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# Applications of capillary electrophoresis and laser-induced fluorescence detection to the analysis of trace species: from single cells to single molecules

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Applications of capillary electrophoresis and laser-induced  
fluorescence detection to the analysis of trace species:

From single cells to single molecules

by

Qifeng Xue

A Dissertation Submitted to the  
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For the Graduate College

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Applications of capillary electrophoresis and laser-induced  
fluorescence detection to the analysis of trace species:

From single cells to single molecules

Qifeng Xue

Major Professor: Edward S. Yeung  
Iowa State University

Several separation and detection schemes for the analysis of small volume and amount of samples, such as intracellular components and single enzymes, were developed in this work. Laser-induced fluorescence (LIF) detection provides a very sensitive approach for both direct and indirect detection in capillary electrophoresis (CE).

First, indirect LIF detection and capillary electrophoresis were used to quantify lactate and pyruvate in single red blood cells. By choosing a highly efficient fluorophore and adding 1% glucose to the running buffer to stabilize the system, a detection limit of around 20 attomoles was achieved for small anions, which resulted in the easy quantification of targeted anions in single erythrocytes.

The measurement of the activity for sub-attomole enzymes inside single red blood cells presents a high challenge. The assay of specific enzyme activities was achieved by monitoring the highly fluorescent enzymatic reaction product, NADH. By adding proper non-fluorescent substrates into the running buffer, the enzymes will catalyze one specific reaction after they are separated into different zones and the CE flow is stopped. The fluorescent products were

related to enzyme activity. Consequently, the enzyme activity can be quantified by monitoring the fluorescent product. At about biological pH 7.4, lactate dehydrogenase (LDH) isoenzyme activities were assayed for single red blood cells. A detection limit of  $1.3 \times 10^{-21}$  moles for lactate dehydrogenase was achieved by the combination of on-capillary reaction and electrophoresis. The present approach is also applicable to the assay of multiple enzymes by introducing appropriate substrates. Since lactate dehydrogenase activity serves as a good marker for certain diseases, the ability to quantify individual isoenzymes at the single cell level is of clinical importance. Leukemia cells were analyzed to evaluate the value of LDH activity as a marker for the diagnosis of leukemia. From the single cell analysis, we found that LDH activity is not a unique marker for diagnosis of leukemia, although the LDH activity in leukemia cells is lower than that in normal white blood cells.

Reactions of single LDH-1 molecules were investigated by monitoring the reaction product with LIF detection. By filling a narrow capillary tube with a very low concentration of LDH-1 and excess lactate and  $\text{NAD}^+$ , discrete product zones of NADH associated with individual LDH-1 molecules are formed. We can quantify molecular concentrations down to  $10^{-17}$  M, and can also measure their activities. From the products formed during two consecutive incubation periods, each LDH-1 molecule maintains the same distinct activity over a 2-hour period. We found that the same kind of enzyme molecules can have different activities, which vary in a factor of 4. The differences in activity might be caused by different stable conformation of LDH-1 enzymes.





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## GENERAL INTRODUCTION

### **Dissertation Organization**

This dissertation begins with the general introduction of literature related to this work, in which the background concepts and the most recent progresses in this area are presented. The following chapters are arranged in the way such that the research publications of the author are each presented as separate chapters. Finally, a general summary presents the final comments on this work and a listing of the cited references for the general introduction concludes the dissertation.

### **Capillary Electrophoresis**

Electrophoresis as a very powerful separation technique<sup>1</sup> has been well known and applied in the separation of charged species based on differential migration in an applied potential field. Since the early 1980's, after Jorgenson and his colleagues laid down the foundation for running electrophoresis in a narrow bore (<100  $\mu\text{m}$ ) fused silica tube (capillary) to separate charged species<sup>2,3</sup>, capillary electrophoresis has begun to achieve scientific recognition and has been undergoing an explosive progress. Capillary electrophoresis (CE) offers several exciting features: 1) highly efficient and extremely fast separations of both ionic species and neutral compounds; 2) requiring very small amount of sample for

one analysis (~nl); 3) using the capillary tube acting as a microreactor to carry out microreactions, after which one microscale separation and quantification are executed. To date, a wide spectrum of analytes has been proven to be feasible in analysis by this technique. Not only can the small ions be separated and analyzed by CE coupled with different detection schemes, but also the neutral molecules and the large biologically interesting molecules can be analyzed with different modes of capillary electrophoresis.

Depending on the analytes, several modes of CE techniques can be selected to perform a quick, efficient analysis. As recently categorized by Knox<sup>4</sup>, four distinct capillary separation techniques are widely accepted and applied in separating different species, although different names and categories are still used by different authors based upon the separation mechanisms. Capillary electrophoresis was originally called capillary zone electrophoresis (CZE)<sup>3</sup>, in order to indicate that the separated analytes migrate as separately independent non-contiguous zones. Since each zone of analytes might migrate at a different speed, both cations and anions can be determined in the same run with CE. Molecules and ions of similar charge to mass (Z/M) ratio, such as DNA and proteins, lead them to have very similar electrophoretic mobilities in free buffer solution. Capillary gel electrophoresis (CGE) can provide the capability<sup>5,6</sup> of resolving these analytes due to a different separation mechanism. The gel filled capillary can obstruct different sizes of analytes to different extents, or the

entangled gel molecules provide a sieving effect, in which different sizes of molecules penetrate the gel medium at different speeds and are separated. The separation efficiency and the fast speed in DNA separation by CGE, coupled with various sensitive detecting methods, have increased the pace of potentially sequencing the human genome <sup>7,8,9</sup>.

Terabe and his co-workers <sup>10,11</sup> first tried to add modifiers (e.g., SDS) into the running buffer for separating neutral compounds, as well as some ions and ion pairs, which are difficult to separate with CE. The buffer modifiers, usually surfactants, form micelles when the concentration is higher than the critical micellar concentration in the solution. When the analytes are injected into the capillary filled with the buffer solution containing micelles, the analytes are partitioned between background electrolyte and micelles. Thereby, the separation is achieved, based upon primarily partitioning differences between analytes. The process is in fact chromatographic and not electrophoretic, even though the electroosmotic flow still plays a very important role in transporting the solution through the capillary. Accordingly, this technique is usually called capillary micellar electrochromatography (CMEC) or micellar electrokinetic chromatography (MEKC). By choosing chiral active micelles, several works<sup>12-14</sup> demonstrated that this technique can be used to separate chiral compounds, such as D,L-amino acids and chiral drug molecules.

Although capillary liquid chromatography, or capillary electrochromatography (CEC)<sup>15-17</sup> does not really belong to capillary electrophoresis according to the separation mechanism, there are still some similarities between CEC and CE, since both of them use an electric field to drive the liquid carrying the analytes through the capillary tube. In CEC, the capillary is packed with a conventional HPLC stationary phase (even though the particles may be much smaller because of the small tube inside diameter). The analytes are separated primarily based upon their different partition ratios between the mobile phase, electrolyte, and the packed stationary phase. With this technique, the analytes of neutrals, ions and ion pairs can be separated, and the electric field might be used to achieve additional selectivity and efficiency.

### **Optical detection in CE**

To detect extremely small amounts of materials is always challenging for various detecting methods. The often nano-liter range of analytes available in the capillary electrophoresis especially requires highly sensitive and fast response detectors, which do not disturb the electric field for running CE. A number of detection methods have been shown to be effective and sensitive for CE analysis. In this part, the absorbance detection and fluorescence detection will be covered briefly in both direct and indirect detection schemes. As a number of excellent and comprehensive reviews have been available that covers

the development of CE and its detection methods<sup>18-22</sup>, more detailed information of CE detection systems can be found in these resources.

### Absorbance Detection

Among the advantages associated with UV-Vis absorbance detection for CE, the nearly universal nature and easy coupling for on-capillary detection prevail. However, the short pathlength inherent with a capillary tube restricts the sensitivity for UV-Vis absorbance detection. Usually, the short optical pathlength (~50  $\mu\text{m}$  capillary i.d.) typically results in concentration limits of detection (CLOD) on the order of  $10^{-6}$  M<sup>23</sup>. The benefits of UV-Vis measurements for CE were discussed in ref. 24. With multiwavelength UV-Vis absorbance detection usually available with the commercial CE instruments, additional chemical selectivity can be obtained.

In order to overcome the low sensitivity of UV-Vis absorbance measurements for CE analysis, techniques and instruments using different capillary geometries and improved detection cell designs were developed and described in many publications<sup>25,26</sup>. According to Beer's law, the optical absorbance of a sample, the signal associated to a certain concentration of sample, is directly proportional to the optical pathlength through which the absorbance is measured. Therefore, the effective extension of optical length for the measurement is obviously leading to an enhancement in the limit of



detection (LOD), if there is no peak broadening effect involved in a specific design. Also, a ball lens, when put close to the capillary, is found the most effective in coupling the light into and through the capillary<sup>27</sup>.

Chervet et al.<sup>28</sup> designed and manufactured Z-cells by bending the capillary to extend the optical pathlength from 75  $\mu\text{m}$  to 3 mm. With this special design, a 5-fold improvement in sensitivity was achieved, although there was a 40-fold increase in the pathlength, because the background noise was also tremendously enhanced. By optimizing the efficiency in light throughput, the enhancement in detection sensitivity was significantly increased to 17-fold. An alternative to increase the optical pathlength was to use a bubble-shaped cell for measurement. Basically, one region of the capillary can be expanded by glass-blowing or controlled local etching to create a longer absorption pathlength cross the capillary. This kind of detection cell is commercially available with 3 time expansion<sup>29</sup> and was extensively studied in Ref. 30 for up to 15x radial expansion. The expansion requires much better focusing optics to make sure that the light beam remains collimated over a longer distance, in order to fully utilize the enhancement provided by the pathlength expansion. By using a laser beam to measure the absorption<sup>30</sup>, an 8 times enhancement of measured absorbance was observed. Another alternative to gain a longer optical pathlength<sup>31,32</sup> was to perform electrophoresis in “flattered” channels (square and rectangular capillaries). This geometrical capillary can provide efficient

heat dissipation. When the narrow separation channel dimension is maintained, at the same time the longer dimension provides the requirement of longer optical pathlength for enhancing absorbance detection sensitivity. However, the fragility of the thin-walled fused silica tubing is a great concern when they are routinely used for doing analyses. Some other techniques and special design for increasing the pathlength included multireflection flow cell<sup>33</sup> and axial-beam irradiation<sup>34-36</sup>. In both cases, the optical pathlength of the capillary can be increased tremendously, while the narrow separation dimension as well as the separation efficiency is maintained. By carefully constructing the multireflection flow cell, a 40-fold increase in sensitivity was demonstrated<sup>35</sup> with a 44-fold increase in pathlength. With the axial-beam irradiation, the absorbance indicates the sum of the absorbance signals resulting from all analytes that existed in the capillary. As components elute out the capillary at different speeds, the total absorbance decreases in a step-like manner. Total internal reflection was used to let the light propagate efficiently along the column. An enhancement of 7-fold in detection sensitivity was also achieved with axial-beam irradiation.

In addition to increasing the pathlength to improve the absorbance detection sensitivity, the double beam optical approach has been the most popular detection scheme in commercial instruments to compensate for the intensity fluctuations. The principle is that a second detection channel of no

analyte is serving to monitor the instantaneous variations in the light source, and then can be used to normalize the measurements in the sample channel. Virtually, the normalization process can eliminate the part of measurement fluctuation caused by the light source fluctuation. Based on this idea, a double-beam laser absorption was used to achieve lower LOD by reducing the noise level<sup>37-39</sup>. With the double-laser beam absorbance system, a LOD of  $2 \times 10^{-8}$  M of malachite green was achieved.

### Fluorescence Detection

Because fluorescence detections can provide the highest sensitivity for CE, and is easy to couple for on-column detection, it becomes more popular, particularly for the determination of biologically interesting molecules that exhibit native fluorescence. Compared to absorbance detection, fluorescence detection typically results in sensitivity gains of 1-3 orders of magnitude, depending on the light source, its intensity and stability, fluorescence efficiency and background interference, as well as the detector configuration<sup>40</sup>. The reason for the big difference in LOD between absorption and fluorescence measurements is the way the signal is monitored. In absorption, the small signal from the transmitted intensity is monitored at the presence of a relatively high background signal, the reference intensity. Generally, it is not easy to measure a very small signal change on a high background, since a small fluctuation on

the background can overlap the small signal<sup>41</sup>. On the other hand, the fluorescence measurements can be performed with a negligible background signal, which means that even a very small signal; can be easily recognized. If stray light and background signal are controlled, increasing the excitation intensity will produce larger signals and improved LOD in fluorescence. Of course, the intensity needs to be carefully controlled to avoid the photo-bleaching. With a high power laser beam to excite fluorescence, the short optical pathlength in CE is less problematic for achieving low LOD, even though the fluorescence intensity is also linearly dependent on the pathlength.

The most popular geometry for constructing a laser induced fluorescence (LIF) detector is orthogonal<sup>42,43</sup>. The construction is simple and rugged. The well-collimated laser beam can be focused to a beam diameter determined by the diffraction limit of light with a short focal-length lens. The focused laser beam can deliver a high photon flux to the interior of a capillary to allow efficient excitation of the analytes. Since the laser beam can be focused to a very small diameter, much smaller than the inner capillary bore, appropriate alignment of the capillary to let the laser beam pass through the capillary center will minimize the background level caused by the scattered light. Appropriate spatial and spectral filterings are always necessary in reducing the background. To collect a large part of the fluorescence, a high numerical aperture (NA) microscope objective is desired for collecting and focusing the fluorescence light

on a photomultiplier tube (PMT). With this design, a LOD of  $3 \times 10^{-12}$  M of fluorescein was achieved with a linear dynamic range of more than 4 orders of magnitude<sup>42</sup>.

One very efficient geometry is utilizing an epi-illumination fluorescence microscope with laser excitation<sup>44</sup>. This geometry offers performance comparable with the best detection schemes in CE. A high numerical aperture objective is used to tightly focus the light into the detection region while increasing the solid angle for light collection. Since this optical system can view a very small field and allow the adjusting of the depth of field, only the interior of the capillary is probed after suitable adjustments. The stray light and background fluorescence can be rejected by spatial and spectral filters. In Ref. 45, this geometry also has been demonstrated to be capable of monitoring separations in multiple capillaries. By laying multiple capillaries in parallel on a translational stage, DNA-sequencing runs have been performed in 24 capillaries and detected with epi-illumination fluorescence detection system. This set-up opens the possibility for fast sequencing DNA in the future, by using two-color fluorescence detection and a two-dye-labeling protocol.

One way to reduce the background noise produced by the capillary wall is to use a sheath-flow cuvette as the detection cell<sup>46,47</sup>. With this construction, the stream flows out the capillary column can be confined and hydrodynamically focused into a narrow stream by a sheath flow. The diameter of the sample

stream is determined by the ratio of sheath flow rate and the sample flow rate. The laser beam can be focused tightly through the sample beam to eliminate any noise contribution from scattered light and fluorescence light originating from the capillary walls. This has allowed detection of  $1.7 \times 10^{-21}$  moles FITC-arginine. Lately, multiple sheath flow is established within a flow cell to run multiple capillary electrophoresis<sup>48</sup>. An intersecting laser beam is used to excite simultaneously the analytes in different capillaries and the detection is performed by a CCD camera. This is one of the approaches toward speeding up DNA-sequencing by running a multiplexed capillary array.

Since most analytes do not fluoresce, or have very low fluorescence efficiency, derivatization of sample is one necessary approach for extending fluorescence detection to determine many analytes. Precolumn, on-column and post column have been reported to be applicable for derivatizing different samples with a variety of fluorescent reagents<sup>49-51</sup>. If a better detection limit is desired, one can always rely on the laser induced fluorescence detection, no matter native fluorescence will be used or derivatization is necessary before undergoing the detection.

### Indirect Detection

For those species without appreciable functional groups for direct measurement, indirect detection stands up as a quite universal and applicable

method<sup>52</sup>. With indirect detection, the analyte physically displaces a chromophore or fluorephore present in the background electrolyte. In capillary electrophoresis, a charged chromophore or fluorephore is used so that the analyte ions of like charge will displace them, while ions of opposite charge may form ion-pairs with them. The response by the detector is a decrease in the signal, which allows detection of many species that would ordinarily be detector inactive. Both indirect absorbance<sup>53,54</sup> and fluorescence<sup>55,56</sup> detection have been demonstrated very practical and quite rugged in analyzing different species, such as small cations and anions.

## **Applications**

### Small Ions

The development of new separation methodologies and sensitive, rugged detection approaches for the analysis of small ions by capillary electrophoresis continues to grow. By forming complexes with a visible light-absorbing chelating agent<sup>57</sup>, 22 metals were separated at different runs of various pH values. In a standard procedure, the buffer solution containing the chelating agent at appropriate pH was used for running CE, and the sample solutions containing excess amounts of the chelating agent were injected for analysis. The separation largely depends on pH, with the elution order changing at different

pH values. Turcat and co-workers<sup>58</sup> used capillary electrophoresis to monitor concentration of organic acids in snow and rain water. They found that CE is suitable for measurements in environment for the reasons of time, sensitivity ( $10^{-6}$  M) and sample amount (nl). Morin and co-workers used imidazole and benzylamine as the electrophoretic buffers<sup>59</sup> to determine several alkali and alkaline-earth cations by indirect detection with high enough sensitivity of 50 ppb to quantify their presence in mineral waters. Lagoutte et al.<sup>60</sup> demonstrated the possibility of using CE to determine several small anions. Other works discussed the optimizations of separation and detection of low molecular mass anions<sup>61,62</sup>. The sensitivity is governed by the molar absorptivity of the chromophore, its charge and transfer ratio. The good match for the mobility between chromophore ions and analyte ions also plays a very important role for the sensitivity and separation efficiency. Suppression or reversal of the electroosmotic flow (EOF) in the capillary was shown to enhance the separation selectivity<sup>63</sup>. Similarly, these same EOF modifiers can be used to dramatically shorten the time required for one analysis<sup>64</sup>. CE has been used successfully to monitor small ions in widely varying samples.

### Chiral Separation

Chiral separation is always very challenging as the samples have almost exactly the same properties except the difference in optical activity. Many chiral



separations have been reported by using chiral selective complexing agents in the CE buffer. Zare and co-workers reported the first chiral separations in CE<sup>65</sup>. The separation was based on complexation of D,L-amino acids with a Cu(II) complex of L-histidine. Later on, cyclodextrins were found to be very effective chiral selector<sup>66-69</sup>. Different chiral compounds were separated using cyclodextrins for inclusion complexation. Garrison and co-workers<sup>70</sup> also demonstrated the separation of three optically active herbicides by adding cyclodextrins into the running buffer. Walbroehl et al.<sup>71</sup> made a comparison of HPLC, with a chiral crown ether stationary phase, and CE, with a chiral crown ether dissolved in the operating buffer, for the separation of enantiomers. When analogs of DOPA and tyrosine were separated, CE and HPLC yielded similar resolution. As mentioned in Ref. 72, the separation and quantification of enantiomers are an important application of CE. Application areas include enantiomer purity testing of pharmaceuticals and herbicides, reaction rate monitoring, stability testing and chemical analysis.

### Proteins and Peptides

Capillary electrophoresis is a very powerful tool for the separation and analysis of proteins and peptides. Considerable work has already been done on the separation of proteins and peptides by CE. Chen et al.<sup>73</sup> developed a simple method for the characterization of food proteins by CE. Major proteins in

chicken eggs and cow's milk were characterized and quantified by the CE technique. Wu and Regnier<sup>74</sup> utilized non-cross-linked linear polyacrylamide gels to perform a size-based sieving separation of SDS-protein complexes. The system could resolve model proteins differing by as little as 10% in molecular mass. The rapid separation of SDS-protein complex in different polymer media<sup>75-77</sup> (e.g., dextran and PEG) was performed, in which the migration speed is related to the protein molecular mass. Due to the low to moderate viscosities, the linear polymer networks could be replaced routinely.

Other modes of CE were also used for protein separation and analysis. A reproducible, quantitative isoelectric focusing capillary electrophoresis method was developed<sup>78</sup>. Strege et al.<sup>79,80</sup> evaluated protein separations in polyacrylamide-coated and C<sub>18</sub>-derivatized silica capillaries with electrophoretic buffers containing micelles. These conditions allowed simultaneous separation of acidic and basic proteins. Pederson and co-workers investigated the separation of three isoforms of *Serratia marcescens* nuclease using free solution CE, MEKC and capillary isoelectric focusing (cIEF)<sup>81</sup>. cIEF gave a resolution superior to MEKC, although both could be used to resolve the proteins; whereas free solution CE was unsuccessful in separation of these 3 isoforms.

CE is an excellent technique for peptide mapping, since only a very small amount of sample is required. Novotny et al.<sup>82,83</sup> developed a variety of different peptide-mapping schemes with emphasis on the procedures that can be done

with limited quantities of proteins. Three protein digestion reagents were used, trypsin, chymotrypsin and cyanogen bromide, to cut the protein at different site. They also used two different amino acid-selective fluorogenic reagents to derivatize the peptides. Both UV-absorbance and laser induced fluorescence detection were used to measure the peptides separated by CE. Other workers<sup>84,85</sup> developed the analysis procedures involving on-column digestion for peptide mapping. One of the procedures immobilized trypsin on the inner surface of a 50-cm length of fused silica capillary for on-line digestion of minute amounts of proteins. The trypsin modified fused silica microreactor was directly interfaced to a CE separation capillary for on-line digestion and mapping of picomole quantities of proteins. Chang and Yeung<sup>86</sup> utilized pepsin for on-column digestion of as little as 10 fmoles of  $\beta$ -lactoglobulin in 1.5 hr. This is the smallest amount of total protein used to produce a peptide map using native LIF detection.

The separation of small peptides by a different type of CE was investigated to optimize the separation conditions and to compare the different charge to size parameters used in correlating peptide migration. For a series of equally charged polyalanines, the best resolutions were achieved at low pH when a large metal ion such as  $Zn^{2+}$  was added into the buffer. The separation of peptides at pH 2.5 improved as temperature was decreased<sup>87</sup>. MEKC was proved to be useful for high resolution separations involving peptides with

similar electrophoretic mobilities. The micelles chosen either have very weak interactions with peptides<sup>88</sup> or are used together with organic modifiers<sup>89</sup> to prevent complete association of the peptides with the micelles.

### DNA Separation and Sequencing

Driven by rapidly sequencing the human genome, DNA separation and sequencing techniques have advanced at a very fast pace. Since DNA fragments have a very similar charge to mass ratio, it is extremely hard to separate them in free solution CE. A variety of water soluble polymers have been tried to promote the separation of DNA<sup>90</sup>. Basically, the polymer is dissolved into the running buffer to form a gel solution. After filling the capillary with the gel solution, the different length polymer chains form different size loops or holes which provide the size selection. When the DNA molecules are injected into the gel filled capillary, they are separated based on their sizes.

Agarose is a popular gel matrix for separating DNA. The selectivity of DNA separation in the agarose gel-filled capillary was found to be a complex function of the temperature<sup>91,92</sup>. The upper limit of the size range of DNA for separation in agarose solution was as high as 12 kilobases (kb). In a 1.7% agarose solution, the resolving power of DNA less than 1 kb in size is high. Resolving power for DNA larger than 1 kb increased when the agarose concentration was increased in the range 1.0 - 2.6%. Polyacrylamide is also a

popular gel matrix used to separate DNA samples<sup>93,94</sup>. With a polyacrylamide gel filled capillary and ethidium bromide as an intercalator, the DNA fragments from 72-1353 bp were well resolved within 12 min. Linear polyacrylamide was also tried as a sieving matrix for separating DNA fragments. Single-base adjacent peaks of FITC-labeled DNA sequencing fragments were resolved and detected up to for 520 base long fragments.

Other polymers were also used for separation DNA fragments. McGregor and Yeung<sup>95</sup> demonstrated that methyl cellulose was very efficient as a sieving agent for separating HAE III DNA fragments. Most recently, Chang and Yeung<sup>7</sup> found that linear polyethylene oxide (PEO) showed different resolving power for different sizes of DNA fragments, depending on the molecular weight of the PEO used. Based on the correlation between PEO molecular weight and resolving power, they developed a series of mixtures containing various molecular weights PEO to optimize the gel composition for the best resolution of DNA fragments and samples for DNA sequencing. There are several advantages for using PEO as gel matrix. First, the separation speed is faster than other gel matrices. Second, the viscosity of PEO is lower than other gel matrices such that it is easier to replenish the gel medium between runs. Third, the background is much lower than that for the other gel medium when using LIF for detection. Lately, Fung and Yeung<sup>96</sup> made a big breakthrough in using PEO for DNA sequencing. By treating the capillary with 0.1 M HCl before filling the capillary

with PEO gel solution, the speed for DNA sequencing is even faster while retaining resolution.

