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# ELECTRONIC IMAGING SYSTEMS FOR QUANTITATIVE ELECTROPHORESIS OF DNA*

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ABSTRACT

Gel electrophoresis is one of the most powerful and widely used methods for the separation of DNA, proteins and other biologically important macromolecules. Most applications, however, use only the spatial information available from a gel -- the location of a band or spot -- while either ignoring quantitative information completely or making only limited -- semiquantitative -- use of it. The quantitative information available from a gel has been ignored, in large part, because of a lack of adequate technology for accurately and quickly obtaining quantitative data on the distribution of DNA on a gel post electrophoresis. To appreciate the progress that has been made in the last decade, the reader is directed to papers describing the quantitation of fluorescence from ethidium bromide stained DNA gels[1-3] that required a person to read optical densities from the chart recorder of a densitometer and enter the resulting values for many positions spanning a band on a gel onto computer cards. Other workers quantitated the distribution of radionuclides in a gel or on a Southern blot by physically slicing the gel and counting the parts in a scintillation counter. Little wonder these did not become routine laboratory procedures!

To be really useful, it is necessary to obtain the quantitative image of the distribution of DNA or other biological molecules on a gel or blot after electrophoresis in digital form so that it can be analyzed by a computer. During the last decade, instruments have been developed that accurately quantitate in digital form the distribution of materials in a gel or on a blot prepared from a gel. In this paper, I review the various physical properties that can be used to quantitate the distribution of DNA

on gels or blots and the instrumentation that has been developed to perform these tasks. The emphasis here is on DNA, but much of what is said also applies to RNA, proteins and other molecules.

# I. PHYSICAL PROPERTIES USED TO QUANTITATE DNA IN GELS

## A. ABSORPTION OF VISIBLE OR ULTRAVIOLET LIGHT

The absorption of visible and, more recently, ultraviolet light has been one of the most frequently used methods for following the separation of biological molecules. Indeed, the term "chromatography" derives its name from the visible absorption spectra of components that were separated. Direct absorption measurements of nucleic acids and proteins in gels are complicated by the optical properties of gels, which tend to absorb or strongly scatter light in the spectral region below 300 nm where these molecules absorb strongly. Procedures exist to "shift" the absorption spectrum of the target to the visible part of the spectrum by adding substances that bind the target molecule and absorb in some portion of the visible spectrum. The most widely used procedure for DNA is silver staining,[4] for which very high sensitivities have been reported. Care must be exercised in the preparation and staining of a gel as agarose tends to bind the stain also, giving rise to a significant background.

There are inherent disadvantages in any absorption measurement compared to the methods described below. Scanning the gel with a densitometer equipped with a single detector makes possible the measurement of high optical densities since stray light can be suppressed, but requires a long time to acquire the entire digital image. In contrast, using a multielement detector, similar to those described below for fluorescence, permits rapid acquisition of the digital image but limits the dynamic range of the measurement since stray light from other parts of the object makes it impossible to read high optical densities. The same argument

holds for photographic negatives as well as gels. The stray light situation for multielement detectors is quite different for absorption and fluorescence experiments. In fluorescence, stray light from a few bright spots is spread over the much larger dark background, while in absorption the large background area is bright and hence contaminates the few dark regions where the sample is located. At the other extreme, measuring very low optical densities is also difficult because a small absorption signal requires taking the difference between two intensities of the transmitted beam. But both light intensities have statistical uncertainties. Perhaps the best compromise between rapid data collection and tolerable scattered light is a linear detector combined with a parallel linear light source in an arrangement which translates the gel or negative between the source and detector.

## B. FLUORESCENCE

Fluorescence is one of the most widely used methods for the imaging of DNA in electrophoretic gels. While DNA is only very weakly fluorescent at room temperature,[5] many fluorescent molecules bind to DNA and can be used as fluorescent labels. The most widely used is ethidium bromide, which intercalates between the DNA bases. In this environment, the quantum yield of fluorescence increases by a factor of about 20,[6] thus suppressing the contribution of background fluorescence of ethidium molecules not bound to DNA or RNA compared to the fluorescence from bound molecules. Ethidium binds to DNA separated as single strands in an alkaline gel provided the gel is neutralized prior to staining.[7] Other fluorochromes such as Hoechst 33258 can also be used to detect and

quantify DNA.[8] Chemical procedures for covalently linking fluorochromes to single stranded polynucleotides have been developed for use in DNA sequencing.[9-11]

