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DNA Sequencing with Capillary Electrophoresis and Single Cell Analysis with Mass Spectrometry

by

Fung, Nga-kit

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Ames Laboratory, U.S. DOE

Iowa State University

Ames, Iowa 50011

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ABSTRACT

Since the first demonstration of the laser in the 1960's, lasers have found numerous applications in analytical chemistry. In this work, two different applications are described, namely, DNA sequencing with capillary gel electrophoresis and single cell analysis with mass spectrometry. Two projects are described in which high-speed DNA separations with capillary gel electrophoresis were demonstrated. In the third project, flow cytometry and mass spectrometry were coupled via a laser vaporization / ionization interface and individual mammalian cells were analyzed.

First, DNA Sanger fragments were separated by capillary gel electrophoresis. A separation speed of 20 basepairs per minute was demonstrated with a mixed poly(ethylene oxide) (PEO) sieving solution. The solution was composed of 1.5 % 8,000,000 Mn PEO and 1.4 % 600,000 Mn PEO. This sieving matrix offers fast separation, high resolution and is easily replaceable. In addition, a new capillary wall treatment protocol was developed in which bare (or uncoated) capillaries can be used in DNA sequencing.

Second, a temperature programming scheme was used to separate DNA Sanger fragments. The temperature was increased from 35 °C to 65 °C at a rate of 2 °C per minute. A separation rate of 30 basepairs per minute was demonstrated. Furthermore, DNA separation in a non-denaturing medium was demonstrated.

Third, flow cytometry and mass spectrometry were coupled with a laser vaporization / ionization interface. An aqueous buffer system composed of 2 mM ammonium chloride at pH 7.3 was used to optimize cell viability and mass spectrometer sensitivity. A mass detection limit of 20 amol (S/N = 3) of serotonin in a single laser pulse was demonstrated. Determination of serotonin and histamine in individual rat peritoneal mast cells (RPMC) was demonstrated. The intracellular contents of serotonin and histamine were found to vary considerably. No correlation was found between the amount of serotonin and histamine.

GENERAL INTRODUCTION

Cells are the fundamental units of living organisms. Complex organisms like humans have different types of cells in different organs. Each type of cell has its own particular shape, composition and function. Each cell has a complex mixture of biomolecules such as proteins and DNA. The direct analysis of these biomolecules at single cell level is rapidly becoming a powerful means with which to investigate questions related to flow of genetic information, diseases, cellular metabolism and aging.

In this work, the analysis of two different types of biomolecules are described. In the first application, DNA Sanger fragments were separated by capillary gel electrophoresis. The development of an ideal sieving matrix has been the focus in the field of DNA separation. The ideal sieving matrix has to provide long read length, fast separation and be replaceable. In this work, we describe a mixed solution of poly(ethylene oxide) solutions which closely resembles an ideal sieving matrix. In addition, a new capillary wall treatment protocol is described. With the use of this protocol, uncoated capillaries can be used to separate DNA Sanger fragments without sacrifying the separation efficiency.

In the second application, serotonin and histamine in individual rat peritoneal mast cells were analyzed by flow cytometry-mass spectrometry. A Kr-F waveguide excimer laser was used to interface the two techniques. One of the major challenges in coupling nanoscale separation techniques with MS is to develop a suitable buffer system. Traditionally, single cell experiments are performed in buffer solutions with more than 100 mM concentration salt. However, this large amount of salt is detrimental to mass spectrometry due to clogging at the tip of the capillary, cation adduction, and reduced ionization efficiency. Here, we developed a suitable buffer solution that can prolong the lifetime of the cells while keeping the resolution

and detection limit of the system. With this method, information on the cell population as well as on individual cells can be obtained in a short period of time.