Capillary electrophoresis is also a popular method for analyzing polymerase chain reaction (PCR) products. A novel molecular technique<sup>97</sup> was developed for the identification of specific bacterial species by using CE to analyze the PCR amplified rRNA product from a mixture of bacteria. Gelfi et al.<sup>98</sup> detected a point mutation in PCR amplified DNA, based on capillary electrophoresis in sieving liquid polymers in the presence of temporal thermal gradients. In the case of an individual heterozygous for a point mutation, the expected four-band pattern is obtained. Capillary electrophoresis was feasible for detection of hybridization between synthetic oligonucleotides and HIV-1 genomic DNA amplified by PCR. The CE method for identifications of HIV-1 and HTLV-I PCR products appears interesting in light of its reproducibility, sensitivity and because it is fast and suitable for detection of DNA/DNA and DNA/RNA hybrids<sup>99</sup>.

### Single Cell Analysis

The capability of handling extremely small volumes of sample and the high separation efficiency inherent with CE make it a unique technique for analyzing single cells. Several groups have developed schemes for analyzing intracellular components in different cells. Jorgenson and co-workers

demonstrated the capability of CE to sample and analyze whole cells<sup>100</sup> and a detailed analysis of single cells by using open tubular liquid chromatography<sup>101,102</sup>. For quantifying different components, they utilized both LIF and electrochemical detectors to determine appropriate intracellular compounds, such as amino acids and neurotransmitters. Ewing et al. has developed several methods for the analysis of single cells with CE<sup>103-107</sup>. Capillaries with i.d. as small as 2  $\mu\text{m}$  have been used to separate cytoplasmic samples. Actually, since only one small portion of the cytoplasm is enough for one analysis, these represent subcellular measurements. A variety of neurotransmitters were quantified by electrochemical detection. They also demonstrated the capability of using on-column derivatization to determine several amino acids in single PC12 cells<sup>108</sup> and using on-line CE-mass spectrometry to identify several intracellular proteins in erythrocytes<sup>109</sup>.

The erythrocytes as the most well characterized cells<sup>110</sup>, with its intracellular components measured by analyzing large numbers of cells, from which the amount in one cell is calculated. The features obtained thus represent the average value. To analyze erythrocytes at the single cell level is very challenging in terms of the total amount of sample available for the analysis. The analysis of single red blood cells can provide more information for better understanding the functions and properties of individual cells. To date, different schemes have been established for determination of intracellular components in

the red blood cells<sup>111-115</sup>. Sodium (Na<sup>+</sup>) and potassium (K<sup>+</sup>)<sup>111, 113</sup> were measured with indirect fluorescence detection. By using laser induced native fluorescence detection, several major intracellular proteins were quantified<sup>112</sup>. Different variants of hemoglobin were determined in fetal, diabetic and normal red blood cells<sup>114</sup>. Derivatization proved to be applicable for analyzing intracellular glutathione (GSH)<sup>111</sup>. Particle counting technique could detect z mole ( $10^{-21}$  mole) levels of glucose-6-phosphate dehydrogenase (G6PDH)<sup>115</sup>. Several single cell analysis techniques were also developed in this dissertation work for analyzing different compounds, such as pyruvate lactate and lactate dehydrogenase (LDH). Chang and Yeung<sup>116</sup> demonstrated that native fluorescence detection and CE separation could be used to analyze single adrenal medullary cells. This method was successfully used to quantify epinephrine and norepinephrine in individual bovine adrenal medullary cells, which provides a promising method for the study of neurochemicals.

#### Analysis Associated with On-Column Reaction

If one short zone of the capillary with a given i.d. is used as a reactor, the volume of the reactor will be well defined, and very small since capillary i.d.s are usually 50  $\mu\text{m}$  or less. The fused silica capillary wall is quite inert which is important for carrying out a chemical reaction. The differences in electrophoretic mobilities for different species provide a chance to bring them



together and mix them in the nanoliter scale, which means that the microscale reaction is possible. Regnier and coworkers established an electrophoretically-mediated microassay (EMMA) scheme for analyzing different enzymes<sup>117-120</sup>. In this technique, the analyte and reagents are introduced into the capillary as distinct zones. Upon applying an electric potential, these zones electrophoretically merge due to differences in their electrophoretic mobilities. The reaction is then allowed to proceed within the capillary either in the presence or absence of an electric field. In the absence of the electric field, after mixing the analyte and reagent zones, the products will be accumulated in that mixed zone. Depending upon the reaction speed, a chemical amplification provided by the reaction will lead to a very sensitive detection of the analyte (e.g., a few hundreds of leucine aminopeptidase<sup>119</sup> can be detected), since usually one of the products or one of the reagents will be monitored for quantifying the analyte. In order to quantify the analyte in this method, the analyte has the catalytic property, and the reagents have to be maintained at a saturated concentration. At this condition, the reaction speed will only depend on the analyte available in this mixture. The most impressive analysis performed with this method is to analyze different enzymes<sup>117,119</sup>. The analysis of the substrates is also possible with on capillary reaction<sup>121,122</sup>. In this case, the substrates are usually compounds that do not have an appreciable property for direct measurement (e.g., alcohol or sugar).

INDIRECT FLUORESCENCE DETERMINATION OF LACTATE  
AND PYRUVATE IN SINGLE ERYTHROCYTES  
BY CAPILLARY ELECTROPHORESIS

A paper published in the Journal of Chromatography <sup>1</sup>

Qifeng Xue and Edward S. Yeung

**ABSTRACT**

A scheme of using fluorescein as the fluorophore for indirect detection of anions was demonstrated. This system is quite stable at a fluorescein concentration of 100 mM even without any other buffer components. Different injection modes affect the limit of detection (LOD). A LOD of about 20 attomoles was obtained for lactate under optimal conditions. Lactate and pyruvate in the intracellular fluid of erythrocytes were measured in this manner. The average amounts in a single erythrocyte for lactate and pyruvate are 1.3 and 2.1 femtomoles, respectively, or a ratio of 1.6 for pyruvate to lactate. Variations of the absolute amounts and the ratios are fairly large among a group of 27 cells examined. This is consistent with the difference of cells in size and composition. Although the migration times changed by up to 20% during a series of runs from the influence of concomitants in the cells, the migration time ratio was maintained around 1.072 with 3% relative standard deviation.

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## INTRODUCTION

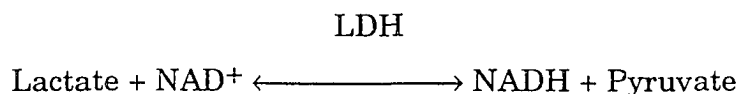
Indirect detection, as a universal detection approach for ions in capillary electrophoresis (CE), is widely used for the determination of both organic and inorganic compounds that do not possess a suitable detection property<sup>1,2</sup>. To a first approximation, this detection scheme is based on charge displacement between the analyte and a background ion. As many interesting compounds do not always possess a physical property suitable for direct detection, indirect detection became a popular scheme in both absorption<sup>3,4</sup> and fluorescence<sup>5,6</sup>. The limit of detection (LOD) for indirect detection is directly proportional to the concentration of the fluorophore used in the running buffer. By lowering the concentration of the background ions without sacrificing the dynamic reserve, the LOD can be further improved. Despite the impressive LOD obtained in Ref. (1), further improvement is necessary for the application of this detection mode to the determination of certain components at the single-cell level. Fluorescein is well-known for its high fluorescence efficiency<sup>7</sup>, and is conveniently excited by visible light. So far, little work has been done to optimize the conditions necessary for using fluorescein for the indirect detection of anions.

Determining the chemical and biochemical composition of a single cell can help to elucidate the detailed functions of some organisms. Potentially, it can provide useful information for diagnosis of diseases at an early stage. Much work has been done recently regarding the chemical analysis of individual cells because of the promising biomedical applications. Various schemes<sup>8-11</sup> were developed for determination of different components in different kinds of cells. The analysis of single neurons conducted by Olefirowicz et al.<sup>12</sup> and Kennedy et al.<sup>13</sup> showed the applicability of capillary electrophoresis to the analysis of individual

cells. Other investigations <sup>13-17</sup> demonstrated the quantitative determination of multiple constituents in individual cells coupled with electrochemical detection modes.

Recent work in this group centered around the determination of several components within single erythrocytes <sup>18-20</sup> with both direct and indirect fluorescence detection. The red blood cell is the smallest cell so far subjected to chemical analysis. The successful analysis of such a small entity demonstrated that laser-induced fluorescence (LIF) is by far the most sensitive approach for direct and indirect detection of intracellular components. The indirect detection method described earlier <sup>18,21,22</sup> provided a general approach for the quantitation of intracellular ions normally with undetectable properties. This is accomplished without the tedious steps associated with derivatization. It also avoids the problems of incomplete reaction and of slow kinetics at low analyte concentrations.

Lactate and pyruvate that exist in the red blood cell play an important role in the glycolysis process <sup>23</sup>. The determination of the amounts of each in a single erythrocyte should provide some information about the carbohydrate metabolism, as these are products during the carbohydrate substrate conversion process. Lactate and pyruvate convert to each other via the following reaction cycle:



This cycle also brings about the conversion of NADH to NAD<sup>+</sup>, and is catalyzed by LDH. The determination of pyruvate and lactate can further be correlated to the concentrations of NADH and NAD indirectly. Also it is possible to relate

these to the activity of LDH. Both lactate and pyruvate do not contain any physical properties amenable for sensitive detection. They are also not easily derivatized.

In the present work, the use of fluorescein as the fluorophore for the indirect detection of anions was evaluated. It was then used as the background ion for the determination of pyruvate and lactate in single human red blood cells. The variations in the amounts of pyruvate and lactate from cell to cell were depicted.

## EXPERIMENTAL SECTION

**Instrumentation.** The CE system used in this work is similar to that described before <sup>21,22</sup>. For single cell analysis, a 70 cm long, 55 cm to detector, 14  $\mu\text{m}$  I.D. and 350  $\mu\text{m}$  O.D. fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) and a high voltage power supply (Spellman Electronics Corp., Plainview, NY, USA) were used throughout the study. The running voltage was kept at 25 kV. An on-capillary detection window was created by burning off a short section of the polyimide coating. The cell injection end was conditioned by removing a 2-mm section of the polyimide coating for visual monitoring. About 3.2 mW of 330 nm laser light (after separation from the 350 and 360 nm lines) from an argon ion laser (Model Innova 90, Coherent, Palo Alto, CA, USA) was used for excitation. The laser beam was focused onto the detection region by a 1 cm focal length lens and the emitted fluorescence was collected using a 20X microscope objective lens at an angle of 90° to the laser beam. A spatial filter and a 400 nm long-pass filter plus an interference filter at 516 nm were used to eliminate the scattered light before imaging onto the photomultiplier tube

(PMT). The current produced was converted to voltage by an electrometer. Then, via a 24-bit A/D conversion interface (ChromPerfect Direct, Justice Innovations, Palo Alto, CA, USA), data was collected and stored on an IBM compatible PC/AT computer.

For evaluation of the fluorescein system, a modified setup was also used. The major change is that a visible argon ion laser (Model 2211-10SL, Cyonics, Uniphase, San Jose, CA, USA) was used. The laser beam was not focused in order to simplify optical alignment. Basically, the original laser beam with a diameter of about 1.5 mm directly irradiates the capillary at the detection window. Since indirect detection involves a fair background concentration of a fluorophore, the signal level is adequate even though only a few percent of the unfocused laser intensity is used.

**Cell Preparation and Injection.** Human erythrocytes were isolated from the fresh plasma of a healthy adult male. Usually 5 mL of plasma was obtained, with heparin and EDTA added to protect the red blood cells from coagulating during storage at 4 °C in a refrigerator for as long as 4 days. Before detection of the anions, the red blood cells were separated from the serum by centrifuging, and also were washed by a process similar to that described in Ref. 18. A different wash solution (8% glucose, 100  $\mu$ M fluorescein disodium salt, 2-hydrate with no further adjustments in pH) was used. Multiple washings were performed so that the cells were free from the extracellular ions.

The cells were suspended in the wash solution at a concentration of about 0.1% by volume. A 50- $\mu$ L droplet of cell suspension was mixed with an identical volume of running buffer already deposited on the glass microscope slide. An individual red blood cell was selected for injection by manually guiding the

capillary orifice close to the cell of interest with the help of the microscope and a 3-dimensional micromanipulator. A vacuum pulse produced by pulling a syringe connected to the ground end of the airtight buffer vial was applied to draw the cell into the capillary. The whole process was clearly monitored and easily controlled under 100X magnification. It is easy to push out any air bubbles from the capillary that are inadvertently introduced during the injection process by squeezing on the piston of the syringe.

In order to move the capillary orifice to the cell as close as possible, the injection end was etched to form a tip of about 50  $\mu\text{m}$  O.D. with HF using the similar procedure as reported in Ref. 17. By using the etched capillary, we can decrease the injected amount of the suspension solution, which was the main interfering component in this detection scheme. Injection of large amounts of suspension solution resulted in instability of the baseline. After a red blood cell was drawn into the capillary, the capillary was immediately moved back into the vial containing the running buffer. After 30 s to allow lysing, the separation begins. In the running buffer, erythrocytes typically lysed in a time shorter than 1 s. The fast lysis is desirable for the determination of intracellular components without extra manipulation. The 30 s waiting period allows the running buffer to mix with the suspension solution and to reach the cell.

**Injection of Standard Samples.** The standard samples were injected hydrodynamically at a height of 20 cm relative to the ground end. The hydrodynamic injection mode is comparable and consistent with the cell injection process. Also, it is not biased for ions with different mobilities and the injected amount is easier to control based on the Poiseuille equation.

**Reagents.** Sodium pyruvate was obtained from Fluka Chemica, Switzerland. Sodium lactate was purchased from Sigma (St. Louis, MO, USA). Fluorescein disodium salt, 2-hydrate ( $C_{20}H_{10}Na_2O_5 \cdot 2H_2O$ , MW 412.3) was purchased from Eastman Kodak (Rochester, NY, USA). Other chemicals were obtained from Fisher Chemical (Fair Lawn, NJ, USA).

**Solutions.** Running buffer was made with 1% glucose (W), 100  $\mu$ M fluorescein disodium salt, 2-hydrate, and 500  $\mu$ M Tris with pH 8.5 without further adjustment. The standards were dissolved in the running buffer. For the evaluation of fluorescein, different running buffer solutions were used as described below. All solutions were prepared in deionized water and were filtered with a 0.22  $\mu$ m filter before using.

## RESULTS AND DISCUSSION

**Evaluation of Fluorescein Performance.** Since fluorescein is a highly efficient fluorophore, we expect that even at low concentration a stable baseline can still be obtained for small capillaries. For a new bare capillary and buffer solutions without CTAB, a positive system peak comes out very early at the running conditions tested. After that the baseline becomes quite stable and clean. When using a DB-1 capillary (J&W Scientific, Folsom, CA, USA) the system peak shows up much later, as the capillary coating and the low concentration of CTAB (20  $\mu$ M) lead to a slow electroosmotic (EO) flow rate. The system is also quite stable and clean. The relationship between anion migration and EO flow is different for these two conditions. In first case, the anions swim upstream. The EO flow rate is greater than the electrophoretic rate so that the anions are carried to the grounded buffer vial. The more mobile anions will



come out later. However, for the second case, the capillary walls are positively charged, leading to EO flow and anion migration in the same direction. The more mobile anions will come out earlier. Both systems are suitable for the detection of common anions.

Fig. 1 shows that 8 anions were nicely separated, except that nitrate and iodide coeluted. Corresponding concentrations and migration times are listed in Table 1. A dynamic reserve of  $> 400$  is easily obtained when using fluorescein as the fluorophore at this low concentration. This is a promising feature for the detection of anions. However, the LOD in indirect detection is not simply determined by the concentration of the background fluorophore and the dynamic reserve. The displacement ratio is also an important factor that affects the LOD. The problems with fluorescein are the relatively large MW and the double charge at pH 8.5, which may degrade the LOD. From the pyruvate peak area and peak height, the estimated displacement ratio is 0.2 to 0.08, which is dependent on the buffer components and the capillary size. The buffer components can introduce additional interaction between the analytes and the fluorescein ions. The effect of capillary size is not clear. Generally, simpler systems (using only 100 mM fluorescein as the running buffer) and smaller capillaries give larger displacement ratios.

Table 2 lists the LOD for different experimental conditions. Certain conditions provide improvements in LOD compared to that reported previously<sup>1,22</sup>. Even though different anions were tested, the LODs are comparable because the MW are similar and the charge is the same. The lowest LOD was obtained for electromigration injection. The lowest LOD was obtained for electromigration injection. A possible reason is that the total injection period is

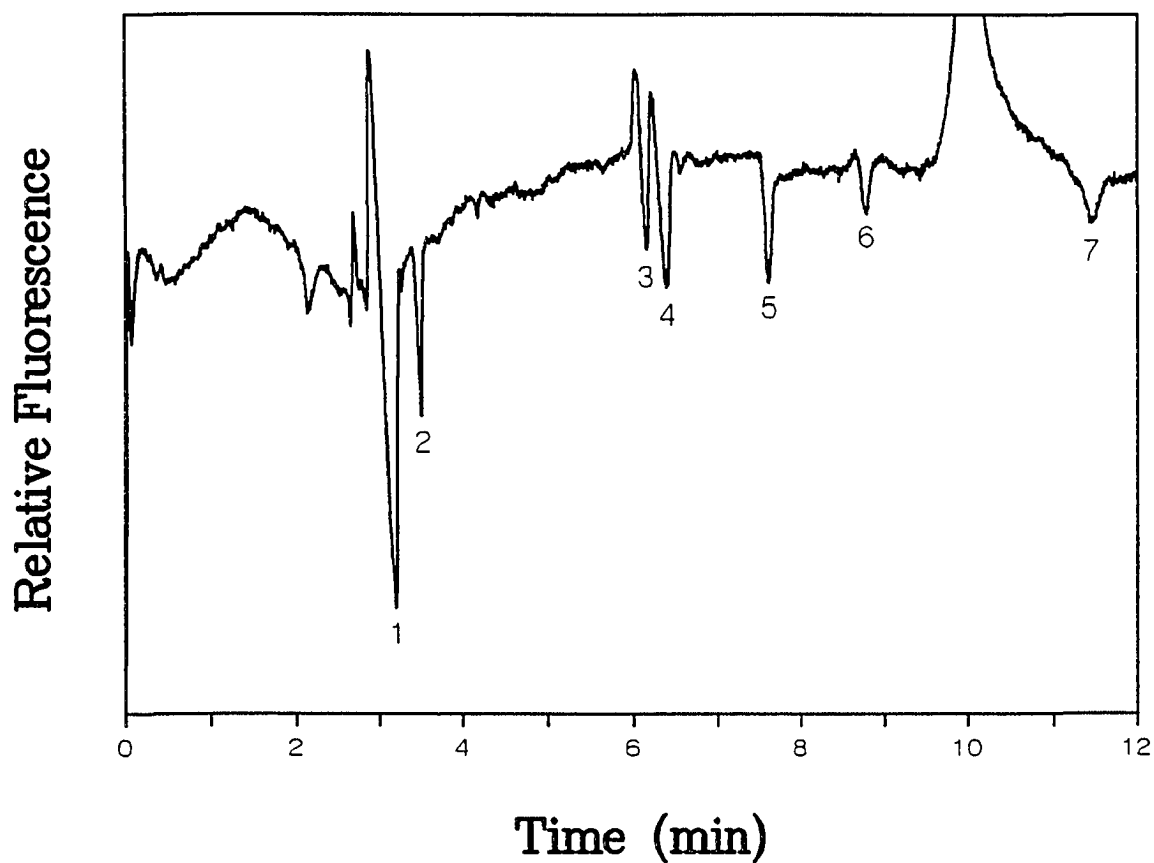


Figure 1. Electropherogram of anion separation. Concentrations and migration times are listed in Table 1. 50- $\mu$ m DB-1 capillary, 76 cm long and 44 cm to detector. Running voltage: 30 kV. Injection: 30 kV, 0.5 s. Buffer: 100  $\mu$ M fluorescein and 20  $\mu$ M CTAB.

much shorter than hydrodynamic injection when injecting the same volume of sample. Therefore the baseline disturbance is smaller in magnitude. However, for electromigration injection, the separation is not as good as that obtained for hydrodynamic injection. The LODs listed in Table 2 confirm the applicability to the detection of pyruvate and lactate in single red blood cells.

Table 1. Concentrations and migration times for the separation shown in Fig. 1

No.	Anion	Concentration $\mu\text{M}$	Migration Time S
1	Nitrate	47.2	191.6
	Iodide	5.1	91.6
2	Chlorate	17.8	210.1
3	Pyruvate	34.5	371.6
4	Acetate	23.3	385.4
5	Benzoate	20.1	460.4
6	Lactate	18.8	533.1
7	Glutamate	16.3	698.1

Table 2. Absolute LOD ( $10^{-16}$  moles) under different operating conditions.

	1	2	3	4	5	6
Pyruvate	4.49	1.58	1.49	0.68	0.32	1.69
Lactate	*	1.35	1.23	0.88	0.21	1.05
Glutamate	7.51	*	*	1.36	0.34	1.40

\*not measured

Conditions:

1. Buffer: 100  $\mu$ M fluorescein and 20  $\mu$ M CTAB, pH 6.3.  
Capillary: DB-1, 350  $\mu$ m O.D., 50  $\mu$ m I.D.; length 76 cm and 44 cm to the detector.  
HV: 30 kV. Injection: 30 kV, 0.5 s. Laser: unfocused 488 nm beam.  
Sample was dissolved in running buffer.
2. Buffer: 100  $\mu$ M fluorescein, 1% glucose and 500  $\mu$ M Tris, pH 8.5.  
Capillary: bare fused-silica, 362  $\mu$ m O.D., 14  $\mu$ m I.D., length 70 cm and 55 cm to the detector.  
HV: 25 kV. Injection: gravity 20 cm, 30 s. Laser: focused 330 nm beam.  
Sample was dissolved in 8% glucose and 100  $\mu$ M fluorescein.
3. Same as 2 except that the sample was dissolved in the running buffer.
4. Same as 3 except that the running buffer did not contain glucose.
5. Same as 4 except that injection was conducted by electromigration at 25 kV, 0.5 s.
6. Buffer: 250  $\mu$ M sodium salicylate, pH 6.0. Other conditions were the same as in 4.

**Determination of Intracellular Pyruvate and Lactate in a Single Erythrocyte.** Bare fused-silica capillaries (14  $\mu\text{m}$  I.D.) were used because this size is suitable for injection of cells with a diameter of 8-9  $\mu\text{m}$  and a volume of 53-87 femtoliters <sup>24</sup>. There is only a small dilution effect after the lysis of the cell and optical alignment is not very tricky. Also, it allows a reasonable vacuum injection time (< 15 s) for introducing one cell into the capillary without drawing in too much extracellular fluid. The separation efficiency and detection sensitivity are still well maintained for both fluorescence detection <sup>21</sup> and electrochemical detection <sup>25</sup>. The results shown in Table 2 provide support for selecting small bore capillaries for single-cell studies.

Laser-induced fluorescence (LIF) <sup>19,26</sup> provides the high sensitivity needed for small amounts of sample when the analyte possesses natural fluorescence. Unfortunately, most ionic components existing within a single erythrocyte do not have appreciable natural fluorescence except proteins, DNA and some amino acids. In-vivo derivatization <sup>18</sup> is one of the possibilities for detecting certain compounds. Nevertheless, the tedious process and possible contamination during treatment are formidable challenges for applying that to such a tiny organism as the red blood cell. Some kind of a specific biosensor is an alternative. The high selectivity however limits its applicability for multiple component analysis. The indirect detection approach <sup>1,18</sup> proves to be practical for determining the ionic analytes inside a cell. Even though it is far from being a perfect method, especially near the LOD due to instability of the baseline and system peak interference, the advantages of easy operation and suitability for monitoring compounds without specific functionality make this a unique technique for the analysis of single cells.