Fluorescence is inherently more sensitive than absorption for quantitative measurements since only the total number of photons is measured rather than the difference between two photon fluxes. The signal can be integrated for as long as necessary to obtain a good image using both analog and photon counting detection schemes. However, the sensitivity of a fluorescence measurement is rarely limited by the instrumentation. The limit of sensitivity is almost always determined by the level of background fluorescence, since it is almost impossible to obtain materials free of contamination by molecules that fluoresce, however weakly. Fluorescence background is particularly critical in ethidium bromide stained gels, since the whole gel matrix is exposed to the fluorochrome as part of the staining procedure, and unbound ethidium molecules are excited by the same wavelengths needed to elicit fluorescence from the bound ethidium.

C. CHEMILUMINESCENCE

Chemiluminescence[12] is a newer approach to recording the distribution of DNA after separation by electrophoresis. It is usually applied to blots rather than gels[13]. Since in a measurement of chemiluminescence there is no exciting UV or visible light, the nonspecific light emission may be reduced compared to fluorescence. However, contamination by nonspecific chemiluminescence is also a possibility. Thus the relative sensitivities achievable with fluorescence and chemiluminescence have yet to be determined. The two approaches may prove complementary, with fluorescence better

suited to gels and chemiluminescence the method of choice for DNA on blots. In any case, the CCD area detectors described below for the detection of fluorescence can also be applied to the detection of chemiluminescence but laser beam scanners cannot.

D. RADIOACTIVITY

Radionuclides have several advantages as labels. The energy released when most nuclei emit a β particle is sufficiently great that the event can be detected with high efficiency. No external excitation source is needed and, with proper shielding and choice of construction materials, backgrounds can be very low. Hence, radionuclides provide the highest sensitivity of detection presently available. Substitution of a radionuclide for a nonradioactive isotope of the same element leaves the structure and chemical properties of the labeled molecule unchanged, so there is no effect on electrophoretic mobility as can happen when fluorochromes are attached to DNA prior to electrophoresis.[10]

Radionuclide labeling also has significant limitations. Like the visible photons emitted in fluorescence, the β or γ rays resulting from a nuclear decay are emitted in all directions. Unlike visible light, however, there are no practical lenses or mirrors to refocus the β's and γ's. Thus an image generated by radioactive decay has poorer spatial resolution than an image generated with visible light -- i. e. the image is "fuzzier". The farther the sensitive region of a detector is removed from the site of the decay the greater the loss of spatial resolution.

A practical, and increasingly important, difficulty with the use of radionuclide labels is the cost and inconvenience of handling, transporting and disposing of them.

## II. QUANTITATION OF FLUORESCENCE FROM GELS

### A. PHOTOGRAPHIC FILM

The limitations of most present methods for analysis of data from gels and blots stem from the use of silver halide emulsions as the primary means of detection. The optical density of a photographic negative is not proportional to the intensity of the light that exposed it. (Positive are even worse.) Procedures for converting the density of negatives to values proportional to ethidium fluorescence, and hence DNA mass, have been described by several workers[1-3,14-17] but all introduce another step in the analysis and hence the potential for degrading the accuracy of the measurement. Other undesirable properties of photography are its limited dynamic range (the ratio of the light intensity that produces saturation to the threshold intensity), lot-to-lot variability, failure of time-intensity reciprocity for long exposures and the time required to convert an image to digital form prior to computerized numerical analysis. One and two dimensional solid state detectors can speed the actual digitization[16-19] but time and labor must still be devoted to developing, washing and drying negatives and accuracy is still limited by the intrinsic properties of photographic film. Reciprocity failure can be accounted for by including a calibration standard in each photograph[20], a procedure that produces quantitatively accurate images, but further increases the time and effort required.

### B. ELECTRONIC IMAGING SYSTEMS FOR RECORDING GEL FLUORESCENCE

Instruments for recording directly the fluorescence from a gel can be classified into two distinct types based on the number of light sensors and the type of excitation source they employ.