Dissertation organization

This dissertation consists of two parts. A general introduction precedes the two parts. Chapter two and three in Part 1 are manuscripts published in the area of DNA sequencing with capillary gel electrophoresis. Chapter five in Part 2 is a manuscript prepared for publication in the area of single cell analysis with mass spectrometry. Each part also contains introduction and conclusions chapters. The dissertation concludes with a general conclusion. The appendix contains the data acquisition software used in the mass spectrometry project. The final reference section lists all references cited in chapters that are not papers.

PART 1. HIGH SPEED DNA SEQUENCING BY USING MIXED POLY(ETHYLENE OXIDE) SOLUTIONS IN UNCOATED CAPILLARIES

CHAPTER ONE. INTRODUCTION

Deoxyribonucleic acid (DNA) is the chemical bearer of genetic information. It is common knowledge now but it has taken many brilliant minds almost a century to understand the structure of DNA, how it controls cell function, and ultimately the structure and function of an entire organism. Yet, we still do not fully understand all these. There remain gaps in the stories. Understanding the storage and flow of genetic information, and applying this information to improve the quality of our lives poses both technical and ethical challenges to scientists of our time.

DNA is a very long, threadlike macromolecule made up of a large number of deoxyribonucleotides, each composed of a base, a sugar, and a phosphate group. The bases of DNA molecules carry genetic information, whereas their sugar and phosphate groups perform a structural role. There are four bases in DNA: Adenine (A), Guanine (G), Thymine (T) and Cytosine (C). Some viruses, however, use RNA (ribonucleic acid) as their genetic material. The covalent structure of RNA differs from that of DNA in two respects: the sugar units in RNA are riboses and one of the four major bases in RNA is Uracil (U) instead of Thymine (T).

A gene is an ordered sequence of nucleotides located in a particular position on a particular chromosome. It is the fundamental physical and functional unit of heredity. The complete set of genes from an organism is called a genome. Some genetic traits are best explained by inheritance of single genes, but most involve combinations of multiple genes with environmental factors.

The growing power and speed of research in molecular biology have led to proposals to apply novel molecular biological methods to the genetics of entire organisms. Research and technology aimed at mapping and sequencing large portions or entire genomes are called genome projects.

Genome projects have several objectives:

- to create maps of human chromosomes consisting of DNA markers that would permit scientists to locate genes quickly.

- to develop new ways to analyze DNA, including biochemical and physical techniques and computational methods.
- to develop new instruments for analyzing DNA.
- to establish and maintain databases containing DNA sequences, location of markers and genes, function of identified genes. (for example, GENEBANK).
- to determine the DNA sequences of human and non-human genomes.