We might expect that many peaks would be present in the electropherograms if the amounts of typical anions within the red blood cell were higher than the LOD obtained in this work (Table 2). In actual fact most of them are at concentrations well below the LOD. The potentially troublesome components were proteins if they could displace enough fluorescein to produce peaks. At the pH and the low concentration of buffer used in this system, proteins interact strongly with the capillary wall and adsorb there. Also, generally the large biomolecules have poor displacement ratios leading to poor LOD<sup>28</sup>. This is in contrast to operation at pH 10 with native fluorescence detection<sup>29</sup>. Actually, for the low absolute amounts of proteins in a cell, they could not produce significant interferences except for changing the EO flow rate due to adsorption. According to Table 2, only pyruvate (~1.29 femtomoles) and lactate (~0.78 femtomoles)<sup>30</sup> are at detectable levels in a single erythrocyte.

Preliminary results showed that the high concentration of glucose used in the cell suspension solution caused an unstable baseline. The disturbance was so strong that no recognizable peak was obtained. To reduce the disturbance, 1% glucose was added into the running buffer. Also, the capillary was equilibrated for 24 hours at the same HV before the analysis of single cells. The system became quite stable and could tolerate the injection of a high concentration of glucose. Furthermore, the mixing of an equivolume of running buffer with the cell suspension on the microscope slide before injection of a cell decreased the actual concentration of glucose to about 4% and reduced the disturbance. Examination under a microscope shows that red blood cells in 4% glucose can last up to 6 hours with no obvious hemolysis. Naturally, leaving cells in the glucose solution for a long time can promote the leakage of

intracellular components. Our experiments are performed immediately after washing of the cells to protect pyruvate and lactate from leaking out.

Figure 2 shows the electropherograms for injection of the running buffer (A), cell suspension solution (B), standards (C), a single cell (D) and a lysed-cell suspension (E). The clean background in (A) and (B) demonstrates a desirable condition for the detection of anions. Considering the detectable components in the cell as well as the consistency of migration times for the standard samples and the cell, one can attribute the two peaks in (D) and (E) to lactate and pyruvate respectively. The stability of the baseline for single cell analysis is dependent on the amount (volume) of suspension solution drawn into the capillary. Sometimes a system peak appeared at the position of the lactate peak, which affects the precise quantitation of intracellular lactate. Therefore, minimizing the volume of the suspension solution injected is crucial for the determination of lactate. With the HF-etched fine tip, the injection orifice can be more easily moved to approach the cell of interest without pushing it away. The other approaches used for controlling the excess injection volume are to use a slightly more concentrated cell suspension and to apply the vacuum pulse gradually after the orifice is near the selected cell.

Nevertheless, the carefully manipulated injection process is still not good enough to make every run informative. Some other factors can make the system unstable, e.g. shaking when moving the capillary in and out of the buffer vial, etc. Several electropherograms for cell separation are shown in Figure 3. Variations among these trials are presumably due to the individual cell volumes

Figure 2. Electropherograms for the analysis of the running buffer (A), cell suspension solution (B), standards (C), a single red blood cell (D) and lysed-cell suspension (E). Peaks 1 and 2 refer to lactate and pyruvate respectively.



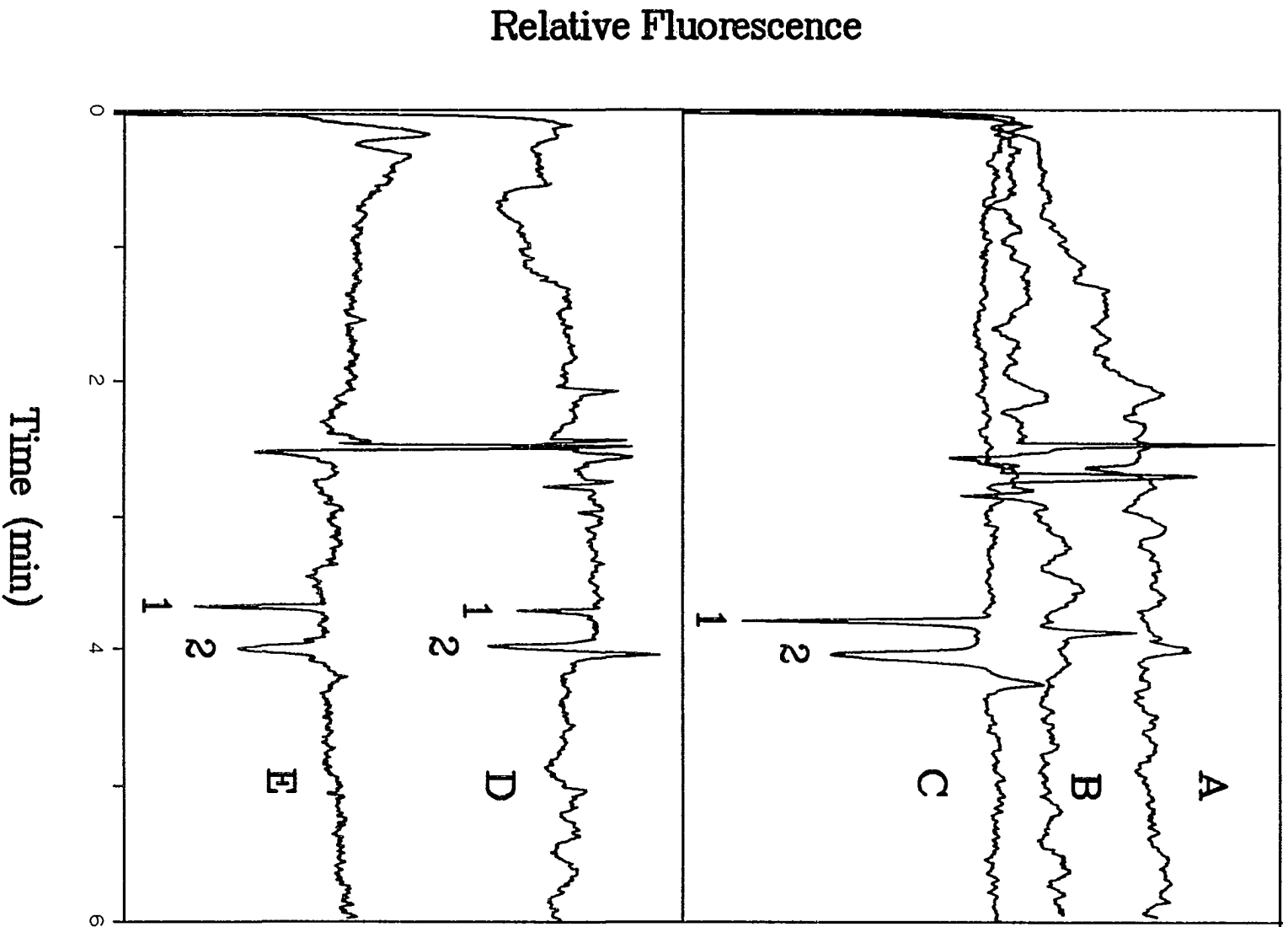
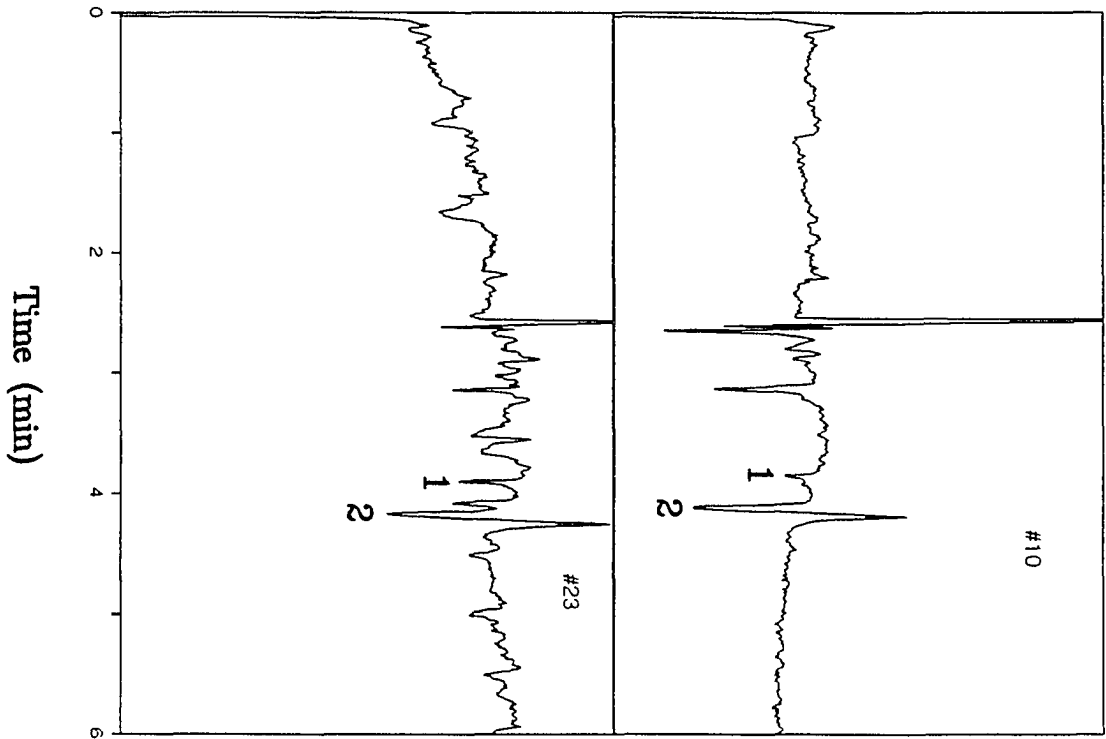
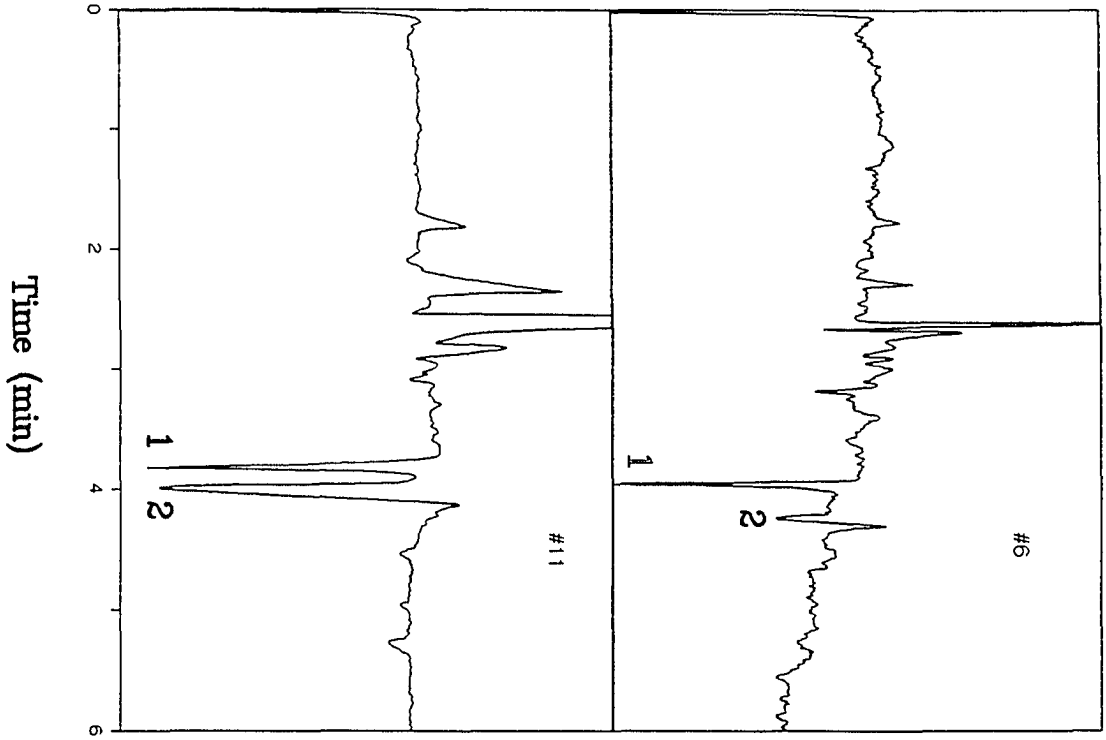


Figure 3. Electropherograms for the analysis of individual red blood cells.  
Numbers (6, 10, 11, 23) refer to chronological order of the experiments.  
Peaks 1 and 2 refer to lactate and pyruvate, respectively.

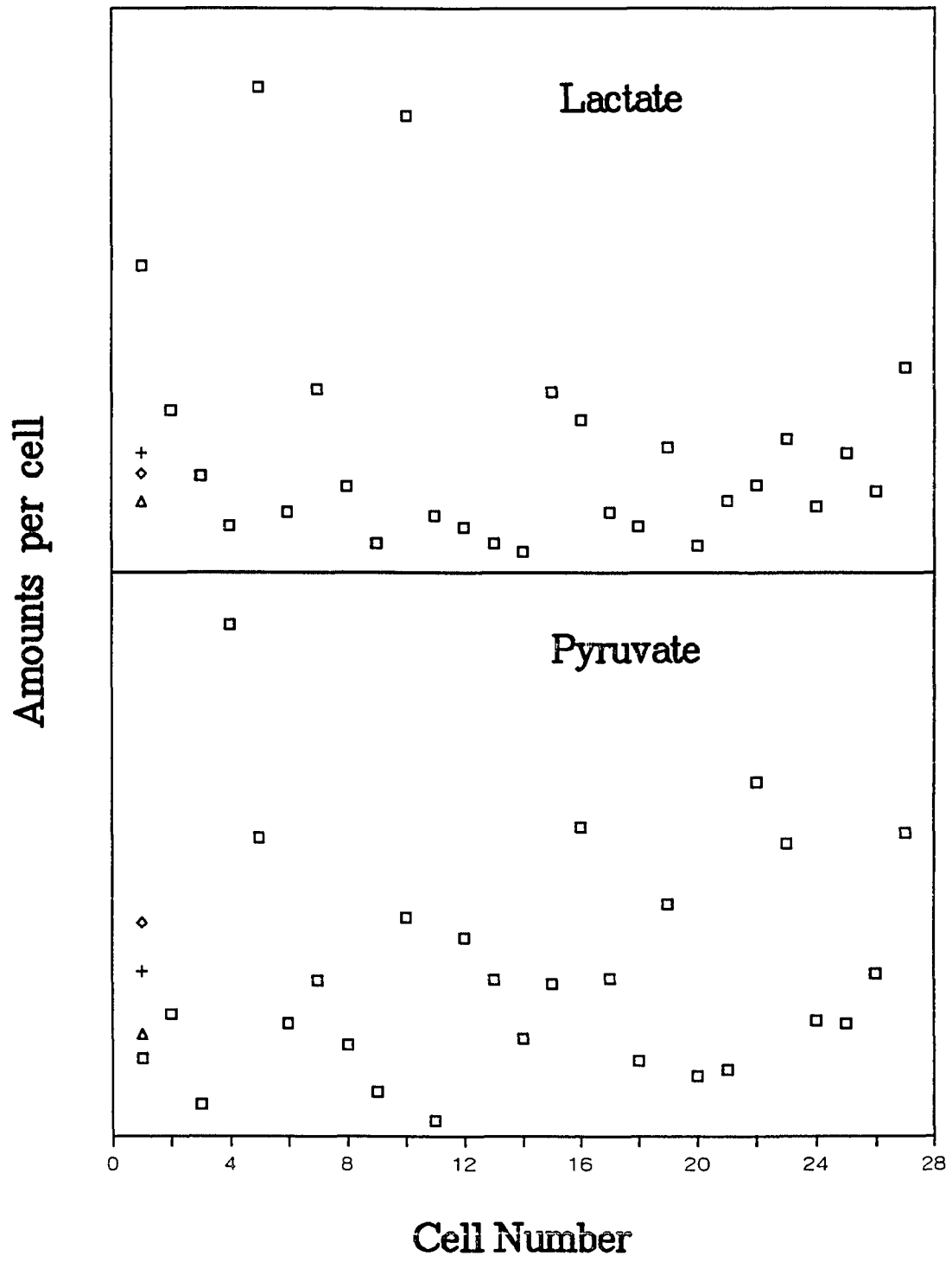
Relative Fluorescence



and actual compositional differences. Runs #11 and #23 show other detectable components, which might denote unusual cells. According to the analysis of whole blood extracts <sup>31</sup>, the chemical composition of normal cells are dramatically different from those of abnormal cells. The common feature for these runs are the peaks for pyruvate and lactate.

In total, 32 cells were analyzed consecutively. Due to unpredictable disturbances, 5 runs were affected by serious baseline jumps and shifts. Therefore, those 5 data files were eliminated from further processing to quantify the individual components. A significant feature is that the individual cells are very different from each other with regard to the contents of pyruvate and lactate, as shown in Fig. 4. This is consistent with earlier observations <sup>18,19</sup>. One of the reasons for the large variability among these results may be that the red blood cells have significant differences in content, since volume and age variations can lead to compositional differences. In fact, lactate and pyruvate reflect enzyme activity that can be very different among cells. Another possibility is associated with the stability and accuracy of the indirect detection <sup>3</sup>. Owing to the low concentration of fluorescein used here, the reproducibility is not very good. Up to 40% deviation is observed sometimes, while typical deviations are below 20%. Another possible cause is the stability of the column after several hours of running. From the result of standard samples examined after 32 cell analyses, the peak area and height of 4 injections yielded a 20% deviation compared to the same standard samples injected before the analysis of cells. The deviation was still within the individual precisions for standard injections. This indicates that the low concentration buffer and the adsorbed material do not damage the capillary. Also, no obvious decreasing or increasing

Figure 4. Distributions of lactate and pyruvate in individual human red blood cells over a series of 27 trials.  $\square$  refers to the amount in individual cells.  $\Delta$ ,  $+$  and  $\diamond$  at the left of each frame denote the literature value, average for all 27 cells, and the amount measured from a lysed-cell suspension, respectively.



trend was observed in Fig. 4. The average intracellular contents of pyruvate and lactate are found to be 2.1 femtomoles and 1.3 femtomoles respectively (a ratio of 1.6 for pyruvate to lactate). These values are different from the literature values of 1.29 and 0.78 femtomoles (a ratio of 1.6) for pyruvate and lactate respectively. The ratio of pyruvate to lactate for each cell is shown in Fig. 5. A considerable variation was observed even though this is a cell-size independent quantity.

The migration time is always a useful marker for identifying the analyte in capillary electrophoresis. The migration times for pyruvate and lactate are shown in Fig. 6 for each cell injection. We note there is no significant change with run number except for the first several runs, where there is a change of up to 20%. A longer equilibration time between runs leads to a smaller change in the migration times. We conclude that the change in migration time is mainly caused by changing  $\zeta$  potential due to the injection of cells, because the running voltage and the buffer ionic strength are kept constant for the entire set of runs. Fortunately, the system peak can be used as an internal standard for migration time calibration and for identifying the sample peaks. The ratios of the migration times for pyruvate and lactate are almost constant, as shown in Fig. 7, with an average of 1.072 and a standard deviation of only 3%. For standard samples, the ratio is 1.078. This provides positive evidence for identification of the two peaks as pyruvate and lactate.

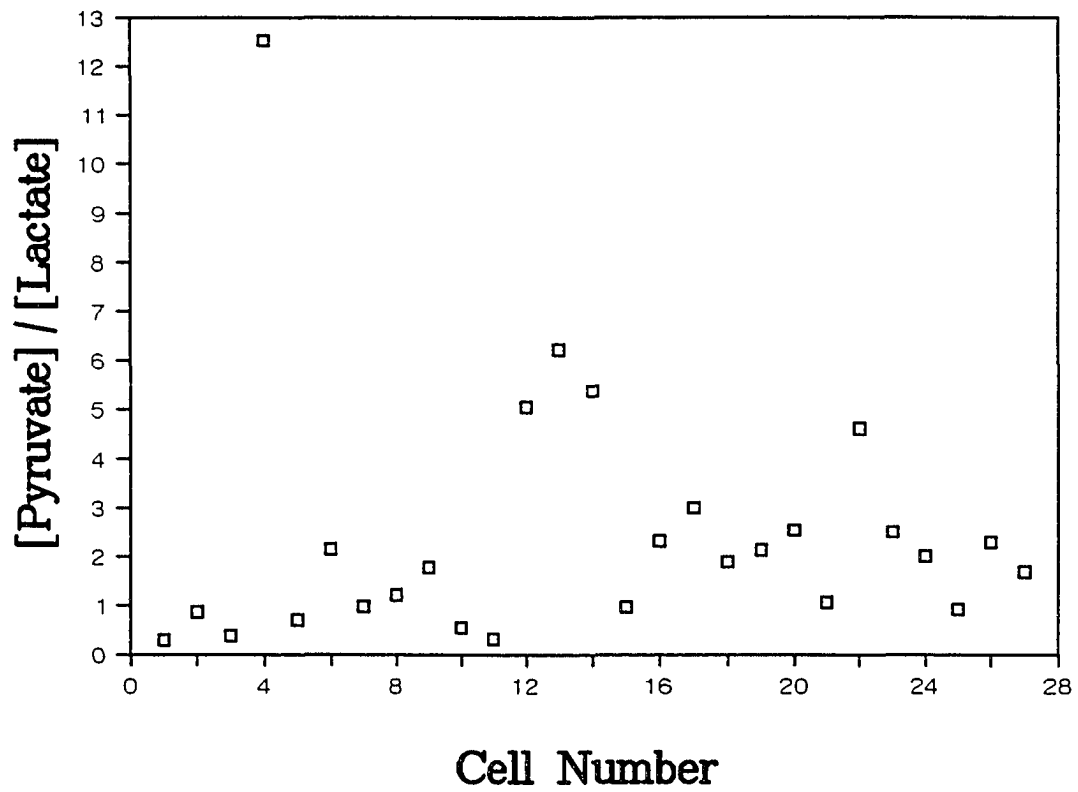


Figure 5. Ratio of pyruvate to lactate in individual red blood cells for 27 trials.



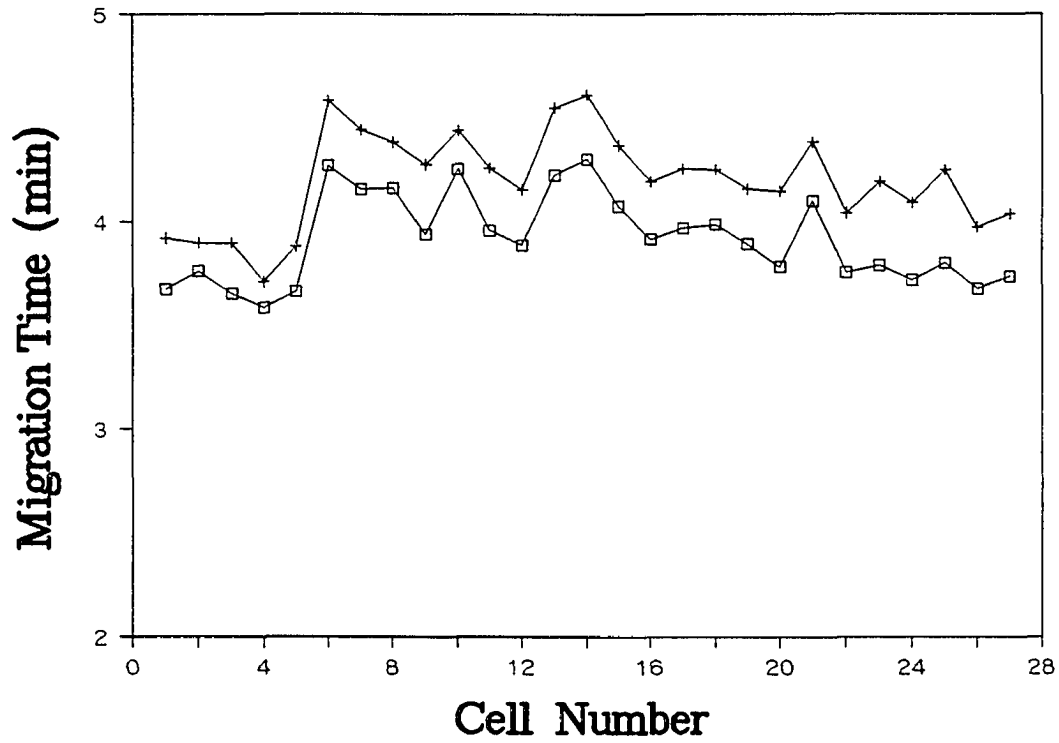


Figure 6. Changes in migration times over the entire experiment.