## 1. MULTICHANNEL SYSTEMS FOR RECORDING GEL FLUORESCENCE

### a. FLUORESCENCE IMAGING SYSTEM USING A TELEVISION TYPE CCD CAMERA

My laboratory has collaborated with Dr. Betsy Sutherland's group to develop a method that uses agarose gel electrophoresis to quantify damage induced in DNA by ultraviolet light and other agents. The method requires determination of the number average molecular length of heterogeneous populations of DNA molecules.[21] This requires measurement of the distribution of DNA mass as a function of its distance of migration plus determination of the electrophoretic dispersion function (i.e. the relationship between molecular length and distance of migration). As a practical matter, these data must be obtained in digital form so that the computation of number-average length can be performed by a computer. Initially, we photographed the fluorescence from gels and used a computer controlled scanner to digitize the photographic negative.[15]

The limitations of photography soon became apparent. Thus, we designed and built an electronic imaging system based on a modified charge-coupled-device TV camera to record directly the fluorescence from gels.[22] Modifications to the camera and other components are necessary because the weak fluorescence from a gel cannot be detected in the 33 msec. exposure period of a normal TV camera. With our modified camera, exposures can last over one minute. The maximum duration of an exposure is limited by the "dark current" of the detector, which saturates the output of our TV camera (operating at -30° C) in about 15 minutes. Both the spatial and

photometric response of our camera are linear.[22] Image data are digitized and stored in computer memory immediately upon completion of the exposure. An important question in the design of the imaging system was whether to store the entire image or only the profiles of the individual lanes. We decided to store entire images since this makes it possible to reanalyze data ab initio and troubleshoot experiments at a later time. The formidable problem of archival storage of complete images was solved by using an optical disk with a capacity of more than a gigabyte. Over 1000 images can be stored on a single dismountable disk.

Images of gel fluorescence intensity are displayed on a color monitor. Pseudocolor coding helps distinguish intensity variations that cannot be detected in a monochrome image. In addition, We display areas of photometric saturation as white and hence make them immediately obvious to the operator. Examples of pseudocolor coding are shown in references 22 and 23.

The programs we have written for the manipulation of image data include those specifically for the analysis of DNA damage plus some that can also be used for the analysis of restriction fragments. For example, we can extract individual rows and columns of an image or average between horizontal or vertical boundaries to give, respectively, band and lane profiles. Examples are shown in ref 22. We also developed a hierarchical data management system to facilitate the systematic naming, storage and recall of images and information about the experiment and sample that produced each image.

b. IMAGING SYSTEMS USING SCIENTIFIC CCD DETECTORS

The original TV camera based system demonstrated the feasibility and the advantages of electronic imaging in the

analysis of restriction fragments separated by gel electrophoresis. Realization of the full potential of this method, however, will require higher performance CCD cameras and considerably more specialized software. Our experience with the original system also suggested modifications in the equipment surrounding the camera and in the configuration of the computer system that will increase experimental productivity significantly.

The photometric performance of our original electronic imaging system is limited severely by the use of television technology. The TV image must be read out of the camera and digitized in 33 msec. This limits the photometric resolution of the digitized image to 256 gray levels (8 bits). The camera malfunctions when cooled below -30° C, hence limiting exposure times and therefore sensitivity. Both of these problems can be overcome with a slow-readout charge-coupled device (CCD) camera (sometimes called a "scientific" CCD camera). Reducing the readout rate by more than two orders of magnitude makes possible increases in photometric precision to as high as 16,384 gray levels (14 bits), with corresponding improvements in the signal-to-noise ratio. These cameras can operate at liquid nitrogen temperature if necessary. Even at -55° C, a temperature attainable with thermoelectric cooling and thus better suited for use in a molecular biology laboratory, dark current is decreased, and hence sensitivity is increased, by about an order of magnitude compared to our TV camera based system.

Scientific CCD cameras have one limitation compared to TV cameras in the analysis of gels. We align gels by operating our camera at the normal TV rate and viewing the position of the gel, illuminated with an intense visible source, on a monitor.[11]

Scientific CCD cameras cannot produce images fast enough to give the impression of continuous motion.

