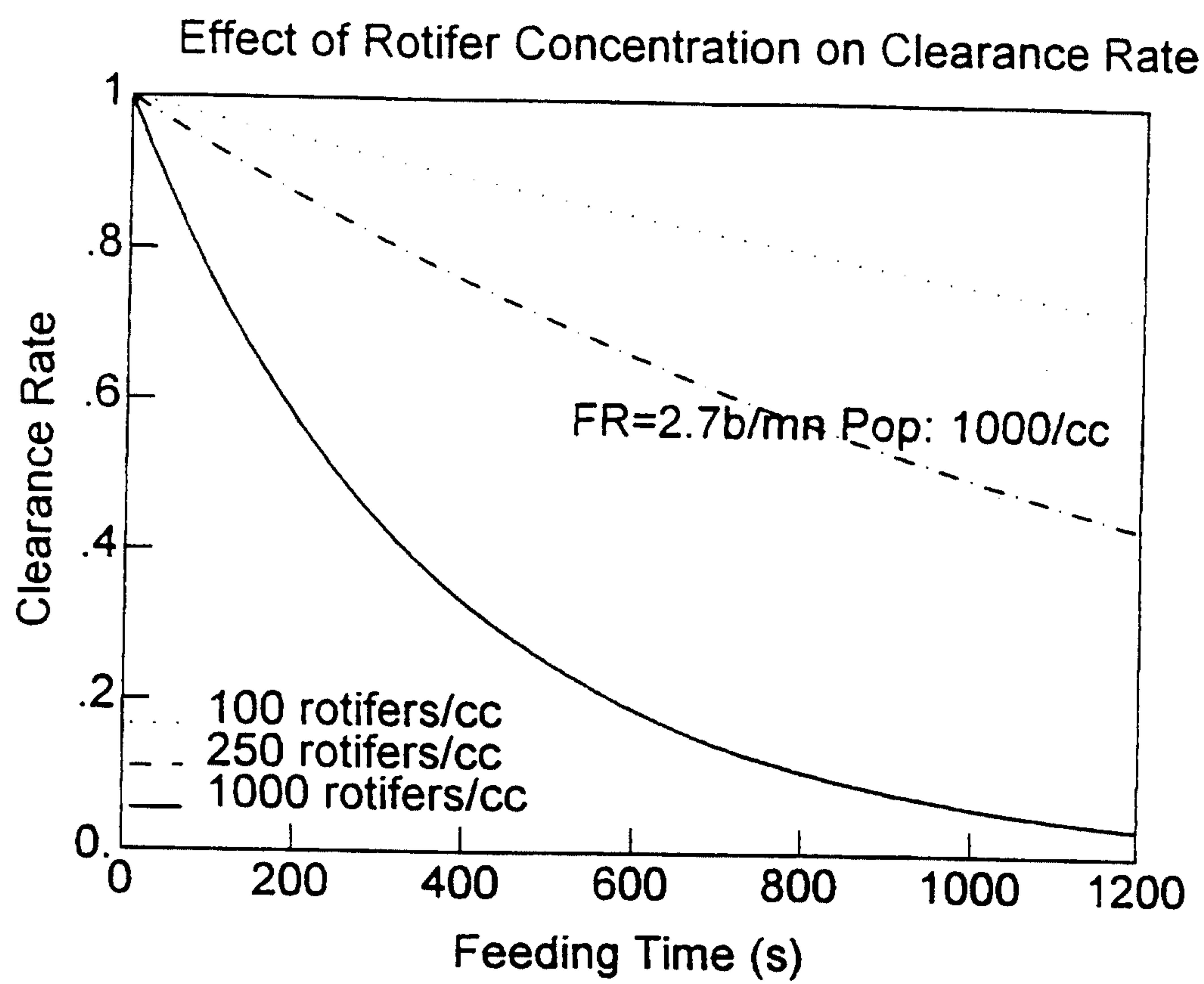