□ = lactate; + = pyruvate.

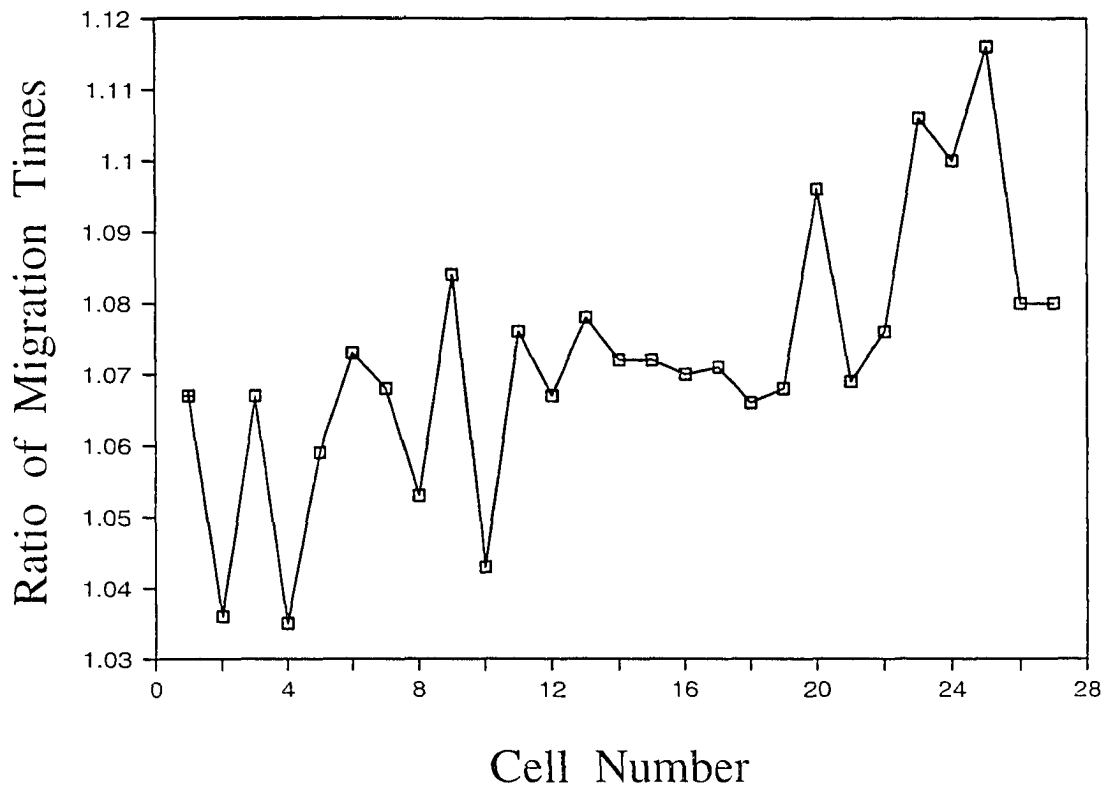


Figure 7. Ratios of migration times for pyruvate to lactate for 27 runs.

## CONCLUSIONS

We have demonstrated the advantages of using fluorescein as the background ion for the indirect detection of anions. The system shows good stability at 100  $\mu$ M fluorescein. LOD of 20 attomoles for lactate was achieved. For the best performance in indirect detection, good equilibration is necessary before running the samples. Fluorescein can also be excited by visible laser sources, paving the way for a more compact and a less costly instrument.

The separation and determination of intracellular anions show an important application of this detection approach in the study of mammalian cells. Variations observed from cell to cell should be useful in understanding the biological functions of different components in cells, and perhaps can help to determine whether cells are normal. This scheme might allow the determination of NADH, NAD or LDH by measuring the amounts of pyruvate and lactate, because these are the products of catalysis by those enzymes. This kind of correlation requires very accurate determinations of pyruvate and lactate. Further improvements in stability and reproducibility for this system would thus be necessary to reach this goal.

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VARIABILITY OF INTRACELLULAR  
LACTATE DEHYDROGENASE ISOENZYMES  
IN SINGLE HUMAN ERYTHROCYTES

A paper published in the Analytical Chemistry<sup>1</sup>

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**ABSTRACT**

Trace amounts of enzymes within single human erythrocytes can be quantified by a combination of on-column reaction and capillary electrophoresis. A detection limit of  $1.3 \times 10^{-21}$  moles of LDH was achieved with laser-induced fluorescence by monitoring the product of the enzyme catalyzed reaction between lactate and  $\text{NAD}^+$ . Single erythrocyte analysis clearly isolates the major forms of LDH. The variation of total LDH activity in a population of cells from a single individual is large but the relative activities of the isoenzymes LDH-1 and LDH-2 are fairly constant. This can be explained by the distribution of cell age in the population. A lower enzyme activity is indicative of senescence. The efficient separation of different LDH forms and the high detection sensitivity opens up the possibility of multiple-enzyme assays with a single mammalian cell.

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## BRIEF

Laser-based fluorescence enzyme assay is developed to detect  $10^{-21}$  moles of lactate dehydrogenase, so that intracellular levels in single erythrocytes can be quantified.

## INTRODUCTION

Besides being intimately related to glycolysis, lactate dehydrogenase (LDH) activity of blood can be used for the diagnosis of liver disease, myocardial infarction, etc. <sup>1</sup>. The determination of LDH in serum is possible with different methods <sup>2-7</sup> in the clinical laboratory. The average LDH activity in erythrocytes has also been measured in hemolysate <sup>3</sup>. The quantification of intracellular LDH activity in individual cells should result in unique information about chemical and biological functions, and help to elucidate whether the cell is in a normal state. Because human LDH is an isoenzyme with five different forms with activities related to metastatic cancers <sup>8</sup> and to cell age <sup>9</sup>, their separation and individual determination may have broad implications.

Capillary electrophoresis (CE) has recently emerged as a powerful separation technique <sup>10,11</sup>. Sub-nanoliter sample volume is an inherent feature of small (5-20  $\mu\text{m}$  i.d.) capillaries <sup>12,13</sup>. CE has already been applied to single-cell studies. Larger cells such as snail neurons or adrenal medullary cells have been probed in conjunction with electrochemical detection <sup>14-18</sup>. Recent progress in single erythrocyte analysis proved that laser-induced fluorescence (LIF) coupled to CE <sup>19</sup> is suitable for the determination of intracellular  $\text{Na}^+$  and  $\text{K}^+$  <sup>20</sup>, glutathione<sup>20</sup>, lactate and pyruvate <sup>21</sup>, and the major proteins <sup>22,23</sup>. However, the components studied so far are those at relatively high concentrations inside the cell. Most of

the several thousand other components in erythrocytes are still inaccessible by the above schemes. In particular, enzymes exist in individual cells in amounts well below attomole levels. Their analysis and characterization in single mammalian cells remain a challenge.

Usually, determining the activity of an enzyme is more important than determining its amount. Hence, enzymes are commonly quantified by their biological activity under selected conditions. As a catalyst, the enzyme is not consumed during reaction, which then provides amplification of the signal with prolonged reaction time. In recent reports <sup>24-26</sup>, Regnier and co-workers utilized capillary electrophoresis to perform enzyme assays. We have modified their approach to allow sensitive detection of LDH in single erythrocytes by using LIF.

### EXPERIMENTAL SECTION

Red blood cells were obtained from a healthy adult donor. Pre-added heparin and EDTA kept the blood from coagulating for several days at a temperature of 0 to 4°C. Before analysis, the cells were washed 8 times with 135 mM NaCl and 20 mM phosphate buffer at pH 7.4, following the same procedure as described previously <sup>23</sup>. The washed cells can be used for several hours afterwards. Cells were suspended in the same wash solution just before injection so that leakage can be neglected. Single cell injection into the capillary <sup>20,21</sup> was monitored under a 100X microscope, and was accomplished by applying a vacuum pulse to the distal end of the capillary with the help of a syringe connected to an air-tight buffer vial.



The experimental set-up is almost the same as before <sup>20</sup> except that a 380 nm cutoff filter and a 480 nm interference filter were used. To limit dilution of the intracellular components, 20  $\mu\text{m}$  i.d. capillaries were used for the separation. Total capillary length is 65 cm and the distance to the detection window is 50 cm.

Detection was based on the large difference in fluorescence efficiency between one of the products, NADH, and all other components in the solution. NADH absorbs light with a maximum at 340 nm, and fluoresces with a maximum at 480 nm. Detection at attomole levels can be easily achieved. Also, this enzymatic reaction is quite fast, with a turn-over number of about 1000 per s <sup>27</sup>. The lactate to pyruvate conversion, in the presence of  $\text{NAD}^+$ , was used to measure LDH activity, as opposed to the normal function of LDH to convert pyruvate to lactate, since  $\text{NAD}^+$  gives a low fluorescence background that is critical for measuring small signals.

The buffer contains 5 mM lactate, 5 mM  $\text{NAD}^+$  and 30 mM phosphate at a pH of 7.3. From published data <sup>3</sup>, at a pH of 7.2 to 7.6 LDH has the highest activity. With these concentrations of substrates, the LDH catalyzed reaction is expected to be pseudo-first order, since the LDH activity is only about 3 nIU <sup>28</sup> in a single erythrocyte. Further, contributions from intracellular lactate, pyruvate,  $\text{NAD}^+$ , and NADH can be neglected. At a given temperature and reaction time, the NADH amount formed is thus directly proportional to the LDH activity. We used a 4-step procedure to measure the LDH activity. First, the standard solution or a single erythrocyte was injected into the capillary. Second, 30 kV was applied for 1 min to separate the different LDH forms into different zones based on their electrophoretic mobilities and to allow them to

migrate into the regions containing the substrates. The red blood cells are easily lysed in the running buffer because of the low ionic strength, requiring only 1 to 2 s of contact as confirmed visually under the microscope. Third, the high voltage was turned off for 2 min. During this incubation period, NADH is accumulated in the different LDH zones due to their enzymatic activities. Finally, high voltage was reapplied to elute the components past the detection window. For the standard solutions, hydrodynamic injection was used and the injected amounts were quantified with the Poiseuille equation. The enzymatic reaction was carried out at room temperature (21°C). No effort was made to measure the internal temperature of the capillary, since the small i.d. capillary is not expected to generate substantial Joule heating.

## RESULTS AND DISCUSSION

The electropherogram for LDH-1 standard is shown in Fig. 1. The step-like increase in background around 7 min is caused by the continuous formation of NADH when LDH moves along the capillary, as discussed earlier<sup>25</sup>. NADH moves slower than LDH under these conditions, leading to an accumulation peak at the trailing edge of the increased background<sup>25</sup>. After the accumulated NADH is eluted, the baseline returns to the original level, which confirms that LDH does not adsorb significantly on the capillary walls. A limit of detection (LOD) of  $1.3 \times 10^{-21}$  moles or 58 pIU LDH was achieved. LDH-1 standards exhibit a linear response from 0.3 to 30 nIU with  $r^2 = 0.995$  while NADH standards exhibit a linear response from  $3 \times 10^{-7}$  M to  $7.5 \times 10^{-5}$  M with  $r^2 = 0.9999$ . Either standard can be used to quantify the LDH activity, which is usually defined with the units of mmoles NADH consumed per min at a certain

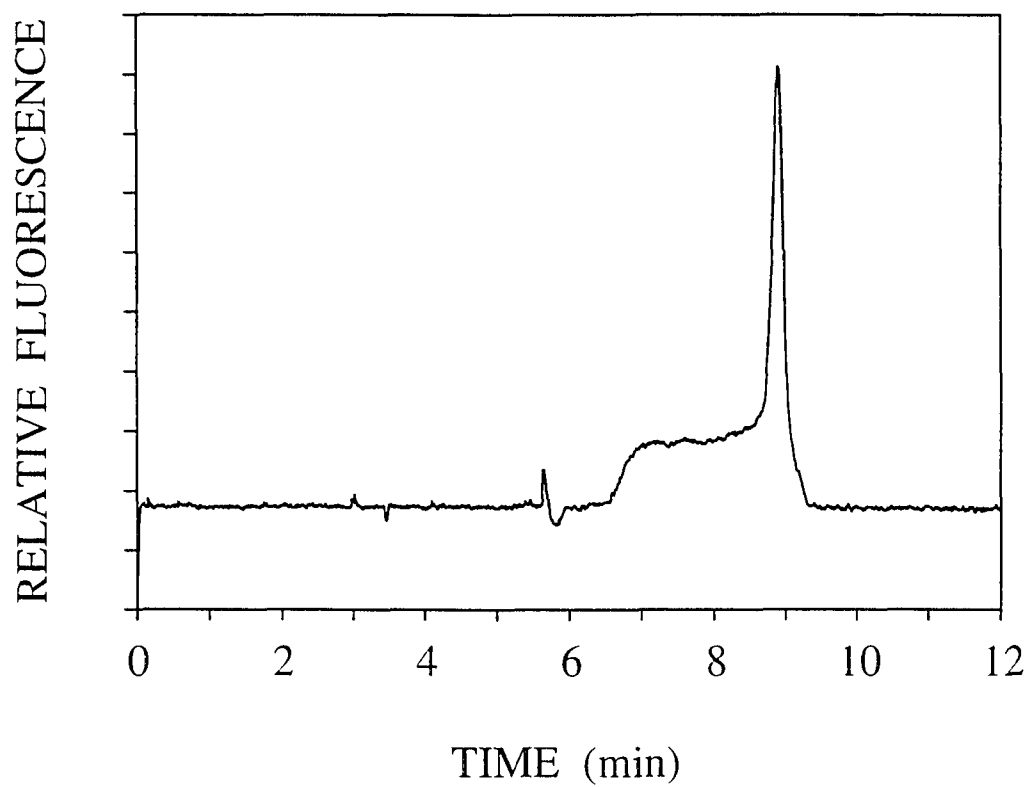


Figure 1. Electropherogram of LDH-1 standard (29.5 nIU).

temperature. Here, the activity for catalyzing the reverse reaction is being measured.

The mixture of LDH-1, LDH-2 and LDH-3 in both a standard solution and a lysed-cell sample was nicely separated. The same features are clearly present in both Fig. 2a and b. This migration order was established by running the individual components separately, and is consistent with slab gel electrophoresis<sup>17</sup>, in which LDH-1 moves fastest towards the anode<sup>29</sup>. Here, each component is brought to the cathode by electroosmotic flow<sup>10</sup> even though the electrophoretic motion of each is towards the anode. The electropherograms of LDHs are shown in Fig. 3 for different separation times before incubation. The spacings between the isoenzymes become larger with increasing separation time. Also, a fourth peak is recognizable for runs with longer separation times, which probably corresponds to LDH-4 or LDH-5. We are not able to positively identify that peak since standards were not available.

The results of analyses of single cells are shown in Fig. 4. Typically, 2 or 3 distinct peaks are recorded for an individual erythrocyte. As stated before<sup>30,31</sup>, LDH-1 (30-40%) and LDH-2 (40-45%) are the major forms of LDH in red blood cells, and LDH-3 (14-16%) is present at a relatively low amount. It is reasonable that not every electropherogram clearly shows LDH-3 as a well defined peak, as in Fig. 4a. For 3 of the total of 36 cells examined, a fourth peak is observed (Fig. 4d and e). This extra feature may indicate the occasional presence of a high LDH-4 or LDH-5 content compared to normal cells, which typically contain these isoenzymes at 3-5% and 2% respectively. Four runs did not result in the clean separation of LDHs, even though the total accumulated NADH amount is quite high. A possible reason is that the injected cell did not

Figure 2. Electropherograms of a LDH standard mixture (a) and LDH in lysed red blood cells (b). The standard solution contains 12.3 nIU LDH-1 (1), 6.7 nIU LDH-2 (2) and 10.2 nIU LDH-3 (3). The amount of lysed cell injected is equivalent to about 9 cells.

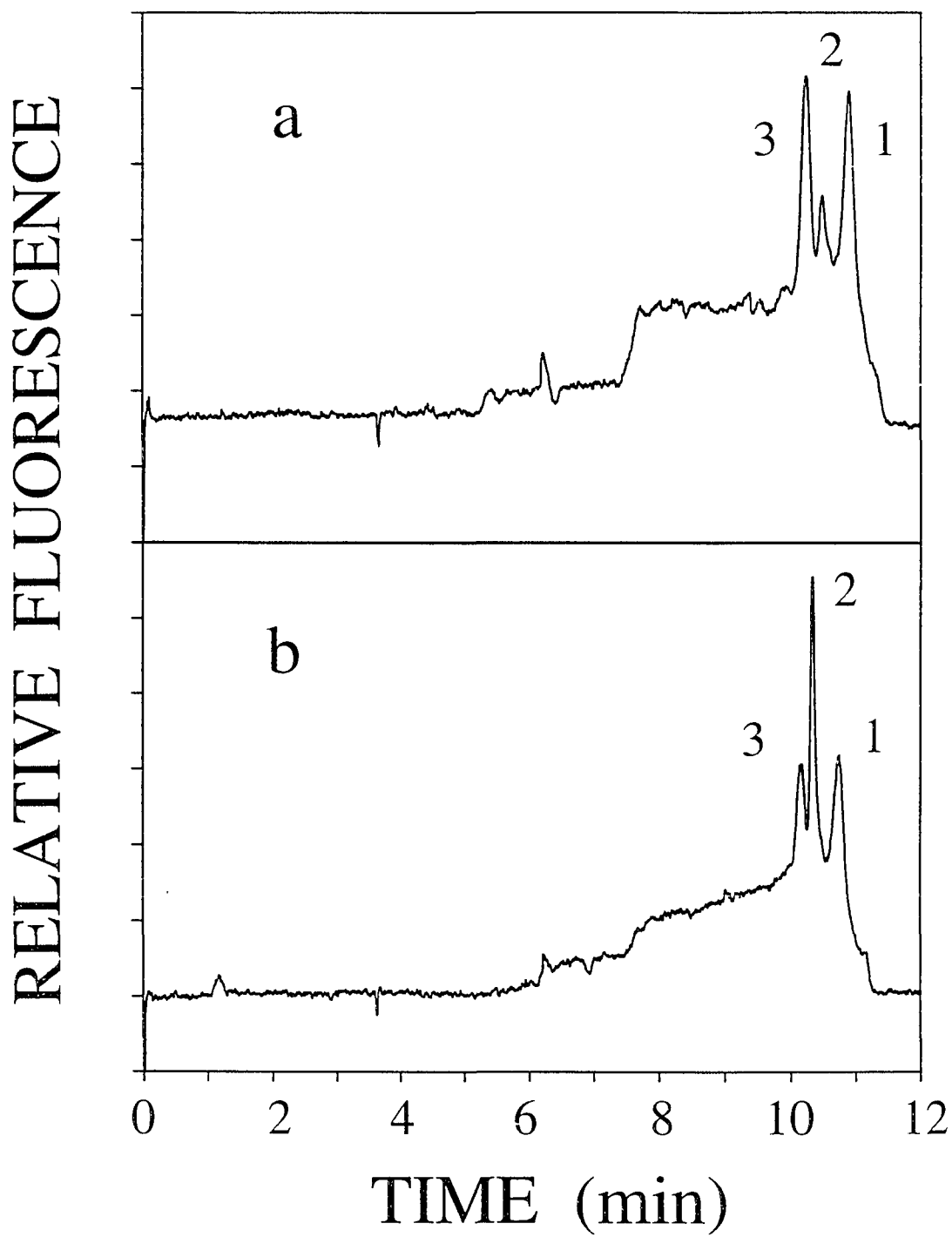


Figure 3. Electropherograms of LDHs in lysed red blood cells with different separation times before incubation. (a) 1 min, (b) 2 min, and (c) 3 min. Peak 4 is probably LDH-4 and/or LDH-5.

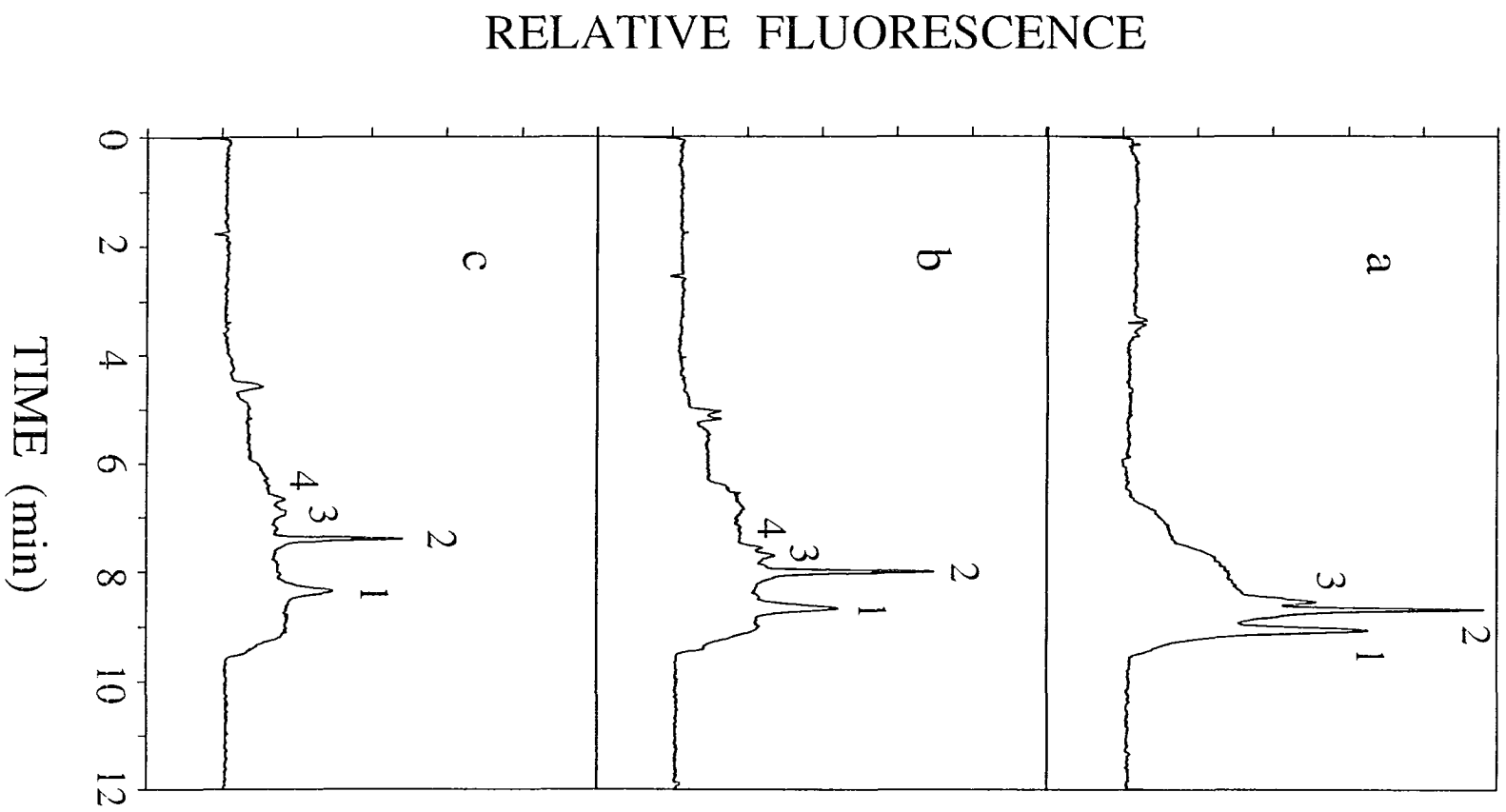
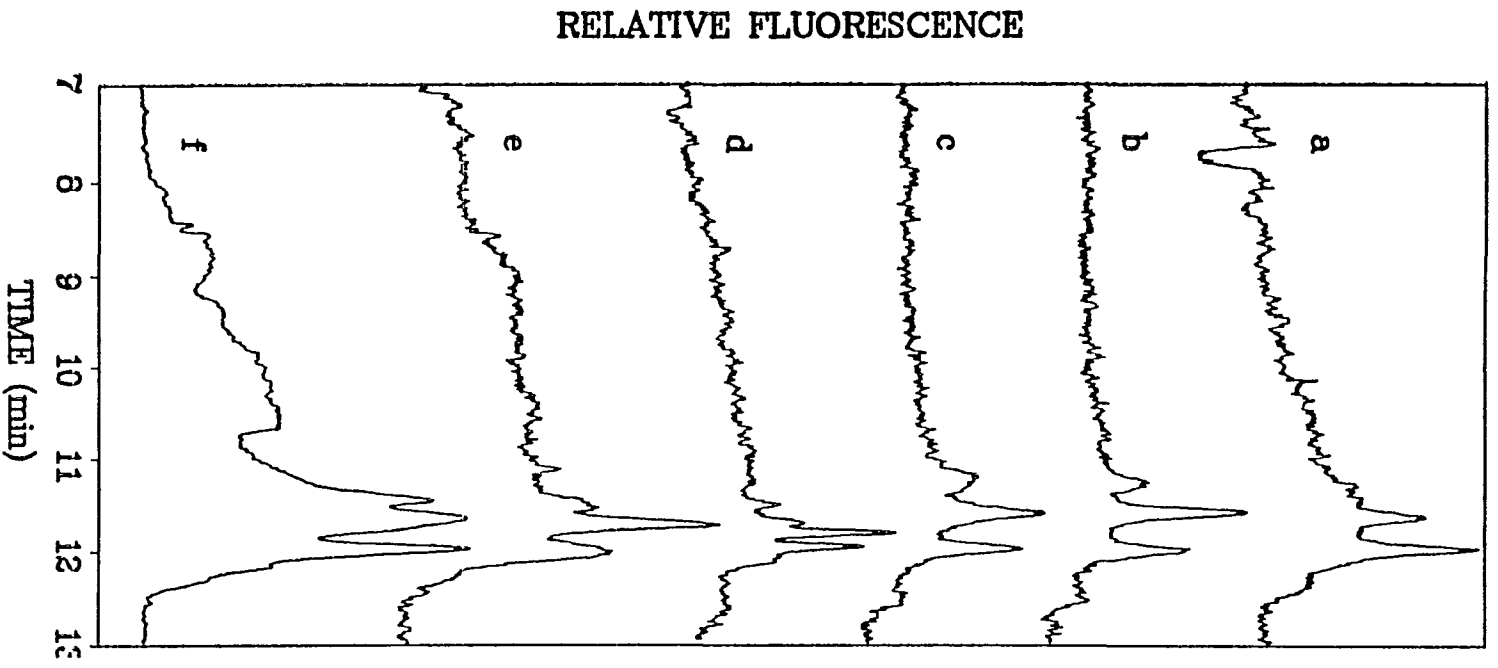




Figure 4. Electropherograms of LDHs in several individual human erythrocytes. (a) through (e), 2 min incubation and (f) 5 min incubation and plotted with a 0.4 scale factor. The migration times have been normalized with respect to that of LDH-1 in each case.



lyse immediately after application of high voltage. Rather, the cell was lysed towards the end of the separation period, and the LDHs were left unseparated. That will be the case if too much of the cell suspension liquid is drawn into the capillary, protecting the cell from lysis. This ambiguity can be avoided by pushing on the syringe piston afterwards to backflush most of the cell suspension liquid out of the capillary. This operation usually does not remove the injected cell as it can adsorb on the capillary wall.