## 2. SINGLE CHANNEL SYSTEMS FOR RECORDING GEL FLUORESCENCE

Single channel systems scan a highly focused excitation beam, usually from a laser, over the surface of a gel in a raster pattern. There are three approaches to implementing a scanning system. First, the excitation beam and the detector can be fixed in space and the gel is moved relative to them -- or visa versa. This design has the advantage that the sensitivity of the system is independent of location on the gel, but results in a painfully slow rate of data acquisition. In the second approach, the laser beam is swept back and forth across the gel in one direction -- the fast axis -- by a servo mirror (or equivalent device) while the gel is translated along the orthogonal "slow" axis. Alternately, the laser beam scans a fixed position on the gel during electrophoresis[10,11]. The latter arrangement, which was developed for reading sequencing gels[10], reduces the amount a gel has to be handled, but requires that the fluorochrome must be associated with the DNA during electrophoresis. It also requires that an expensive instrument be dedicated to "running" each gel. A third geometry is to keep the gel fixed in space and scan the laser beam rapidly over it in two dimensions using dual servo mirrors or some other type of controllable beam deflector.

Fluorescence generated by the moving spot is recorded by a sensitive photomultiplier and associated analog electronics and digitized and stored in a computer for processing. The photomultiplier "views" either the entire gel, or a substantial portion of it. The location of a digitized value in an image is

thus determined by the location of the excitation beam at the time the analog signal from the photomultiplier is digitized. This process assumes that the excited state lifetime of the fluorochrome is much less than the time a given point on the gel is illuminated by the excitation beam or the period the analog signal is summed prior to digitization. For pixel dwell times of milliseconds and fluorescence lifetimes that are typically < 100 ns, this is a valid assumption.

Both the efficiency of exciting fluorescence at a given point on the gel and the efficiency of detecting the resulting fluorescent light may vary for different locations on the gel for any instrument in which the excitation beam is scanned by a servo mirror or equivalent device and the fluorescence detected by a photomultiplier which "views" different points on the gel with varying efficiencies. This variation in optical throughput can be corrected by recording the signal generated by a standard the intrinsic fluorescence of which is known to be constant. These corrections are very much simpler if the excitation beam is scanned along only one axis and the gel translated (or run) in the orthogonal direction since the number of elements in the collection function equals the number of pixels in one row on the image, typically a few hundred to a few thousand. In contrast, if the laser beam is scanned in two dimensions over the surface of a stationary gel, the number of elements in the correction function equals the number of pixels in the image, typically a few hundred thousand to several million.

## 3. COMPARISON OF SINGLE- AND MULTICHANNEL FLUORESCENCE IMAGERS

The performance characteristics of CCD multichannel and single

channel laser gel imagers are very different. CCD imagers are much faster since all of the pixels in an image are acquired simultaneously. In addition, the time required to acquire an image is insensitive to the number of pixels. The time required to read the accumulated charge in each pixel is linearly dependent on pixel number, but the time to align the gel and expose the CCD are independent of the number of pixels. On the other hand, the cost of an imaging system increases rapidly with the maximum number of pixels in an image, since the yield of high quality CCD chips decreases as their complexity and size increase. In complete contrast, the time required to scan a gel with a laser beam increases with the size of the gel, since the fluorescence is read one pixel at a time, but the cost of the scanner is insensitive to the number of pixels in an image and the size of the gel. These concepts are indicated schematically in Figs. 1 and 2.

At present, CCD TV are limited to about 768 by 480 pixels because of the bandwidth available for television signals. Higher resolution may become available as a result of high definition TV. Scientific CCD's are available with up to 2048 by 2048 elements, but these chips are very expensive! For images requiring less than 1024 by 1024 pixels, the speed and convenience of CCD imagers probably make them the system of choice. For higher resolutions, however, the lower cost of laser scanners outweighs the inconvenience of the longer time required to acquire the image. This "cross-over" point may increase with time as manufacturing techniques for CCD's evolve. Of course, larger gels can be imaged with a CCD at a given spatial resolution by acquiring multiple images, but this requires great care in reproducibly aligning the gel after each move if spatial information spanning more than one

segment is needed.

## III. QUANTITATION OF RADIONUCLIDES ON GELS

### A. AUTORADIOGRAPHY

The distribution of radiolabeled DNA on a blot is usually measured by autoradiography, a process that has all of the disadvantages of photography, discussed above, plus exposure times that can extend to days or even weeks! Alternately, the "spots" located by autoradiography can be cut from the blot and counted in a scintillation counter. This approach is tedious, labor intensive, gives poor spatial resolution and prohibits any form of background subtraction or deconvolution of overlapping bands.