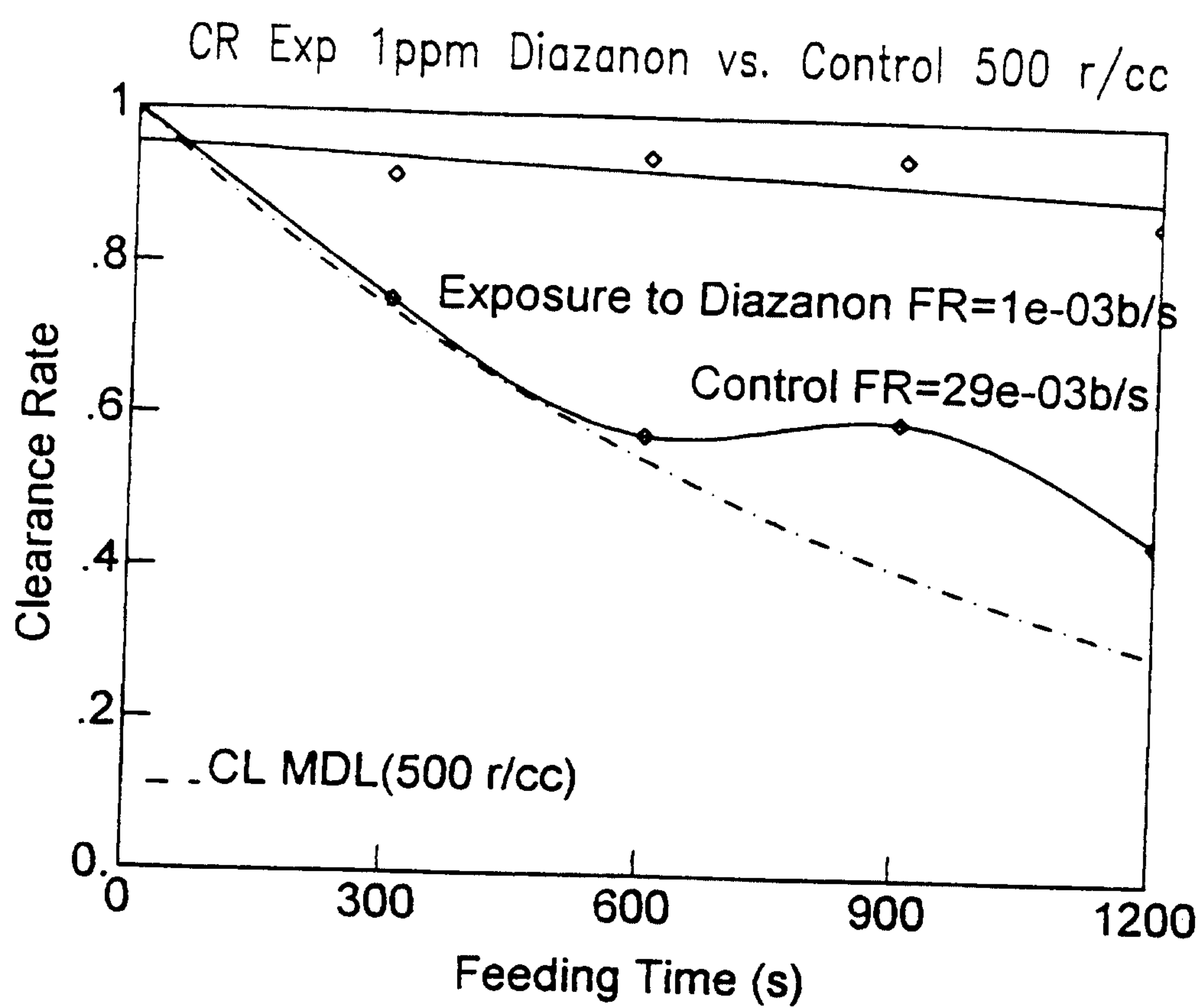
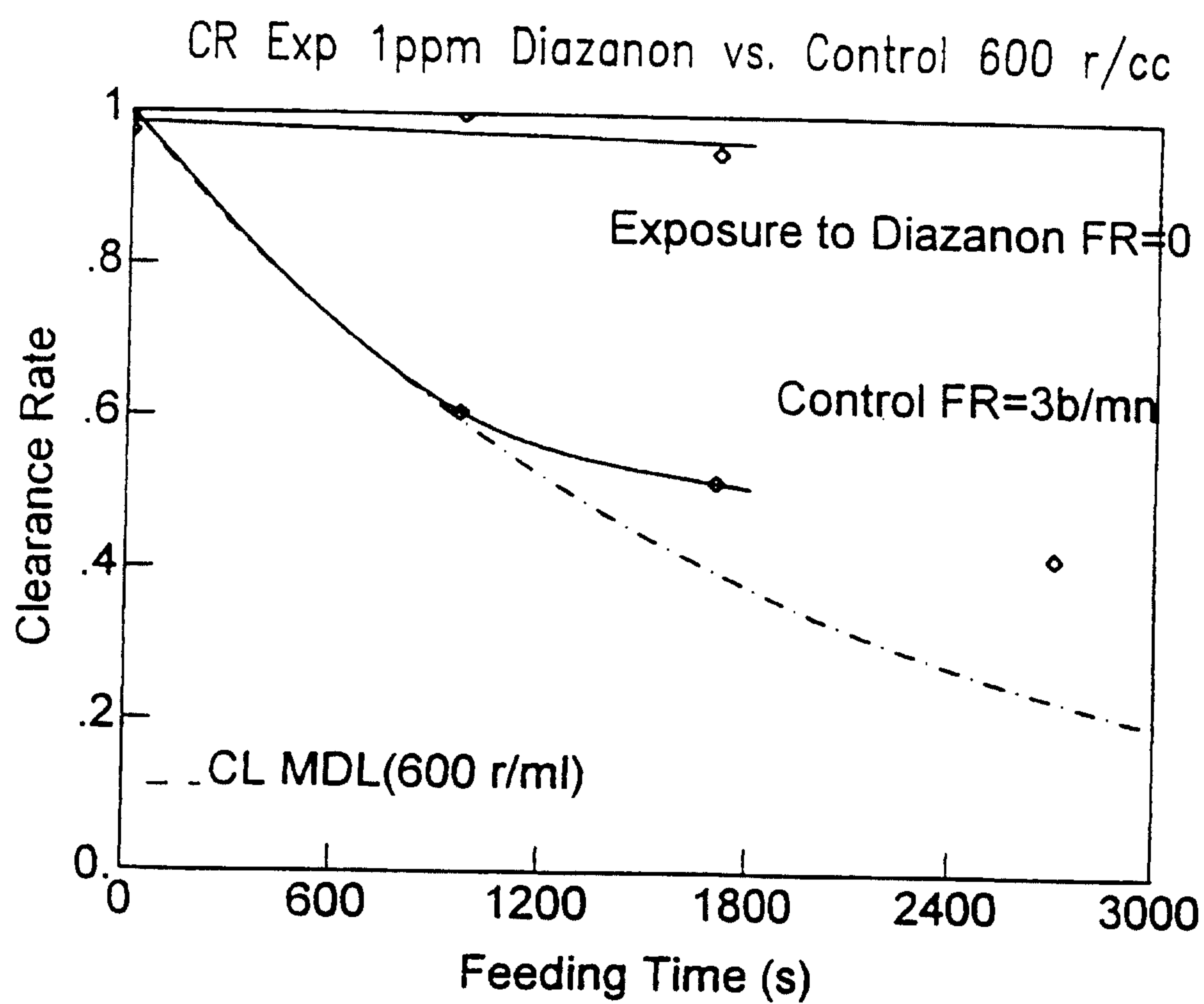