A longer on-capillary incubation time was also attempted, as shown in Fig. 4f. The peaks become higher and broader. A larger peak is desirable for measuring low enzyme activities. However, a broader peak will compromise the separation. We have shown <sup>32</sup> that large biomolecules do not diffuse significantly under similar conditions even after one hour. Hence, broadening was not due to diffusion of LDH but was caused by diffusion of the relatively small molecule, NADH (MW = 709.4). The combined results shown in Fig. 3 and 4f indicate that if a longer reaction time is needed for sensitive detection, the separation time for the enzymes before incubation should be increased accordingly to maintain good spacing between the individual product peaks.

During the series of cell analyses, the migration times changed from run to run. One of the probable causes is the vacuum pulse used for injection. Because of our enclosed system, residual hydrodynamic flow during separation will lead to changes in migration time. This affects all the components to the same extent, such that the migration time difference between LDH-1 and LDH-2 will be a constant. We found that this difference is  $0.37 \pm 0.09$  min and that the ratio of the two migration times varied by only  $\pm 1\%$  over the entire data set. This confirms our assumption that residual hydrodynamic flow is primarily

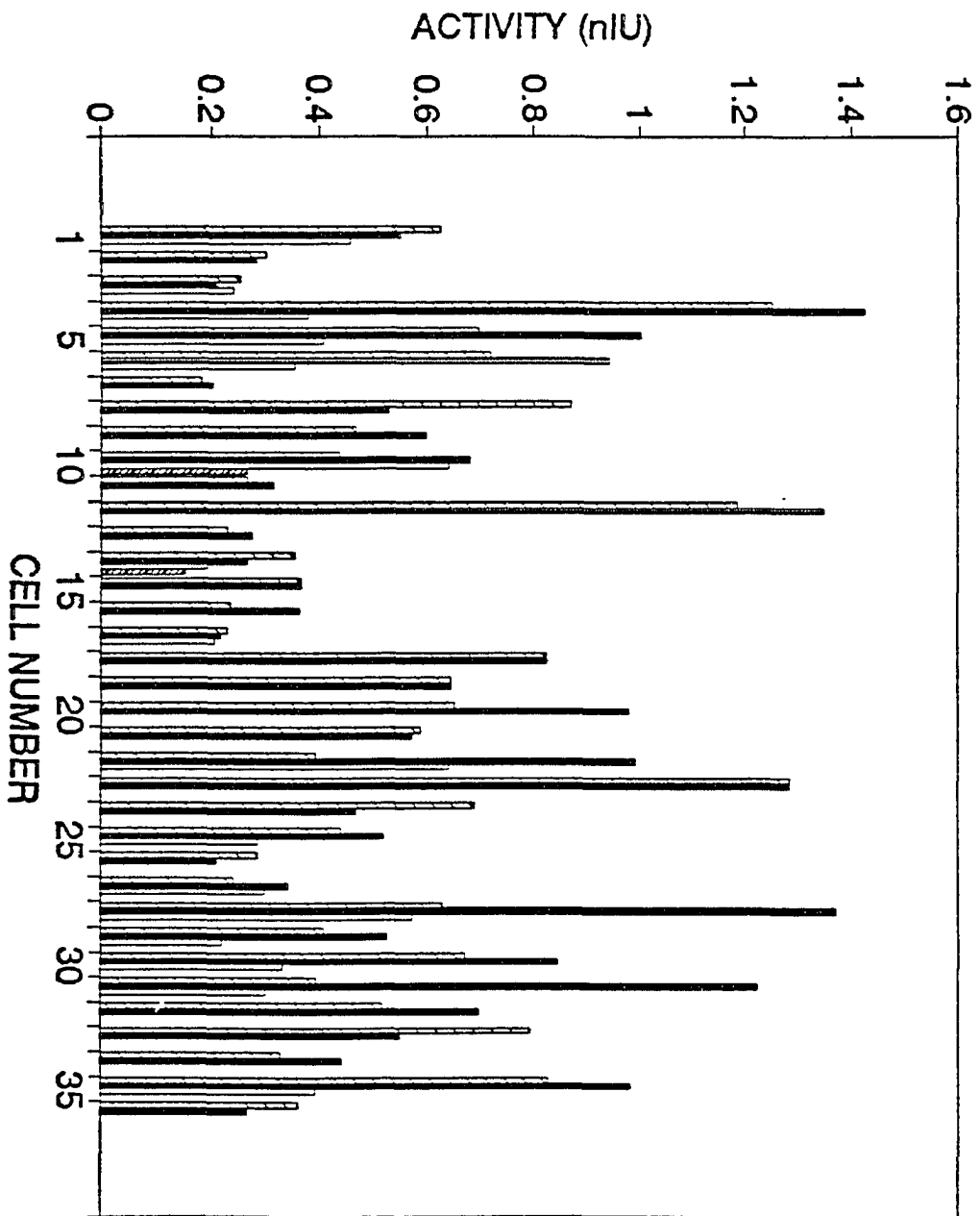
responsible for these variations. Naturally, other effects can also be involved. Usually, proteins and phospholipids have serious adsorption interactions around pH 7. Adsorption on the capillary wall changes the electroosmotic flow rate, which will cause the migration time to change. However, the small total amounts of material injected, even for 36 runs, should only have a minor cumulative effect on the net charges on the capillary walls. We have shown previously that the migration time for hemoglobin changed by only 20% over a 40-cell experiment<sup>23</sup>. In the experiments here, selectivity is provided by the enzyme reaction, and only the separation (relative positions) between isoenzymes is important.

The absolute amounts of LDH in individual erythrocytes were quantified for the 36 cell analyses. Calibration based on LDH standards resulted in a very high calculated LDH activity in the erythrocytes, about 10 times higher than the literature value. The reasons for the unusual result are several folds. During transportation and storage, some LDH activity might have been lost, as LDH is highly susceptible to degradation. On the other hand, when the cell is lysed, its LDH is fresh and is completely injected. It is therefore not surprising that the same *amount* of LDH can show very different *activities* for the standard solutions and for the intracellular fluid. Another factor is that the manufacturer uses different conditions to calibrate LDH activity. The conditions are significantly different from those used in this work (buffer components, pH, temperature, and a reversed direction of enzyme reaction). A third factor is the injection process. The amount of standard solution injected is calculated from the concentration and the injection volume. It is known that proteins in the solution can be lost through adsorption, resulting in the injection of a lower

effective amount. Any of these three factors will lead to a higher calculated activity in single erythrocytes. Consequently, NADH is a more reliable standard for calculating the LDH activity in single cells. The activities thus derived for individual erythrocytes are shown in Fig. 5, with different bars representing the different LDH forms. According to these 36 runs, the average total LDH activity is  $1.36 \pm 0.7$  nIU in a single cell, and is 1.26 nIU/cell as determined from a sample of lysed cells (Fig. 2 and 3). Both are lower than the literature value of 2.9-3.5 nIU/cell<sup>28</sup>. The deviation is expected, and is consistent with a factor of 2 decrease in reaction rate for every 10°C decrease in temperature.

The observed 10-fold variations in LDH between cells are quite large, but are consistent with our studies of other components in single erythrocytes<sup>20,21,23</sup>. Such variations cannot be entirely explained by changes in cell volume, which for human erythrocytes is less than 10% in an individual. The LDH activity has been reported to be related to cell aging<sup>33</sup>, with a declining trend for increased cell age. The older cells are less likely to be able to maintain enzymatic activity, as proteins are not replaced in erythrocytes once formed<sup>34</sup>. So, Fig. 5 reflects the age distribution of erythrocytes in our sample. The relationship between the individual amounts of LDH-1 and LDH-2 was also examined. The ratios for LDH-1 to LDH-2 for single cells, a volume-independent quantity, varied from 0.39 to 1.64 (average = 0.93,  $\sigma = 0.30$ ). There is however a common trend for the LDH-1 and LDH-2 activities. A positive linear correlation exists, with a correlation coefficient of  $r^2 = 0.64$  for 31 cells. This further confirms the hypothesis of cell age, since both isoenzyme forms would degrade simultaneously with age. While total LDH activity serves as a good marker for age<sup>33</sup> and certain diseases<sup>1</sup>, the ability to quantify the individual isoenzymes, as

Figure 5. LDH activities in 36 individual erythrocytes. Light-shaded bars, LDH-1; solid bars, LDH-2; open bars, LDH-3; and heavy-shaded bars, LDH-4 and/or LDH-5. In cells #15, 18, 19 and 23, the peaks are not well resolved. The total activities in these 4 cases are thus equally divided between LDH-1 and LDH-2. Cell #31 was assayed with a 5 min incubation time and therefore gave a much larger response. These activities were multiplied by 0.4 before plotting.



demonstrated here, is important. An elevated LDH-5 level <sup>35-38</sup> or a high LDH-4:LDH-2 ratio <sup>39,40</sup> in serum and in tissues have been correlated with various types of cancer. It will be most interesting to investigate the degree of heterogeneity in neoplastic cells or even benign tumor cells to establish chemical markers for early diagnosis. After all, chemical changes are likely to precede physical changes in the development of diseases. The ability to study individual cells is thus vital.

In summary, we demonstrated a novel approach for rapid assay of ultratrace enzymes within an organism as small as a red blood cell (90 fL). The detection limit here is 800 molecules of LDH for 2 min incubation. It should be possible to detect other enzymes with higher turn-over numbers down to the single molecule level by incubating even longer times. A higher temperature (e.g., 37°C) will also increase the reaction rate and help the detection of even lower enzyme activities. Throughout this work, a 65-cm capillary was used for separation and detection to conveniently interface with the microscope for injecting single cells. Considering that the separation process only took one minute, a much shorter capillary could have been used to perform the assay. The present approach is also applicable to the quantification of multiple enzymes in single cells by introducing multiple substrates. One can rely on electrophoretic separation of the enzymes before incubation or on spectroscopic or electrophoretic differences in the products for simultaneous determinations. Finally, since CE can be run simultaneously in thousands of capillaries <sup>41</sup>, screening a large number of cells in a reasonable time is feasible.



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DIFFERENCES IN THE CHEMICAL REACTIVITY  
OF INDIVIDUAL MOLECULES OF AN ENZYME

A paper to be published in Nature

Qifeng Xue and Edward S. Yeung

**ABSTRACT**

Reactions of single lactate dehydrogenase isoenzyme-1 (LDH-1) molecules can be monitored by using laser-induced fluorescence. We can not only quantify molecular concentrations down to  $10^{-17}$  M of LDH-1, but also measure their reactivities. Large variations ( $\pm 69\%$ ) in activity are found among the 79 molecules studied. The activity for individual electrophoretically pure enzyme molecules can vary by up to a factor of four, and the activity for the same molecule remain unchanged over a two-hour period. We suggest that the origin of the activity difference may lie in the presence of several stable forms of the enzyme.

**INTRODUCTION**

While much recent attention is concentrated on the detection and physical characterization of single-molecules<sup>1-11</sup>, the *chemical* characterization of single molecules can provide unique insights, for example whether the individual reactivities in a population are identical. Further, the time scale over which any inhomogeneties persist can help elucidate structure-function relationships of the

active sites. In previous work <sup>12,13</sup>, enzymes were quantified at the zeptomole level via fluorescence microassay. Single enzyme molecules have also been detected in 14- $\mu\text{m}$  droplets dispersed in oil by fluorescence microscopy <sup>14,15</sup>.

In this work, we show that reactions of single molecules can be monitored by filling a narrow (20- $\mu\text{m}$ ) capillary with very low concentrations ( $10^{-17}$  M) of electrophoretically pure lactate dehydrogenase (LDH-1), excess lactate, and nicotinamide adenine dinucleotide ( $\text{NAD}^+$ ), and observing the discrete fluorescent zones of NADH that are formed together with pyruvate. Unexpectedly, large variations in activity are found among the molecules studied. We also demonstrate how to manipulate each molecule electrophoretically. From the products formed during two consecutive incubation periods, we monitor the reactivity for each LDH-1 molecule over a 2-hour period.

## EXPERIMENTAL

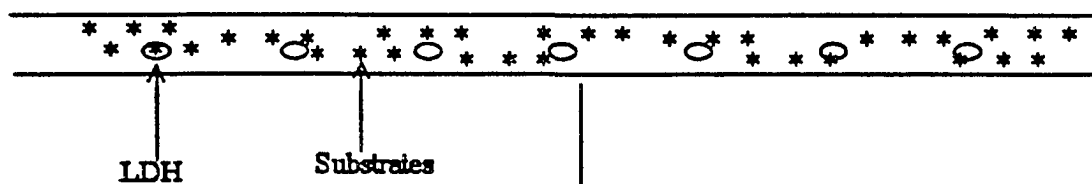
The experimental set-up here was adapted from ref. 12. At  $\text{pH} = 9.1$ , LDH-1 shows maximum activity for catalyzing lactate to pyruvate conversion, and NADH shows higher fluorescence efficiency compared to that at  $\text{pH} 7$ . By increasing the incubation temperature from  $21\text{ }^\circ\text{C}$  to  $40\text{ }^\circ\text{C}$ , the reaction rate is increased without denaturing the enzyme <sup>16</sup>. NADH left in the capillary for 1 hr does not show serious axial broadening (diffusion coefficient  $D \sim 10^{-5}\text{ cm}^2\text{ s}^{-1}$ ), even though it will be well-mixed radially. So, we are able to increase the incubation period, relative to ref. 12, from 2 min to 1 hr; this enhances the sensitivity of detection, as more NADH is produced per molecule of enzyme. In all, we have improved the previous <sup>12</sup> detection limit for LDH-1 by a factor of 1000.

To avoid contamination, all sample solutions were carefully filtered (0.22  $\mu\text{m}$ ). A  $7.6 \times 10^{-6}$  M enzyme solution was diluted in 3 steps such that only a few molecules existed in the capillary when it was filled. Before filling the capillary, the diluted enzyme solution (concentration is at the order of  $10^{-17}$  M) is mixed well with the substrates of lactate and  $\text{NAD}^+$ , with concentration 1 mM and 3 mM for  $\text{NAD}^+$  and lactate respectively. The substrate concentration is saturated comparing to the LDH-1 concentration, at which the reaction speed is only depending on the LDH-1 activity. The process for the filling capillary and incubation to accumulate NADH is shown in Figure 1. After one hour incubation, one zone of NADH molecules will be formed corresponding to each LDH-1 molecule. The activity of each of these molecules can be monitored individually by driving each product zone electrophoretically past an argon-ion laser beam (which excites a region of 10 mm in width). Each enzyme molecule is represented by a product zone of NADH, the concentration of which (typically containing  $\sim 2 \times 10^7$  molecules of NADH) is determined by the activity of enzyme molecule. Fluorescence from the NADH, at a wavelength of 460 nm, is monitored by a photomultiplier tube, via a 20X microscope objective lens. The intensity of fluorescence thereby provides an indication the enzyme's activity.

## RESULTS AND DISCUSSION

The detection of single molecules is shown in Figure 2 for two different concentrations of LDH-1. When the capillary was filled with only the substrates immediately after an enzyme assay, incubation for the same period did not produce any recognizable peaks as shown in Figure 3. This shows that memory

1. Fill the capillary with mixture of LDH and substrates (NAD + lactate)



2. Incubate for 1 hr to accumulate NADH

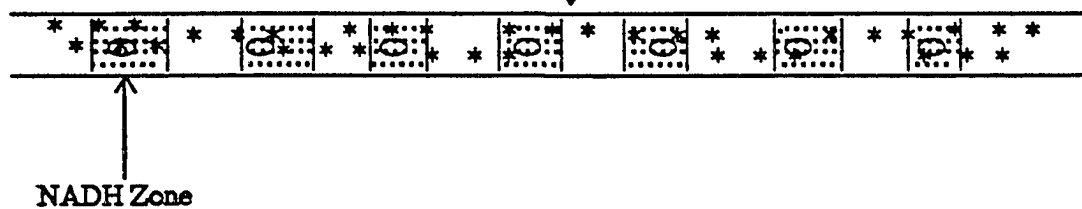
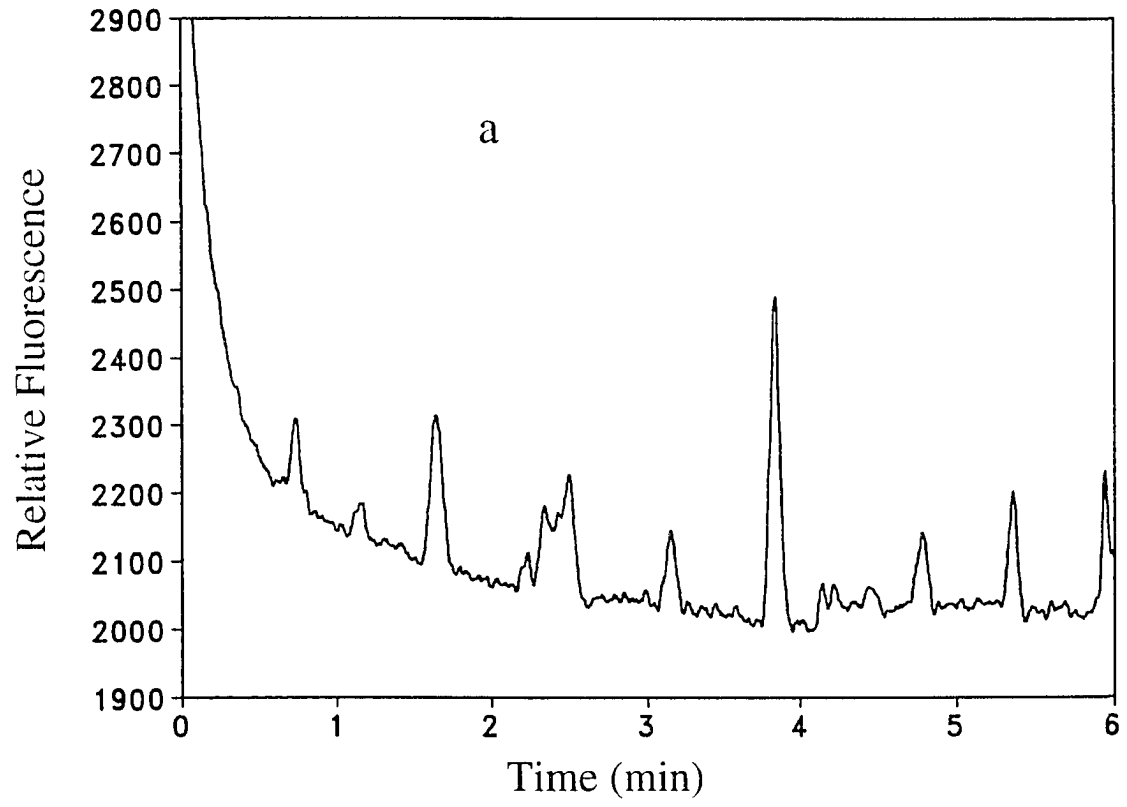


Figure 1. Schematic diagram for the experimental process: fill the capillary with the mixture of substrates and LDH-1 enzymes, and then incubate 1 h.

Figure 2. (a). Detection of single LDH-1 molecules. A LDH-1 solution at  $7.6 \times 10^{-17}$  M was incubated at 40 °C in a 20- $\mu$ m capillary tube together with 1 mM NAD<sup>+</sup>, 3 mM lactate and 20 mM Tris (pH = 9.1) for 1 hour. Afterwards, fluorescence peaks at 460 nm were collected by a 20 $\times$  microscope objective and recorded by a phototube as the individual NADH zones (containing roughly  $2 \times 10^7$  molecules) created within the 70-cm section were electrophoretically driven at 24 kV (11 cm/min) past a 50 mW argon-ion laser beam which forms a 10  $\mu$ m spot at 305 nm.

(b). All the conditions were the same as in (a) except that the LDH-1 concentration was  $1.5 \times 10^{-16}$  M.