### B. INSTRUMENTS FOR THE DIRECT IMAGING OF RADIONUCLEOTIDES ON BLOTS

Several instruments that image the distribution of radionuclides from gels and other planar surfaces have been reported. One-dimensional array detectors using proportional chambers are commercially available, but two-dimensional devices collect data many times faster. Two-dimensional imagers are also many times faster than autoradiography and have the additional advantage of letting the experimenter see the image "grow" during the course of an exposure. Two-dimensional radiation imaging systems have been built using image intensifiers[24], micro-channel plates and a resistive anode detector[25] and multiwire proportional counters[26-30].

#### 1. MULTIWIRE PROPORTIONAL COUNTERS

The inherent difficulty with multiwire proportional counters is loss of spatial resolution due to the distance between the sensitive volume of the counter and the radioactive source. This separation allows some electrons to travel laterally before being detected. The severity of this problem increases with the energy, and hence the range, of the beta particle. Anderson et al.[29] went to heroic lengths to improve spatial resolution of $^{35}S$-methionine labeled proteins in O'Farrell gels by applying a 2 Tesla magnetic field from an electromagnet (with a 10 kilowatt power supply!) normal to the plane of the counter, but the "spots" they recorded were still about 50% larger than the same features in an autoradiograph. The higher energy of the beta particles from $^{32}P$ compared to those from $^{35}S$ proscribes even this approach for $^{32}P$-labeled nucleic acids.

Sullivan et al.[30] recently reported an innovative approach to improving the spatial resolution of $^{32}P$ labeled nucleic acids on Southern blots. They use two multi-wire counters in series. The planes of both counters are parallel to the plane of the blot. The points where an electron crosses the plane of each counter are recorded electronically and used to extrapolate (in real time) the trajectory of the particle back to its origin in the plane of the blot. Unfortunately, the absolute spatial resolution reported by Sullivan et al. is only 2 mm. They presented data demonstrating that the images they obtain are poorly resolved compared to autoradiography. There are two likely causes for the limited spatial resolution. First, the β's may not travel in a straight trajectory as a result of collisions with gas molecules and the force of the electric fields it encounters within the multi-wire counters. Second, the extrapolation of the trajectory of the β

back to the plane of the blot magnifies errors in locating the points of intersection of the β's with the detector planes.

## C. STORAGE PHOSPHOR PLATES

Photostimulated phosphor storage imaging systems are also candidates to replace autoradiography in the imaging of radiolabels on gels and blots. While the potential of these approaches have yet to be fully determined, vis-a-vis autoradiography and multiwire proportional counters, phosphor storage plates are the only technology, other than autoradiography, that can produce a complete, two-dimensional image of the distribution of radiolabels on very large blots and gels (e.g. sequencing gels). In addition, they are efficient since the time required to "read" the latent image from a plate is much less than the time required to expose the plate. Thus, a single instrument can collect images sequentially from many plates that are exposed simultaneously.

X-rays, β-rays, and other forms of ionizing radiation passing through crystals such as BaFBr that has been doped with $Eu^{++}$ can form metastable excited states called "color centers". Absorption of a photon by a color center (633 nm light from a HeNe laser is ideal) generates an excited state which, with high probability, decays to the ground state of the crystal by the emission of a shorter wavelength photon (peak of the emission is near 390 nm). A phosphor imaging plate consists of a thin layer of suitable microcrystals bonded to a plastic backing. X-rays or β-rays incident on the plate produce a latent image that is read by scanning a focused laser beam over the surface and recording the intensity of the photostimulated (390 nm) luminescence with a photomultiplier. Phosphor plate imaging systems were originally developed for

diagnostic radiology, but recently have been explored as detectors of x- and β-rays in scientific applications, particularly as detectors for x-ray scattering experiments using synchrotron radiation sources.[31-36]

The principal advantages of photostimulated phosphor imaging compared to autoradiography are improved dynamic range and sensitivity. The sensitivity of a phosphor plate is more than 10 times greater than x-ray film.[33-36] The response of the phosphor material is linear over more than four orders of magnitude and usable over five orders of magnitude.[33] Amemiya et al.[32] built a two detector image reader that fully exploits the sensitivity range of the phosphor medium.