FIG. -6C

*FIG. - 7A*



*FIG. - 7B*

*FIG. - 7C*

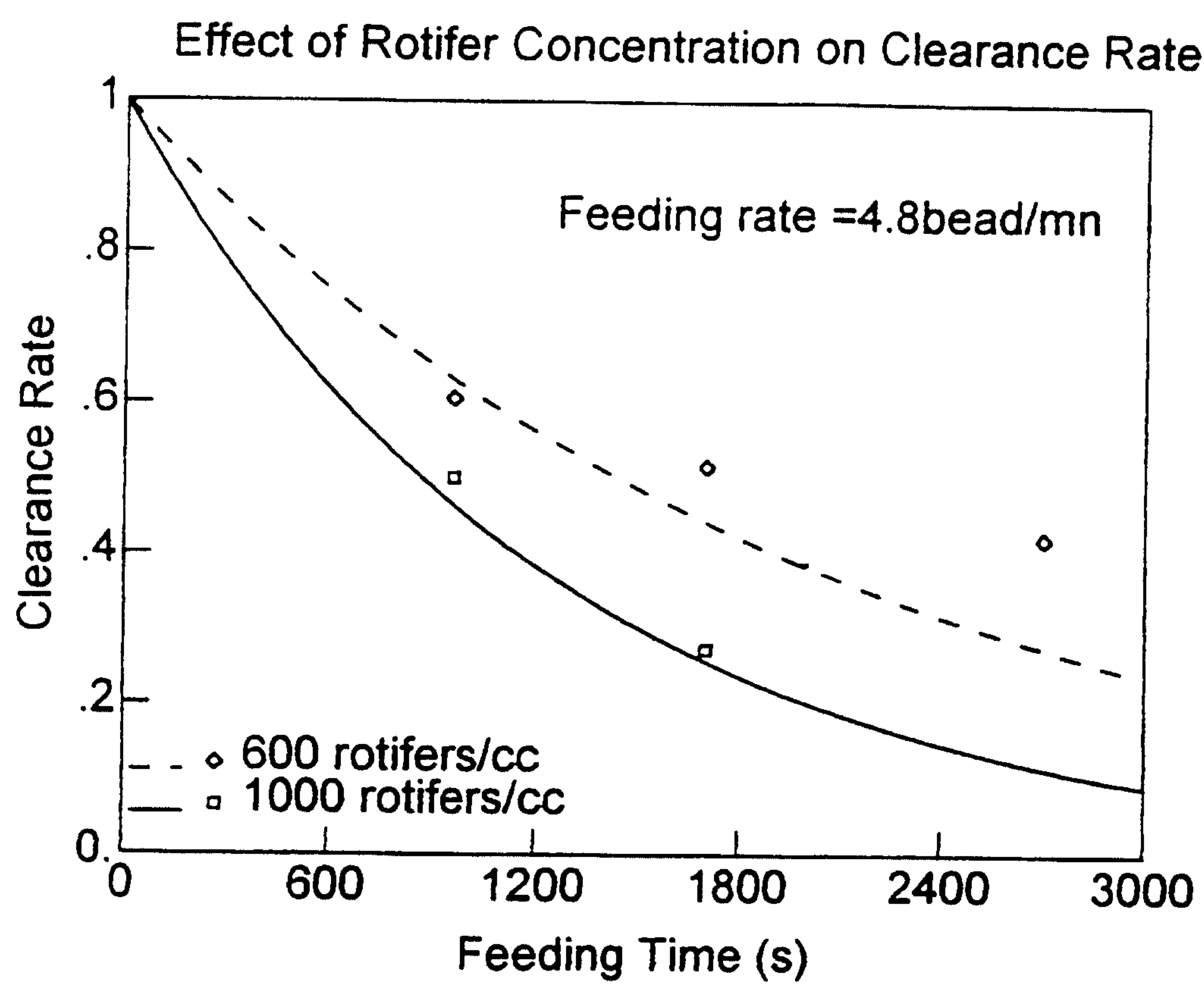


FIG. - 7D