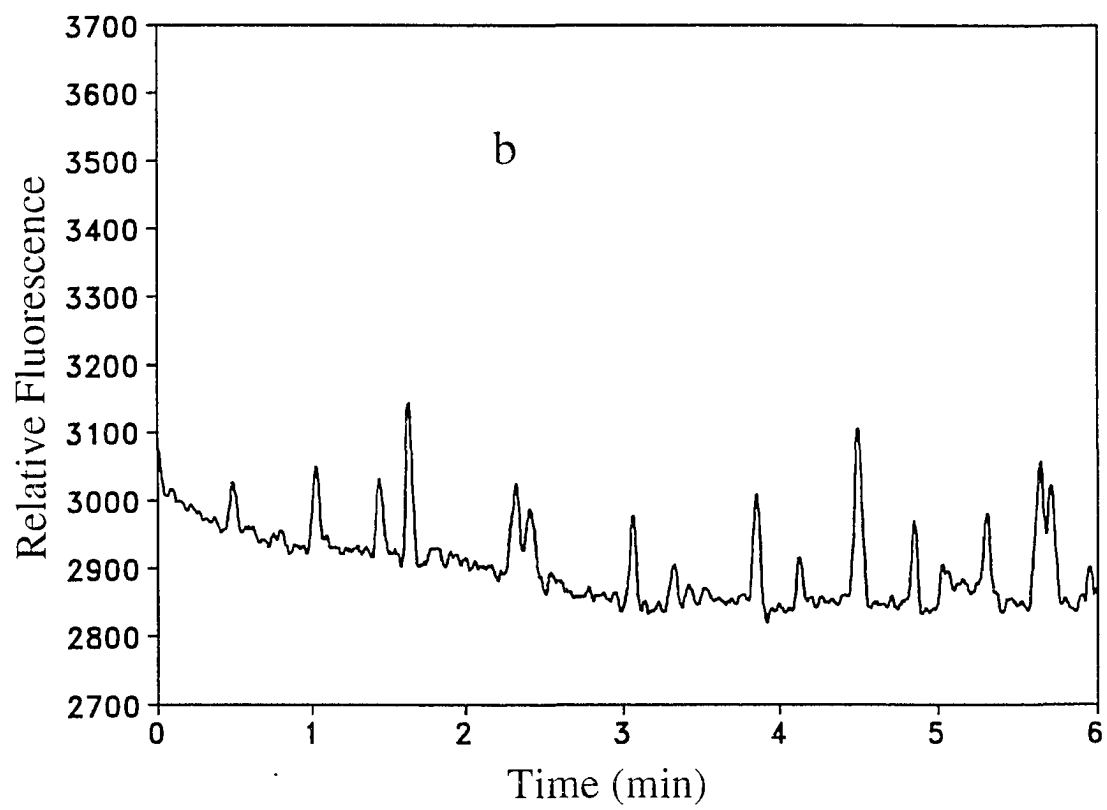


Figure 2 (continued)

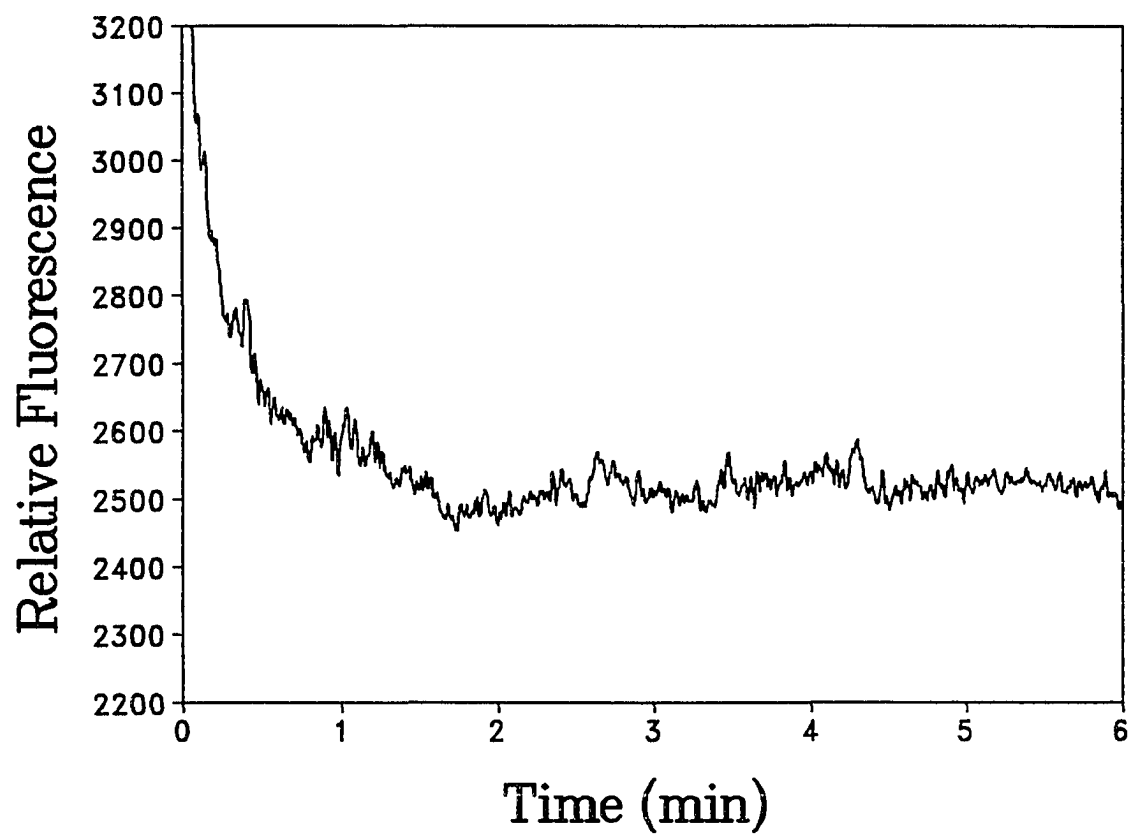


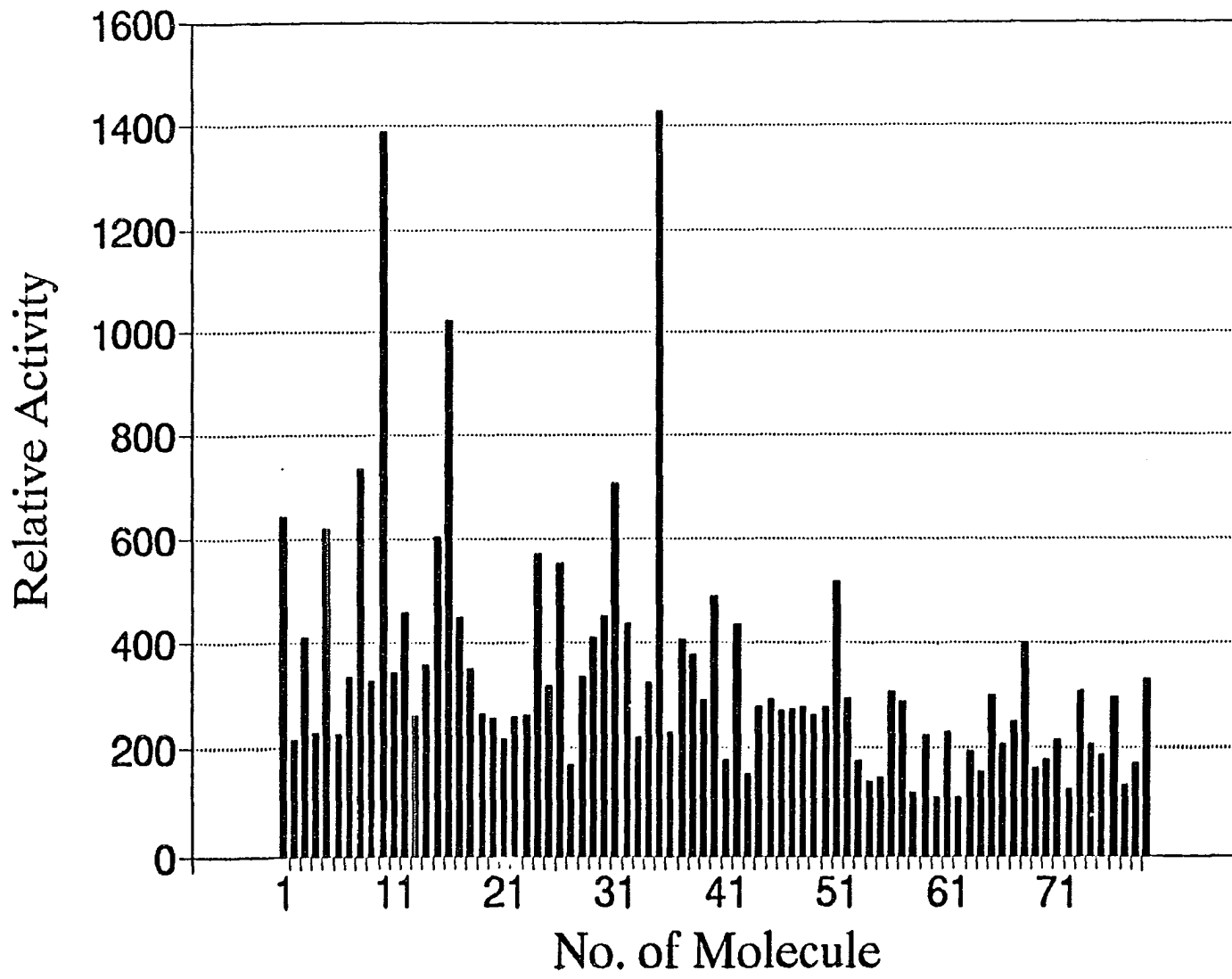
Figure 3. Blank experiment: fill the capillary with substrates only, then incubate at the same conditions as used for Figure 2.

effects (adsorption) and other artifacts are negligible, and proves the peaks observed in Figure 2 are really associated with LDH-1 molecules. The numbers of LDH-1 molecules that exist in the capillary (290 nL volume) are predicted to be 15 and 30, respectively, for the concentrations of  $7.6 \times 10^{-17}$  M and  $1.5 \times 10^{-16}$  M. The average numbers of peaks found were  $8 \pm 2$  and  $13 \pm 1$  (10 separate experiments) respectively, both less than calculated but in a 1:2 ratio. This is partly due to difficulties in preparing very dilute solutions precisely.

Furthermore, by weight, the commercial enzyme preparation is not free of inactive molecules or other proteins. The individual peak areas (average = 343 units), which are independent of axial diffusion, are also consistent with experiments performed at high LDH-1 concentrations when extrapolated to single molecules (308 units).

The widths of the individual peaks in Figure 2a are nearly identical, as would be expected from the time available for axial diffusion. The feature at 2.4 min is unusually broad, indicating possible overlap of two product zones. The probability that any zone is due to two enzyme molecules residing in the exact region is given by the ratio between the zone length and the capillary length, which is about 1/30 here. For the observed 79 molecules, the relative activities were quantified as shown in Figure 4. Even though most of the individual molecules have the similar activity, we did observe several molecules have much higher activity as indicated by the NADH fluorescence intensity. We would predict that around 3 in the set of 79 are double-molecule zones. In Figure 5 of the single molecule reactivity histogram, there are indeed three peaks (> 1000 units) that are much larger than the others. Unfortunately, these cannot be independently verified as double-molecule zones. The relative standard

Figure 4. Relative reactivities of the observed single LDH-1 molecules. The background-normalized peak area in Figure 2 is proportional to the activity of individual LDH-1 molecules, since the presence of excess substrates guarantees a pseudo-first-order reaction.



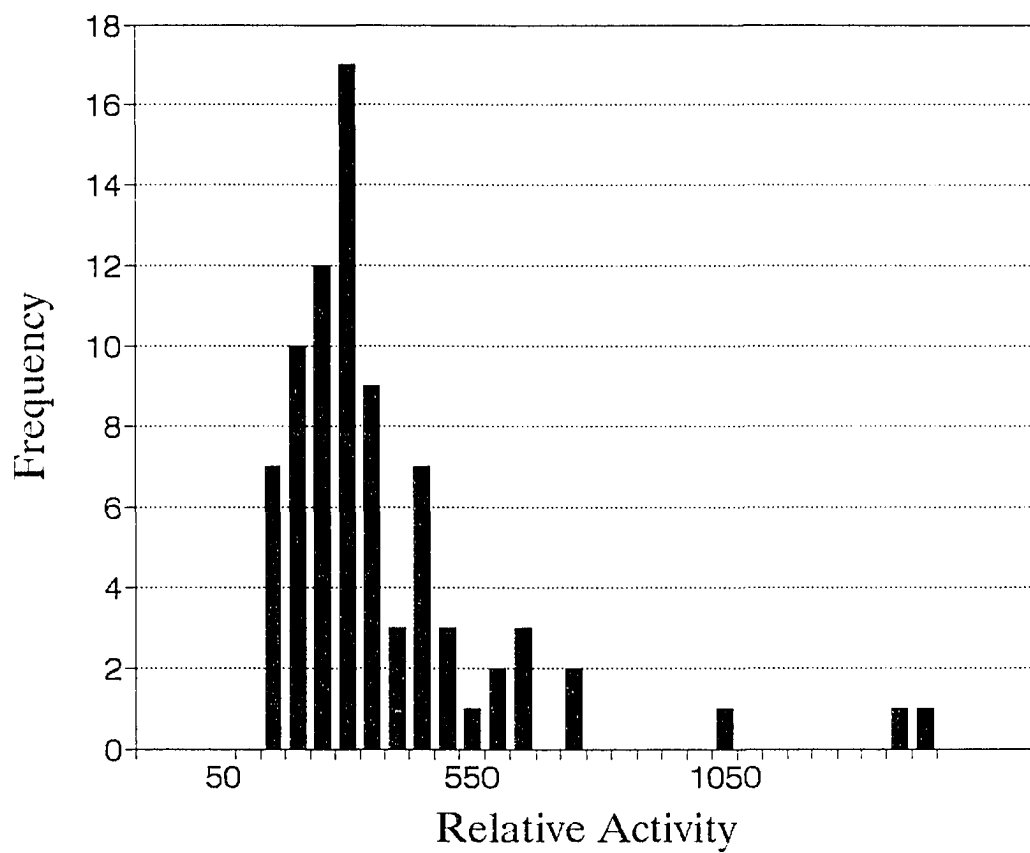


Figure 5. Histogram of single-molecule reactivities for the studied 79 molecules in Figure 4.

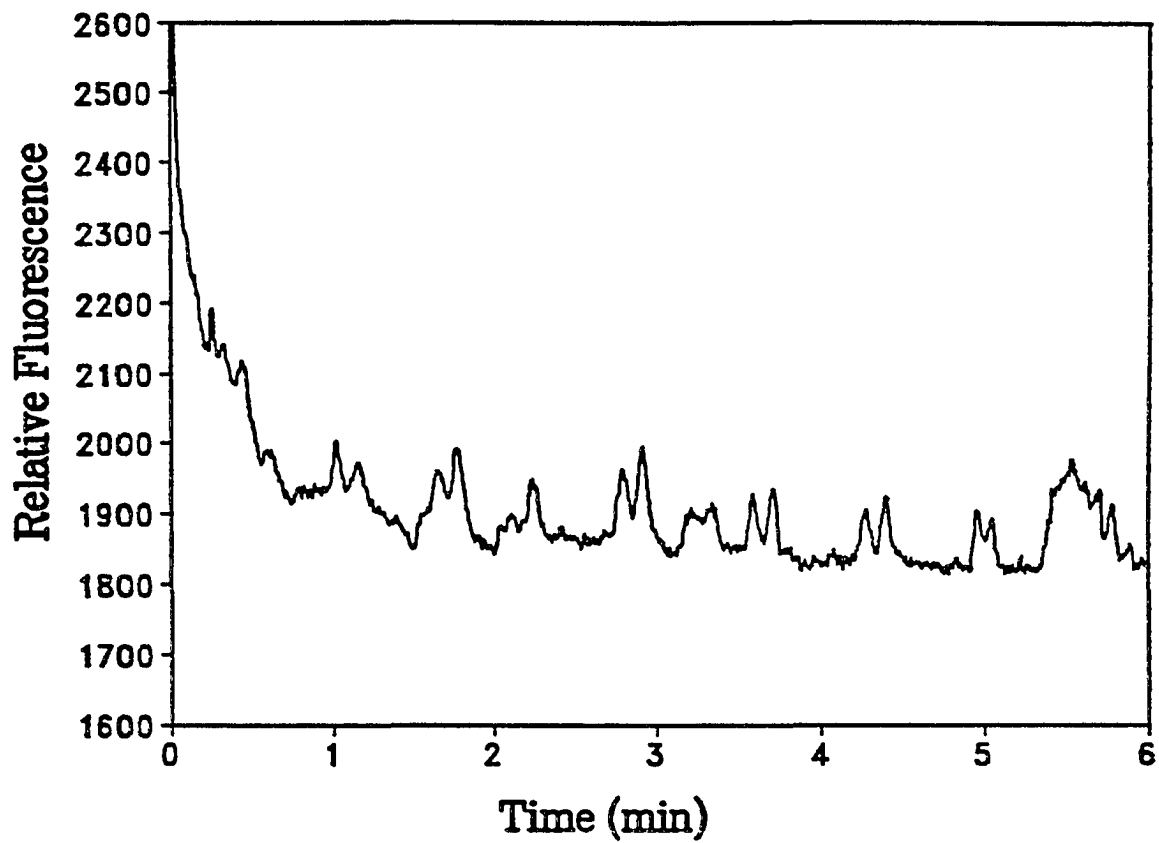
deviation (RSD) of the areas is 69% for 79 clearly isolated peaks. If the 3 questionable peaks are excluded, the RSD becomes 46%. The revised average area (306 units) agrees even better with the extrapolated value. The RSD for areas in single runs ranged from 32% to 72%, showing that the variations are characteristic of individual molecular properties rather than differences in reaction conditions. About  $2 \times 10^7$  NADH molecules are produced in each zone, so fluctuations in substrate concentration around each molecule cannot be responsible for these variations.

It should be noted that we have actually determined the concentration of molecules in a solution at  $10^{-17}$  M, since the total count and the total volume are known. The precision is limited by molecule-counting statistics, and can be improved by using longer capillaries. Previous reports involving single-molecule detection<sup>8,17</sup> are based on samples at  $10^{-14}$  M or higher concentrations. Furthermore, the molecules here need not be fluorescent or pretreated in any way, since the product of a specific reaction is monitored.

We can monitor the activity of individual molecules as a function of time, by electrophoretically separating each LDH-1 molecule from its first NADH product zone, then incubating for another hour, and finally measuring the fluorescence from the two product zones. Such observations are depicted in Figure 6. Several *pairs* of peaks were observed for each analysis. The temporal separation between the peaks in each pair ranged from 0.12 to 0.15 min, comparable to the 0.14 min predicted from standards of LDH-1 and NADH. The fact that a second peak is seen at all indicates that our observations are indeed due to enzyme reactions and not impurities, and that adsorption at the capillary walls is negligible. Also, the first peak of each pair is always broader than the



Figure 6. The activity change of individual LDH-1 molecules with time at 21 °C. Two 1 hour incubation periods separated by electrophoresis for 3 min were employed in the experiment, creating pairs of peaks. The solution concentration was  $4.5 \times 10^{-17}$  M. Other conditions were same as in Figure 2 except that a 75 cm section was monitored.



second because of the longer time available for axial diffusion. The peak widths are narrower than those in Figure 2, which is consistent with viscosity differences at the two temperatures. Finally, the peak areas in Figure 2 are 3-4 times larger than those in Figure 6, as predicted from typical activation energies within this temperature range.

Most importantly, the intensities of the pairs of peaks in Figure 6 remain constant, with an average ratio for 18 molecules of  $1.03 \pm 0.11$  as shown in Figure 7. Clearly, the reactivities are not influenced by the local environment, e.g. wall interactions. This also shows that the differences in reactivities among LDH-1 molecules, which can be as large as a factor of four, are real. This constancy of LDH-1 activity over a two-hour period is consistent with expectation<sup>18</sup>.

Differences in activities amongst individual LDH-1 molecules can best be explained by specific stable conformational arrangements of the 4 identical subunits<sup>19</sup>, making certain active sites<sup>20</sup> less accessible than others for reaction. Indeed, reassociation of LDH at similar temperatures after denaturation is known to lead to structural variants that exhibit different physical and chemical properties<sup>21</sup>, enzyme activity<sup>22</sup>, and spectroscopic properties<sup>23</sup>. The continuous distribution of peak areas (Figure 5) indicates the presence of several conformers. Such insights are unique to single-molecule studies.

This concept can be extended to any species that can catalyze the production of a suitable fluorophore. With the appropriate linkage of LDH-1 to a hybridization tag or to an antibody, practically any molecule can be counted in this way. Each molecule can be characterized, not just counted. The protocol described here also demonstrates how single molecules can be selected and

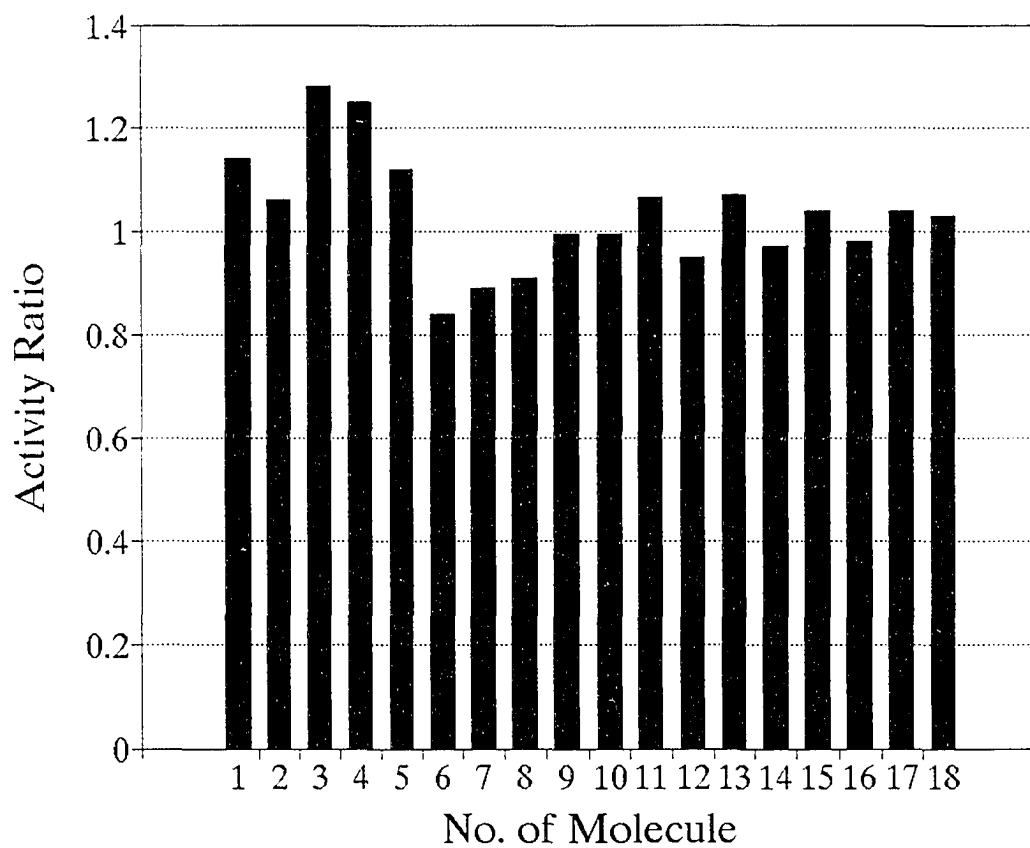


Figure 7. Activity ratios between consecutive two 1-h incubation periods for the observed 18 molecules.

collected in a small volume, e.g. in diagnostics and in biotechnology <sup>17</sup>, because one can extrapolate from the electrophoretic mobilities when each molecule will elute out of the capillary.

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DETERMINATION OF LACTATE DEHYDROGENASE  
ISOENZYMES IN SINGLE LYMPHOCYTES  
FROM NORMAL AND LEUKEMIA CELL LINES

A paper submitted to Analytical Biochemistry

Qifeng Xue and Edward S. Yeung

**ABSTRACT**

This work demonstrates that our previously developed technique for single erythrocyte analysis by capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) can be applied to study individual lymphocytes, with some modification in the cell lysing procedure. A tesla coil was shown to be capable of lysing the lymphocyte cells inside the capillary. The electromagnetic field induced by the tesla coil was believed to be responsible for breaking the cell membrane. The lactate dehydrogenase (LDH) isoenzyme activities and the relative ratios between different LDH isoenzymes were measured for normal lymphocytes as well as B-type and T-type acute lymphoblastic leukemia cells. Both the LDH activity and the isoenzyme ratios show large variations among individual cells. The former is expected due to variations in cell size. The latter implies that single-cell measurements are less useful than the average values over a cell population as markers for leukemia.

## INTRODUCTION

The timely diagnosis of cancer (or carcinoma) is very important for the treatment and even cure of the disease. It is well known that before morphological changes are detectable, cytochemical changes have long undergone gradual transformation. The concentrations of many molecular markers or biomarkers (e.g. growth factors, proteins, polyamines, DNA adducts, etc.) are constantly changing during carcinogenesis. Much effort therefore has been and will continuously be put into finding and defining potential molecular markers for different kinds of cancers.<sup>1-6</sup> Amongst these markers, the enzymes attracted much attention because they control the balance of cytochemicals and actively participate in the cell proliferation process. As investigated previously,<sup>3,4,7-9</sup> enzymes might show different activity in tumor cells compared to that in normal cells, and some isoenzymes even showed different patterns.

Lactate dehydrogenase (LDH) has been found to be a very valuable enzyme in diagnosing different kinds of diseases, such as liver disease, myocardial infarction, etc.<sup>10</sup> More significantly, many researchers noticed that both the LDH activity and the LDH isoenzyme patterns in serum are potential biomarkers for different cancers<sup>11,12</sup> and leukemia.<sup>13</sup> In the sera collected from cancer patients, the LDH-2 form generally showed an elevated activity, even when the total LDH level was normal. However, the results were not very consistent among different groups of patients. The intracellular LDH activity and the ratio for isoenzyme activity in lymphoblastic leukemia cells were also quantified<sup>14</sup> from large amounts of lysed cells. The measured activity indicated that LDH-4 has a relatively higher activity while the total LDH activity is lower than that in the normal lymphocytes. Also, the LDH-4/LDH-2 activity ratio is



higher in lymphocytic leukemia cells than in the normal lymphocytes.