Compared to multiwire proportional counters designed for imaging blots and gels, storage phosphor imaging has better spatial resolution since the image is recorded in a thin layer that - like film - is placed immediately adjacent to the source object. Practical storage phosphor systems can also record larger images, hence making them useful for sequencing gels. They also have the advantage in comparison to other forms of non-photographic imaging of radioactivity on blots and gels in that an expensive instrument is not tied up waiting for radionuclides to decay. Multiple plates can be exposed apart from the scanning system for whatever period is required. Reading a plate typically requires 15 to 30 min depending on its size and the power of the laser beam, hence the instrument is used efficiently. The same comparison holds with DNA sequencing instruments that dedicate an expensive instrument to "running" a single gel.

The scanners required for "reading" a phosphor plate are generally similar to those discussed for obtaining gel

fluorescence, except that an inexpensive HeNe laser is sufficient, whereas fluorescence scanners usually require a more expensive ion laser.

FIGURE LEGENDS

1. The cost of a CCD imaging system increases very rapidly as the number of pixels in the image increases. Although shown in this schematic diagram as linear, the increase in cost is, in fact, greater than a linear function. On the other hand, the total time required to acquire an image is linear, but the fractional increase is not a large fraction of the total since the readout of signals from the CCD chip is the only one of several steps in the generation of an image.

2. The cost of a single channel laser scanner is insensitive to the number of pixels since many components (photomultiplier, power supply, computer, etc.) are the same regardless of image size and resolution. The only components that cost more if larger gels are to be scanned or if spatial resolution is increased are those involved with translating the gel or scanning the laser beam. On the other hand, the time required to record an image increases linearly as the size of the gel for given spatial resolution.

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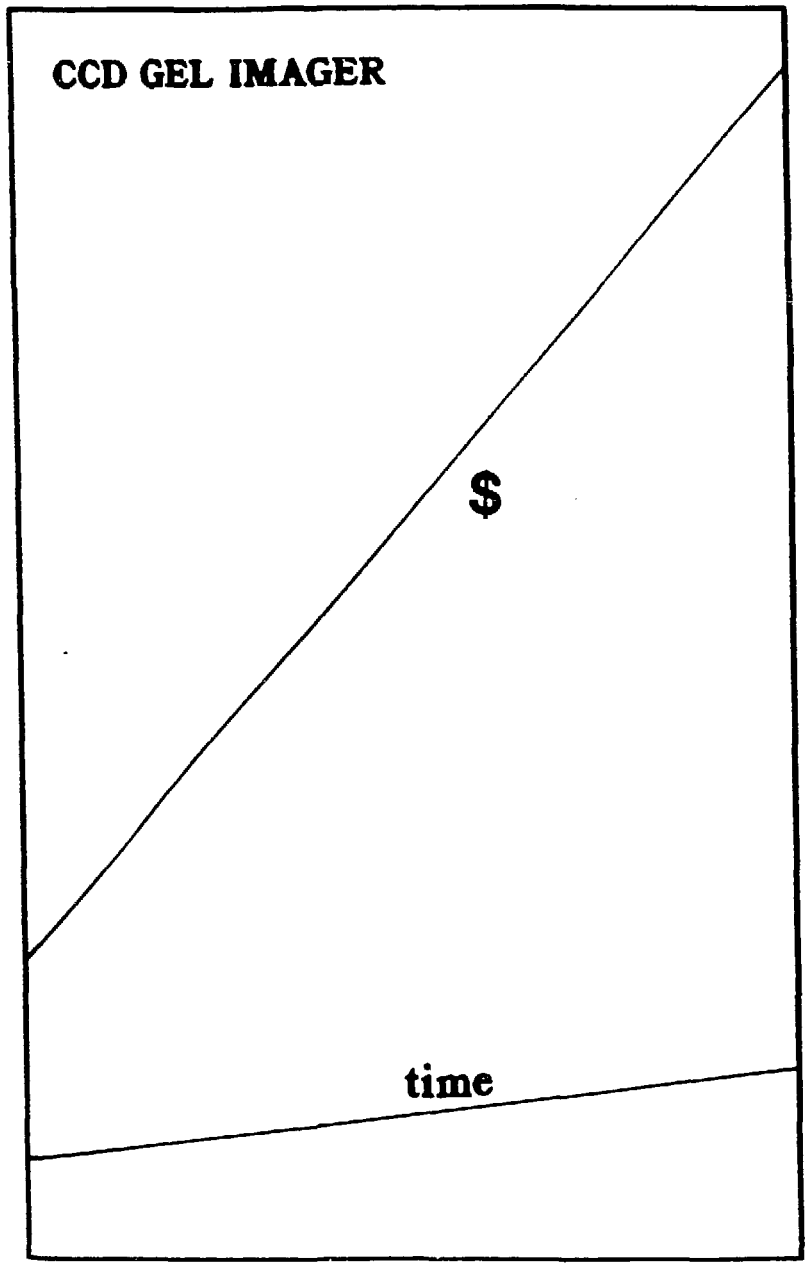