Furthermore, the investigation by Bottomley et al.<sup>15</sup> indicates the possibility of using LDH activity to classify leukemia.

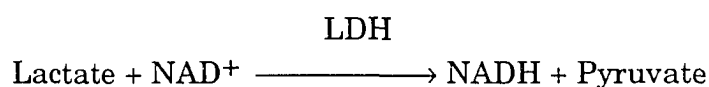
Recently, capillary electrophoresis has become a powerful tool for studying intracellular components in different kinds of single cells.<sup>16</sup> By coupling to different detection methods, a large variety of intracellular components were probed, e.g. neurotransmitters and amino acids in neurons and adrenal medullary cells with electrochemical detection,<sup>17-19</sup> and proteins and small ions in red blood cells with laser-induced fluorescence detection.<sup>20-24</sup> More recently, the catecholamines in single adrenal medullary cells were successfully determined with LIF detection by carefully controlling the pH.<sup>25</sup> The techniques developed for quantifying the LDH activity in single red blood cells<sup>22</sup> and the sensitivity achieved for monitoring the reactivity of single enzyme molecules<sup>26</sup> should enable us to quantify accurately the LDH isoenzyme activities for single lymphocytes. Therefore, the side-by-side analyses of normal lymphocytes and acute lymphoblastic leukemia cells will lend some insight about the difference in LDH activity between these cells and the possibility of using these as markers for leukemia.

## EXPERIMENTAL SECTION

**Instrumentation.** The same home-made capillary electrophoresis apparatus as described before<sup>22</sup> was used throughout this study. Briefly, the mixture of 350 nm and 360 nm laser lines from an Ar ion laser (Model Innova 90-6, Coherent, Palo Alto, CA) was used as the excitation source at a power of 100 mW. A 1-cm focal length quartz lens was used to focus the laser beam to the

detection window on the capillary. Fused-silica capillaries, 22  $\mu\text{m}$  i.d. and 350  $\mu\text{m}$  o.d. with total length of 75 cm and effective length of 55 cm, were used for separating the LDH isoenzymes and as the micro-reactor for carrying out the enzymatic reactions. To minimize the background from stray light, a 380-nm cut-off filter and a 465-nm interference filter (10 nm bandwidth) were used in front of the photomultiplier tube window. A voltage of +30 kV was applied across the capillary for separation and for driving the individual product zones through the capillary.

**Detection method.** In the running buffer (20 mM phosphate, pH 7.4), 1 mM  $\text{NAD}^+$  and 3 mM lactate were added as the substrates for the enzymatic reaction. The reaction direction was chosen as follows:



which is opposite to the natural reaction direction. One of the major reasons is the low background associated with the low fluorescence efficiency of  $\text{NAD}^+$ . Another is to avoid the inhibiting effect of pyruvate at high concentration on LDH.<sup>27</sup> Since the average volume of lymphocytes is at the pL range and the LDH enzyme is one of the minor proteins, the total LDH amount is much less than the amount of substrates available for the catalyzed reaction. At these conditions (pseudo-first-order reaction), the amount of NADH formed during a given period of time at a fixed temperature is linearly proportional to the LDH amount, more precisely to LDH activity. Therefore, the LDH activity can be quantified by measuring the amount of NADH formed during the fixed incubation period. In this work, longer separation times (3 min) and incubation

times (5 min) were used to achieve a better S/N ratio for the enzyme assay to improve the measurement accuracy.

**Cell treatment and injection.** Normal human lymphocytes, T-type and B-type lymphoblastic leukemia cells were purchased from American Type Culture Collection (Rockville, MD). The cell lines were analyzed as received without further culturing. Usually, the cell lines were used as soon as possible after we received them. If not analyzed immediately, the cells were stored at a frozen state. Before doing the single cell experiments, the cell lines were first thawed. Then, the same washing procedure and injection process as before<sup>22</sup> were used to isolate the cells and inject a single cell into the capillary for analysis.

## RESULTS AND DISCUSSION

In the previous work on red blood cells<sup>20-24</sup>, the running buffer readily lyses the cells in a short period of time (~ a few seconds) such that electrophoresis was able to be carried out immediately after cell injection. However, the lymphocytes are so rugged that special techniques are required to break them apart. For lysing a relatively large amount of cells, several methods had been demonstrated to be applicable, e.g. mechanical homogenizer,<sup>12</sup> ultrasonic device,<sup>14</sup> quick freeze-thaw cycle,<sup>15</sup> chemical reagents.<sup>25</sup> When we tried to apply these techniques to lyse a single lymphocyte inside the capillary, there are some problems preventing these methods from being directly applicable.

From our experience, although the ultrasonic device can be used to lyse the cell inside the capillary, it also produces large disturbances, which dilute the intracellular components after their release in the buffer. This makes

quantification very difficult. In the quick freeze-thaw method (with liquid N<sub>2</sub>), lots of bubbles were produced inside the capillary after going through several cycles. The bubbles make it impossible to run capillary electrophoresis.

Surfactants (e.g. SDS) were also tried for lysing the cell. These can lyse the cell in a very short period of time even at as low a concentration as 0.1%.

Unfortunately, SDS also denatures the LDH isoenzymes, making it impossible to quantify the LDH activity.

Teissie<sup>28</sup> discussed that an external electric field could increase the cell membrane permeabilization and even break the cell membrane if the external voltage across the membrane is above ~200 mV. This prompted us to find a way to apply a voltage to induce an external electric field on the cell membrane for cell lysing. We tried to use a tesla coil to induce an electric field and found that it was able to lyse the cell in a reasonable period of time (~15 to 20 s). The lysing process is shown schematically in Figure 1. After a single cell was injected into the capillary, the capillary was moved away from the microscope and fixed on top of a plastic rod. The capillary inlet was then covered with a short piece of teflon tubing to avoid introducing bubbles into the capillary. On touching the tip of the tesla coil to the outside of the teflon tube, the induced electric field on the capillary wall passes on to the cell membrane. After the cell was lysed, the capillary was moved back to the buffer vial to initiate the on-column enzyme assay.

To investigate the effect of the tesla coil on the LDH activity, a LDH standard mixture was analyzed at conditions with and without applying the tesla coil after sample injection. The same conditions as used for single cell analysis were used for the standard assay. As shown in Figure 2, the LDH

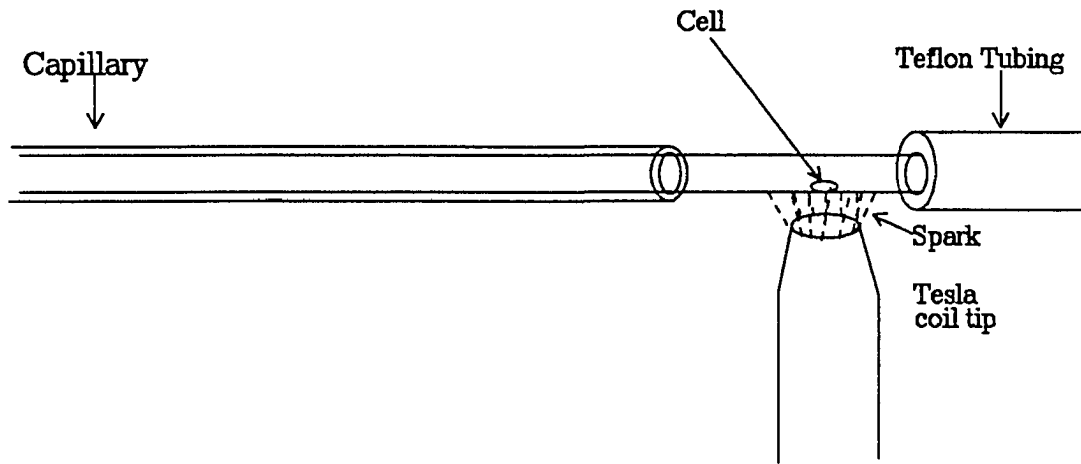
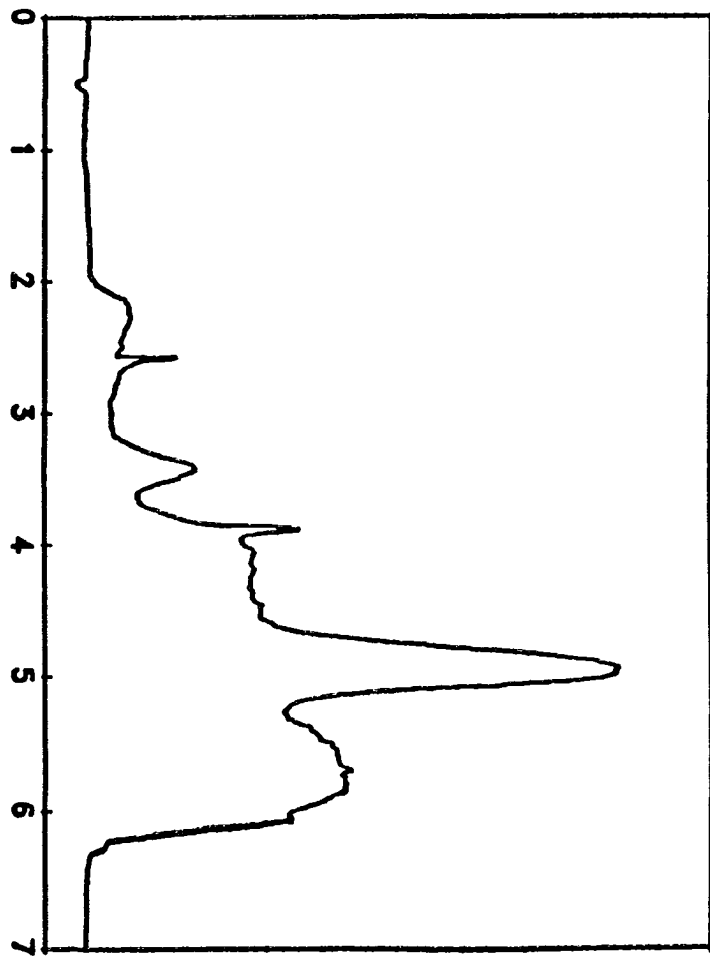
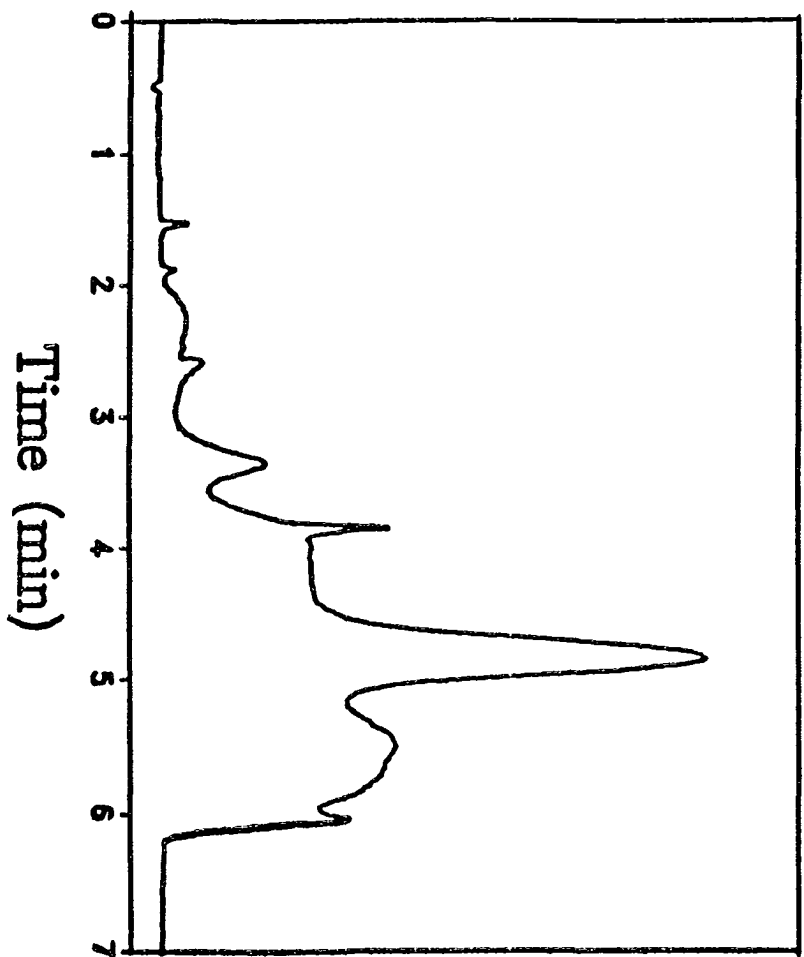


Figure 1. Method for lysing single lymphocytes with a tesla coil.

Figure 2. The effect of tesla-coil treatment on LDH activity. The conditions for on-capillary LDH assay of the standard mixture are described in the text. Bottom, with, and top, without tesla-coil treatment.

# Relative Fluorescence



activity did not show noticeable differences between the experiments with and without the tesla coil treatment. The tesla coil is thus a good tool for lysing individual lymphocytes and similar cells for analysis by CE.

Figure 3 shows electropherograms for the analysis of a LDH standard mixture (a) and lysates of normal lymphocytes (b), and T-type (c) as well as B-type (d) acute lymphoblastic leukemia cells. The LDH isoenzymes are nicely separated by running electrophoresis for 3 min, as confirmed by the LDH standards. Both the migration times and the peak patterns indicate that the major LDH isoenzymes in lymphocytes are LDH-3 and LDH-4, which are quite different from the patterns in red blood cells where the major forms are LDH-1 and LDH-2. The distribution of LDH isoenzymes are organ dependent.<sup>29</sup> The LDH isoenzyme abundance follows the order: LDH-3 > LDH-4 > LDH-5 > LDH-2 > LDH-1, which is different from the order (LDH-3 > LDH-2 > LDH-4 > LDH-1, LDH-5) reported in ref. 27.

In Figure 4, electropherograms of a single-cell analysis for each cell line are shown. Usually, three or four peaks were observed for most of the individual cell analyses. In several experiments, we observed 5 peaks, corresponding to the 5 LDH isoenzymes. The percentage for successful single-lymphocyte analysis as a fraction of all cell injections is about 40%, substantially lower than that of about 80% for red blood cell analysis. The likely reason is related to the cell lysing process, which is not as gentle or as complete as the hypertonic lysing process. The tesla coil can sometimes cause a relatively large disturbance that moves the cell out of the capillary. At other times it may not produce enough shock to break the cell membrane. Also, since the tesla coil only breaks the cell membrane, the undissolved membrane debris may produce extra peaks.



Figure 3. Electropherograms for the analysis of a LDH standard mixture (a), lysed normal lymphocytes (b), lysed T- type (c) and lysed B-type (d) lymphoblastic leukemia cells.

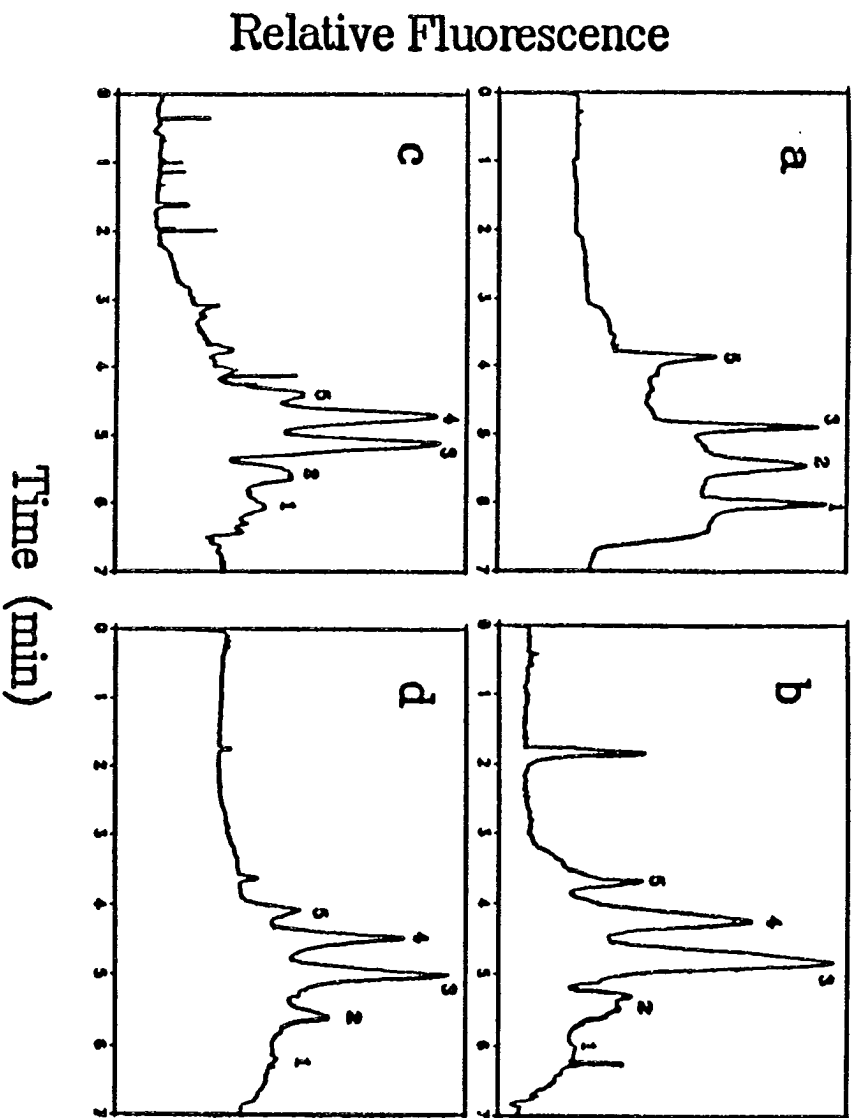
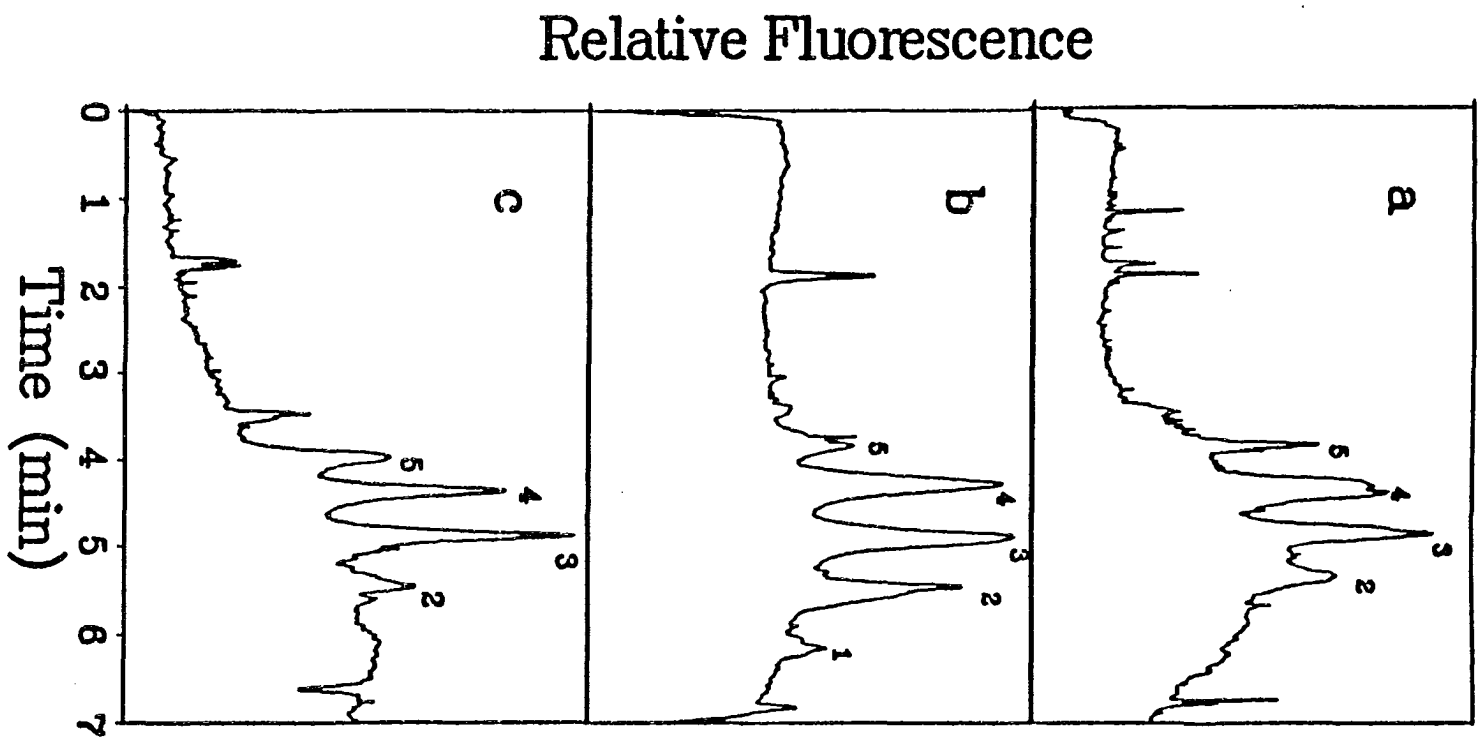


Figure 4. Electropherograms for single-cell analysis. (a) normal lymphocyte;  
(b) T-type lymphoblastic cell; and (c) B-type lymphoblastic cell.

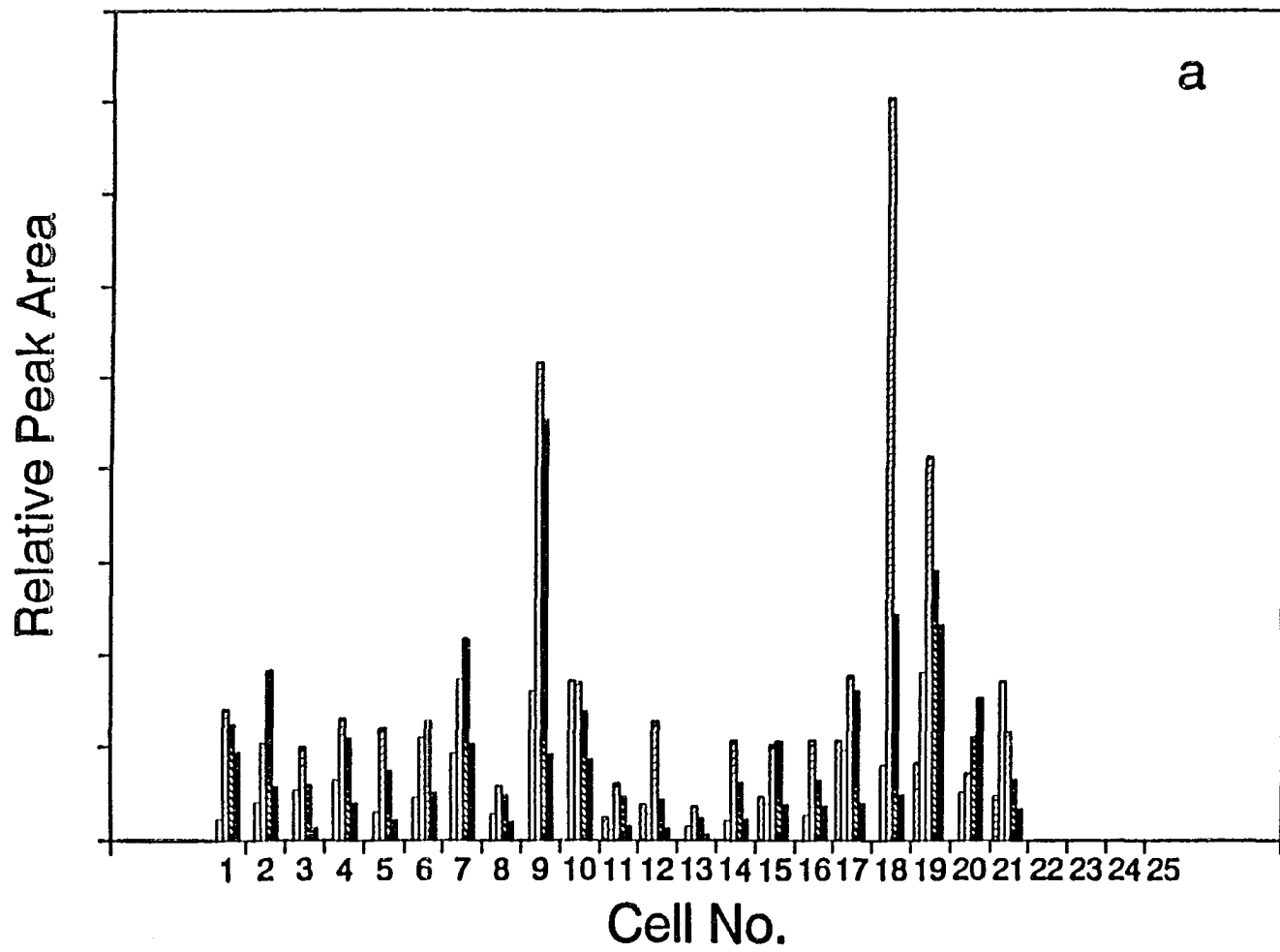


As we observed in ref. 22, the migration time changes after several cells are analyzed due to the adsorption of intracellular proteins and the cell membrane materials. We found that the capillary can be reconditioned by simply flushing with running buffer and re-equilibrating for 30 min.

The size of the lymphocytes varied in a fairly large range, with diameters of 6  $\mu\text{m}$  to 14  $\mu\text{m}$ .<sup>30</sup> The size variations can be confirmed by using a 100 $\times$  microscope objective during the process of single-cell injection. It is very difficult to pick up only cells of a uniform size to be injected for analysis. As we expected from the large size variations, the intracellular LDH activities showed quite large variations, as shown in Figure 5. If a size-sorting method was used to put the lymphocytes into groups with uniform sizes, the absolute intra-cellular LDH activity would be more meaningful. The cell age might also be a big factor for the large activity variations because of the long life span of the lymphocytes.<sup>30</sup> The average total LDH activity for the normal lymphocytes is higher than that in both B-type and T-type lymphoblastic leukemia cells. From the cells studied, the average LDH activities are 6.3, 5.2 and 5.0 nIU respectively for the normal lymphocytes, T-type and B-type leukemia cells.

Even though there is large size variation ( $> 10 \times$  in volume) among the cells, the ratio of different LDH isoenzymes might remain constant if the cells were at the same health state and metabolic state. The LDH-4/LDH-2 ratio was observed to be elevated in the serum of different cancer patients,<sup>31,32</sup> and in the leukemia cells from the analysis of cell lysates.<sup>14</sup> Therefore, we also examined this ratio for individual normal lymphocytes (Fig. 6a) and for individual acute lymphoblastic leukemia cells (Fig. 6b, c). During single-cell analysis, the on-

Figure 5. Relative LDH activity in individual cells. (a) normal lymphocyte; (b) T-type lymphoblastic cell; and (c) B-type lymphoblastic cell. Cross hatched, LDH-1; empty, LDH-2; light-hatched, LDH-3; heavy hatched, LDH-4; and solid, LDH-5. The vertical scales are (a) 0-18,000; (b) 0-14,000; and (c) 0-10,000.



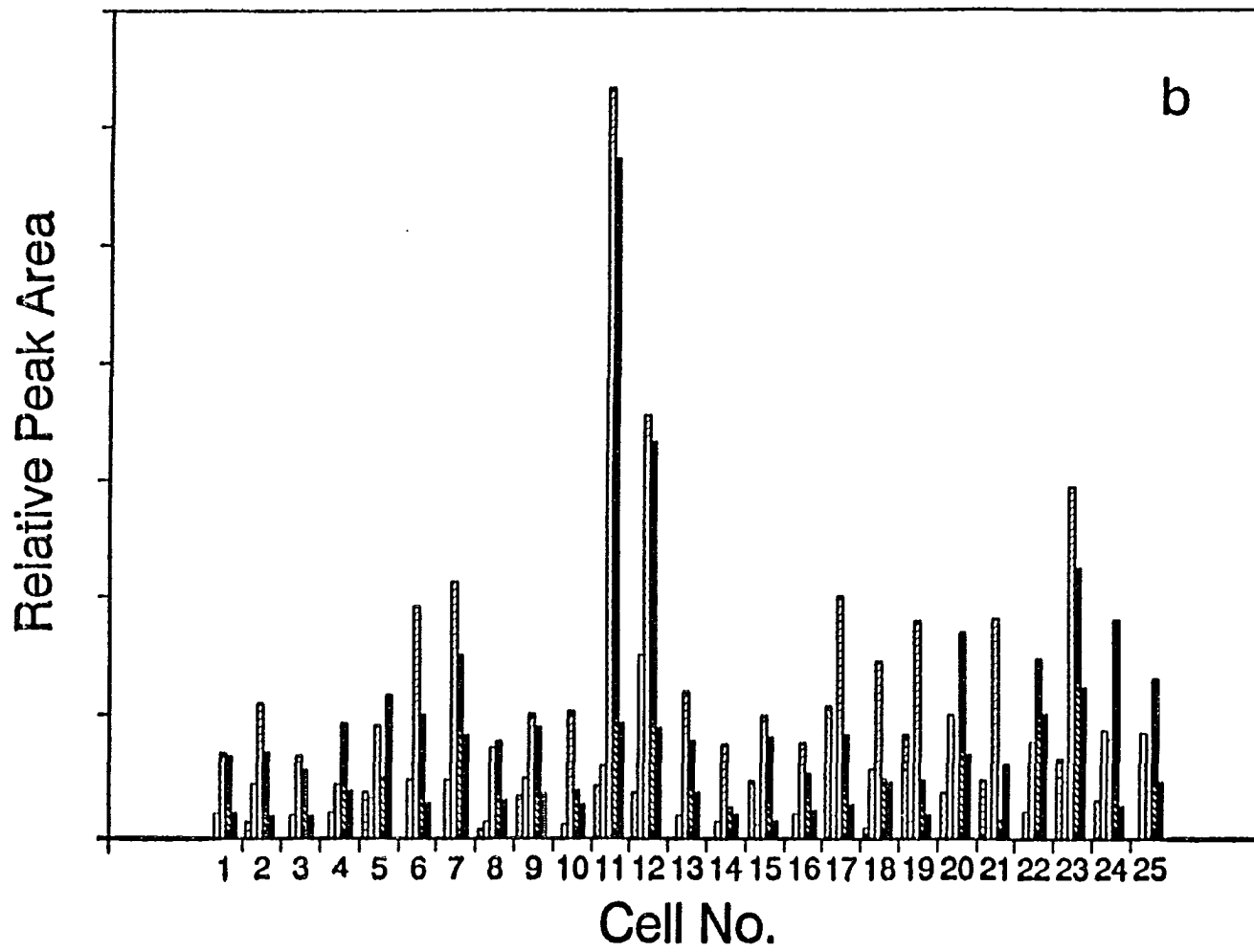


Figure 5 (continued)



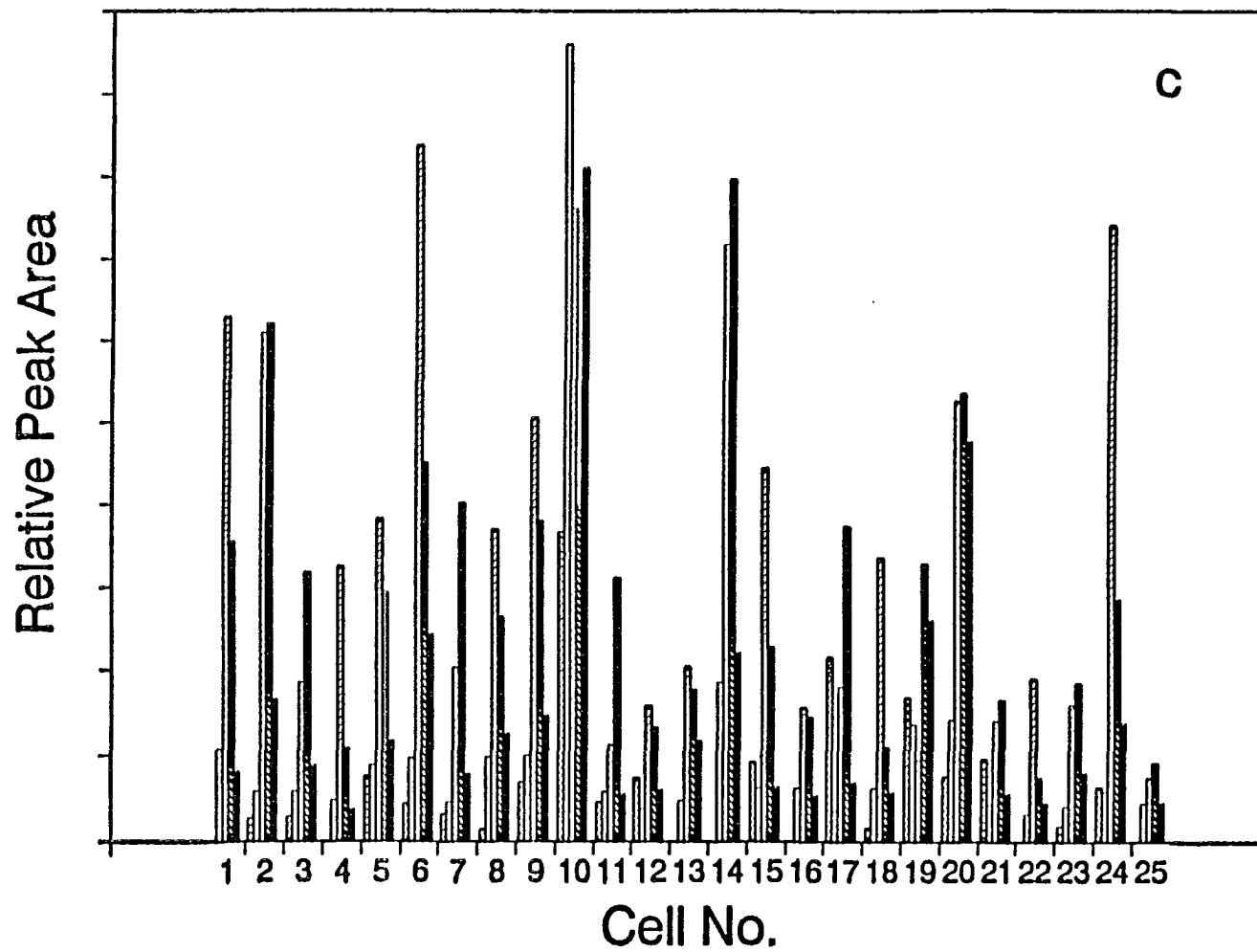
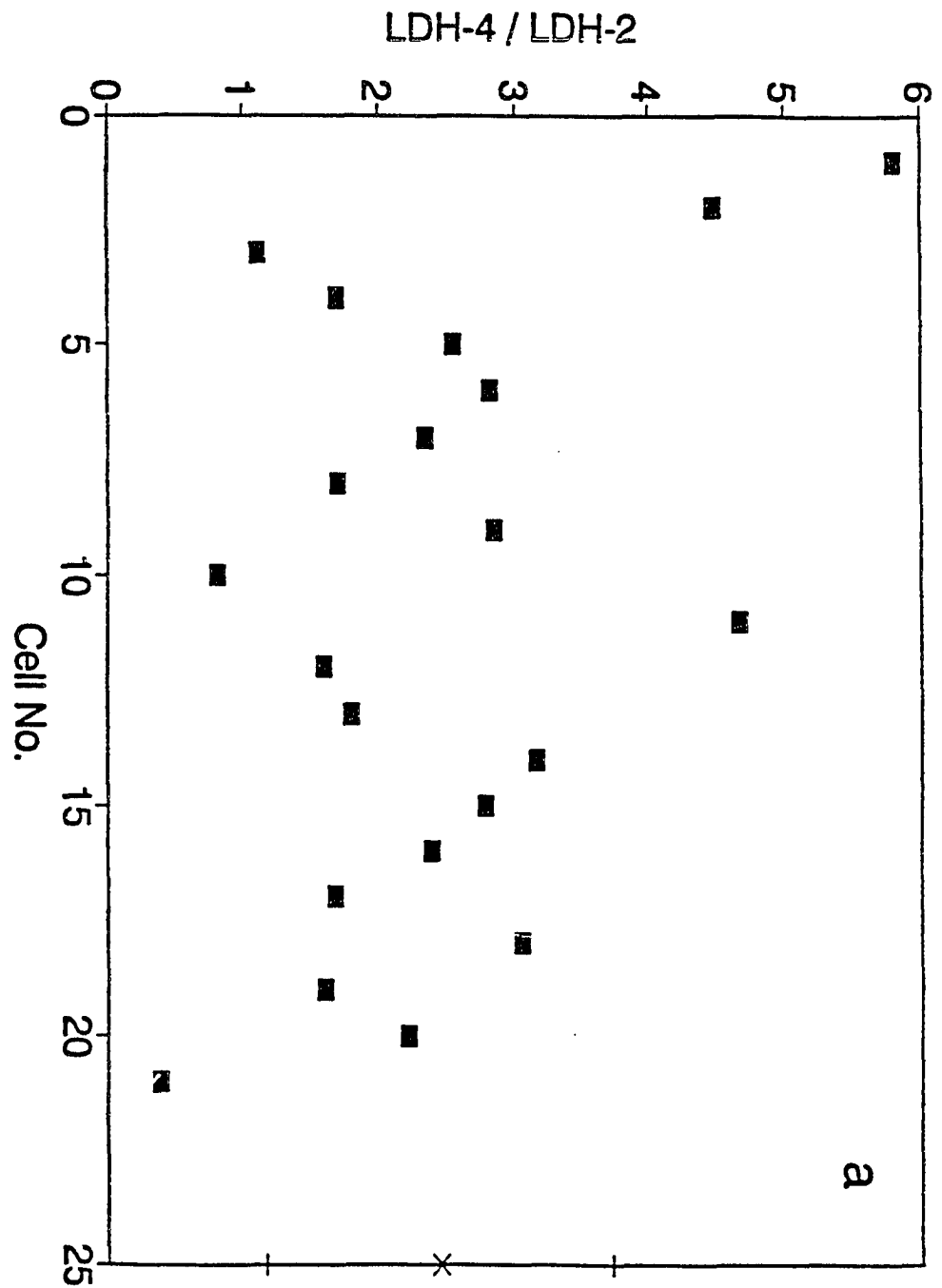


Figure 5 (continued)

Figure 6. LDH-4/LDH-2 ratios of individual cells for different cell lines. (a) normal lymphocyte; (b) T-type lymphoblastic cell; and (c) B-type lymphoblastic cell. To the right of each plot, the average ratio (cross) and the standard deviation (horizontal lines) are also shown.



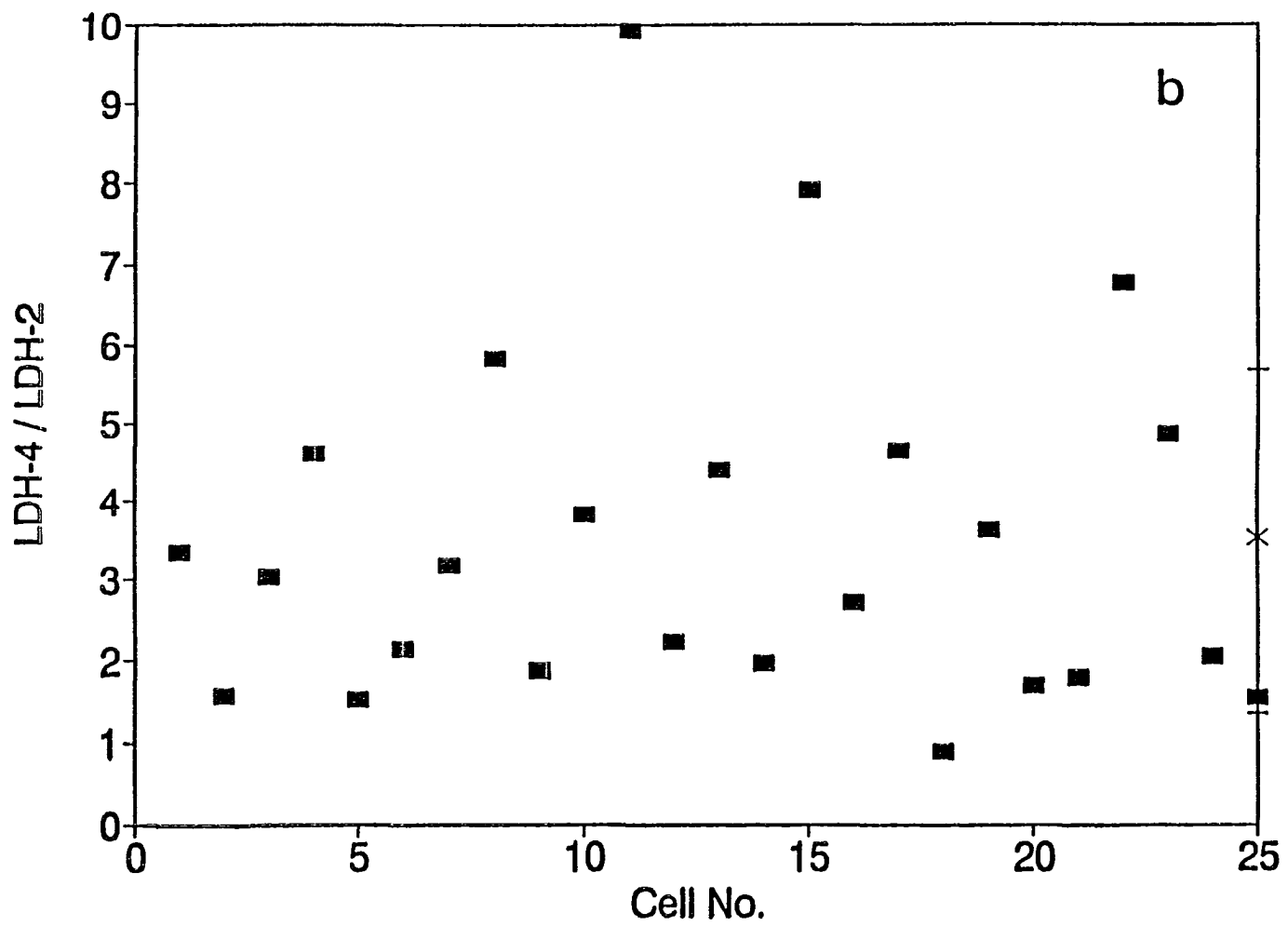


Figure 6 (continued)

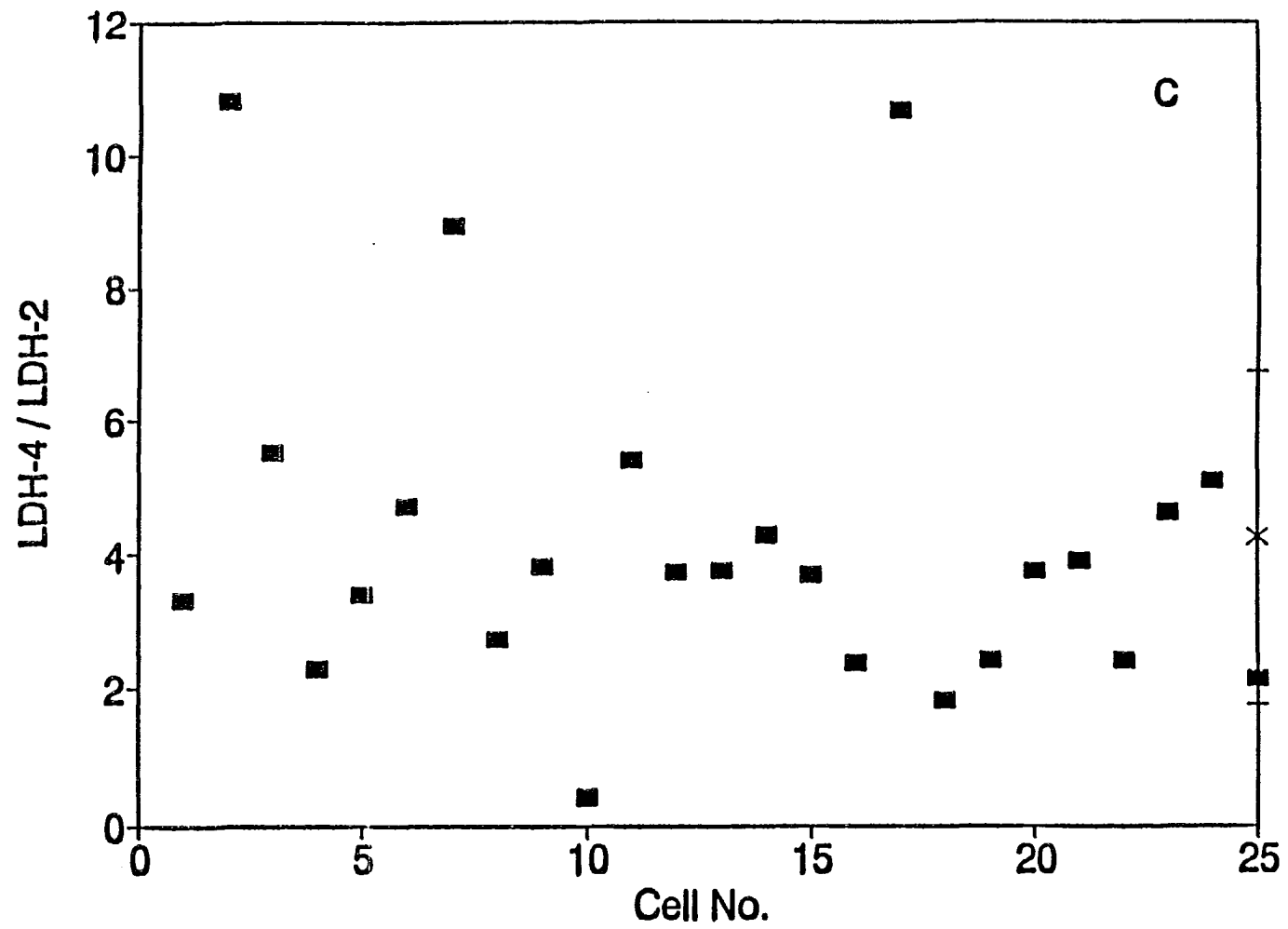


Figure 6 (continued)

capillary enzyme assay was calibrated frequently by using LDH standards. The variations were less than 15%, which indicated that the method is reliable and reproducible. On the average, the LDH-4/LDH-2 ratios for both T-type and B-type leukemia cells were 43% and 73% higher than that for the normal lymphocytes. The difference between T-type and B-type leukemia cells is also reasonable, as they have different functions and are formed in different organs.<sup>30</sup> Nevertheless, Figure 6 shows that the observed elevation did not apply to every single cell in a given cell line. It appears that a reasonable number of cells need to be analyzed before any conclusions can be reached about their state of health. If many cells ( $> 10^6$ ) are available, the analysis of cell hemolysate will be advantageous in terms of providing an average value, convenience, and accuracy. Of course, if there is only a limited number of cells available for analysis, the single-cell technique here will stand in for providing statistical information about the intracellular components. The low detection limit offered by this technique and the small amount of material required for one measurement are still beneficial, particularly when applied to the routine monitoring of intracellular enzymes for less abundant entities, such as white blood cells.

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## GENERAL SUMMARY

Capillary electrophoresis has experienced rapid advancements in the last few years in both instrumental developments and applications. The progress recently achieved in biotechnology and biodiagnostics make CE a more widely acceptable technique as a bioanalytical separation tool. The successful applications of CE in DNA separation and sequencing make it viable to sequence the human genome, and to be widely used in disease diagnosis by monitoring the PCR amplification products. Scientific progress is advancing at a dramatic pace, and revealing lots of challenges in a variety of areas. Many research projects appeal the cooperative efforts of scientists working in different disciplines. The requirements for more sensitive detection schemes and versatile separation methods create many opportunities for the future.

This dissertation work has described developments in the separation schemes and detection approaches for analyses of trace amounts of analytes in a complex matrix such as cytoplasm. The developed techniques for the analysis of intracellular components established the possibility to extend these techniques to analyze other single cells and extremely small amounts of samples. The capability of monitoring single enzyme molecules makes it possible to analyze any species tagged with the enzyme, and to investigate catalytic reactions at the single molecule level. Based on the protocols developed in this work, future studies could be extended to disease diagnosis with single cell analysis and to

the single molecule analysis and manipulation. Environmental effects on enzyme molecules also could be investigated at the single molecule level.

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