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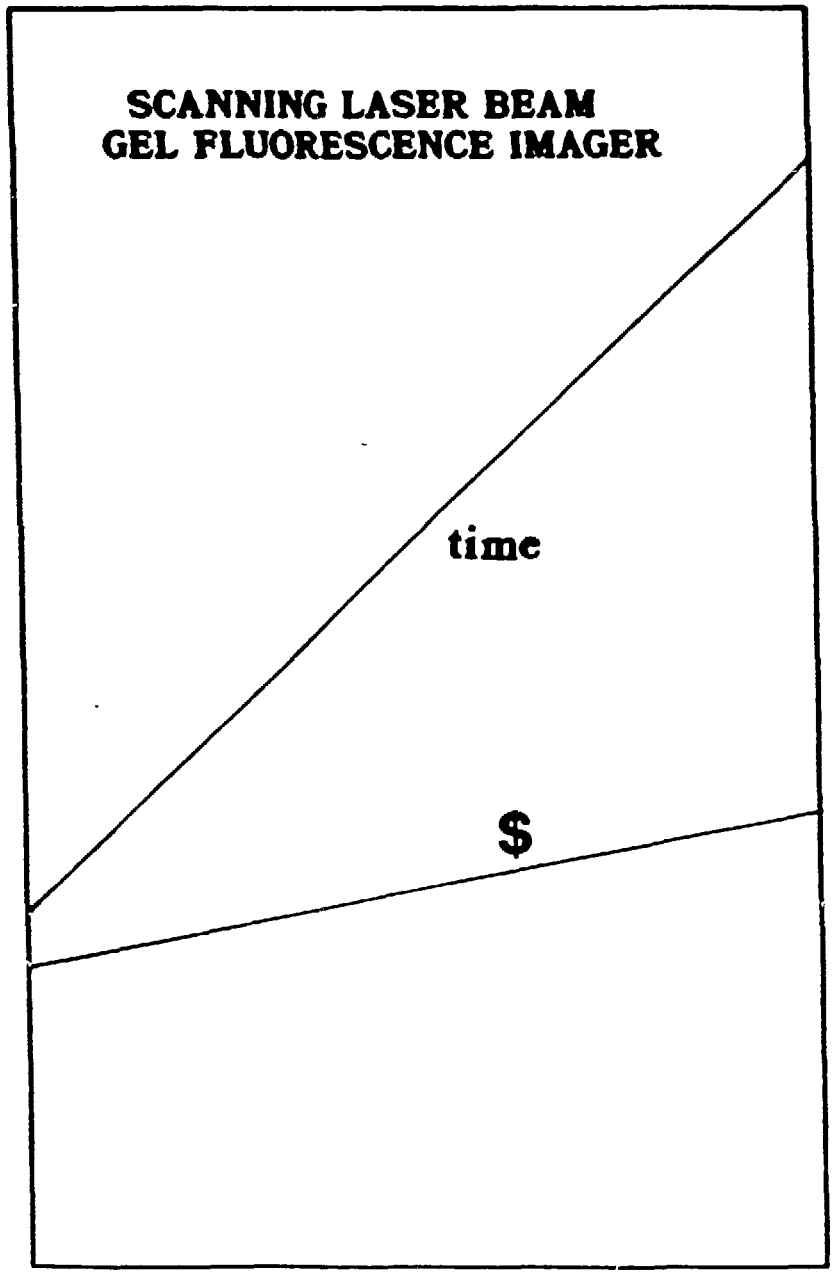


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