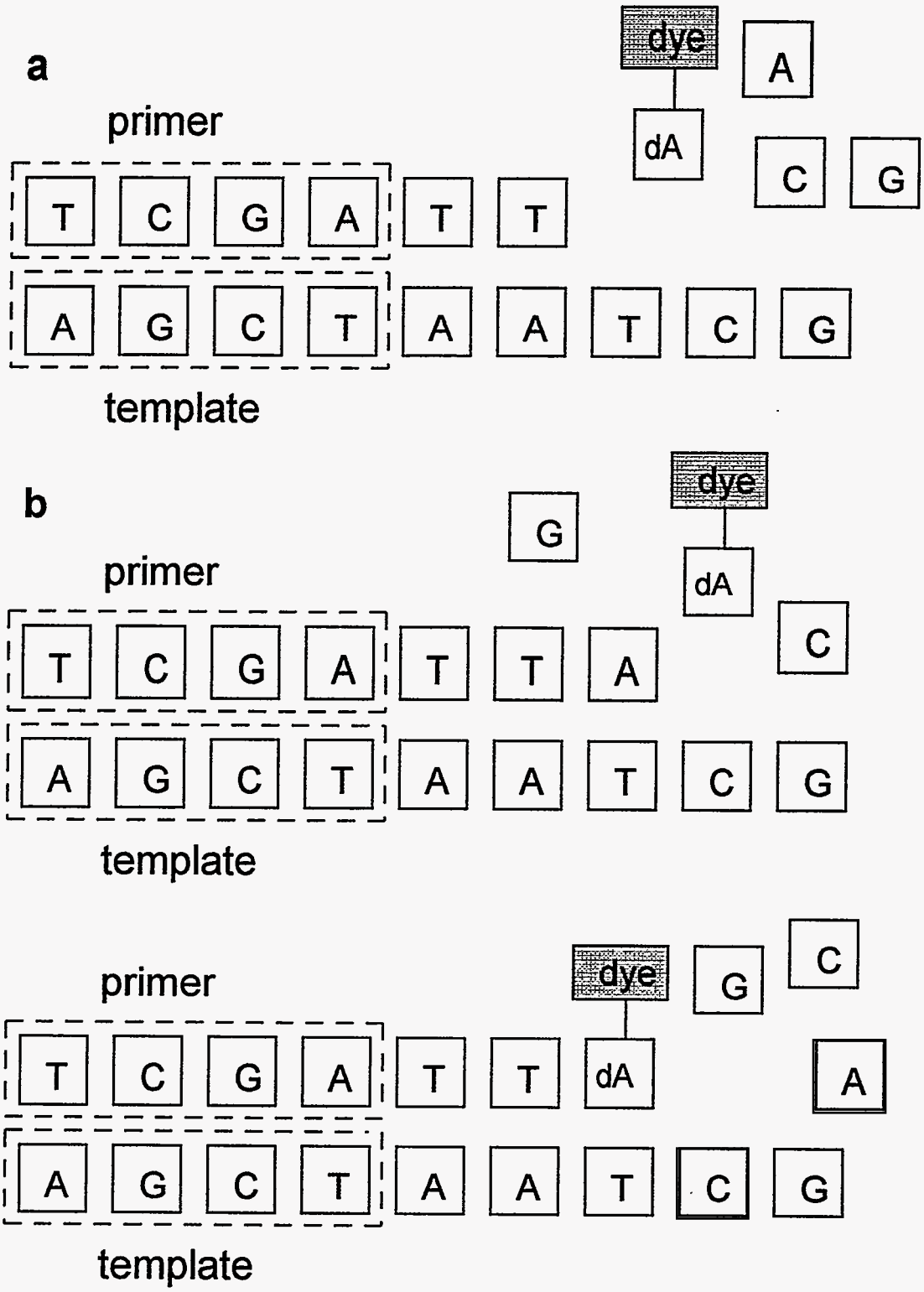
Besides fulfilling our quest to understand humans (and in the process, different species) in a scientific sense, the genome projects have tremendous impact on medicine. With the development of new technologies, research tools and fundamental knowledge, we may eventually understand and even control many human genetic diseases. Also, we may be able to tackle diseases caused by environmental effects such as exposure to radiation or chemicals. In fact, the advancement of molecular biology techniques in the past decades has already have great impact on fields like evolution, agriculture and animal science.

There are numerous techniques available to analyze (or sequence) DNA. The general idea is the separation of the DNA fragments according to their sequences or lengths. The method of choice depends on the required accuracy. For example, a resolution of 1 bp is necessary for sequencing[1,2] while a resolution of 10 bp may be sufficient for gene mapping and restrictive fragments analysis.[3,4] Electrophoretic separation of the DNA Sanger ladder developed by Sanger is the most widely adopted method in DNA sequencing.

Traditional method

Separations of the DNA Sanger ladder are routinely performed by slab-gel electrophoresis with autoradiographic detection. The Sanger method is the most commonly employed method for DNA sequencing due to its ease of automation. The key to this method is a continuous generation of a series of DNA fragments, the so called Sanger ladder as illustrated in Figure 1.1.

Fig. 1.1 A diagram to illustrate DNA sequencing by the Sanger method.
(a) Elongation of the Sanger fragment with normal dNTP's;
(b) Chain termination by ddNTP's;
(c) Separation of the Sanger fragments on a slab-gel.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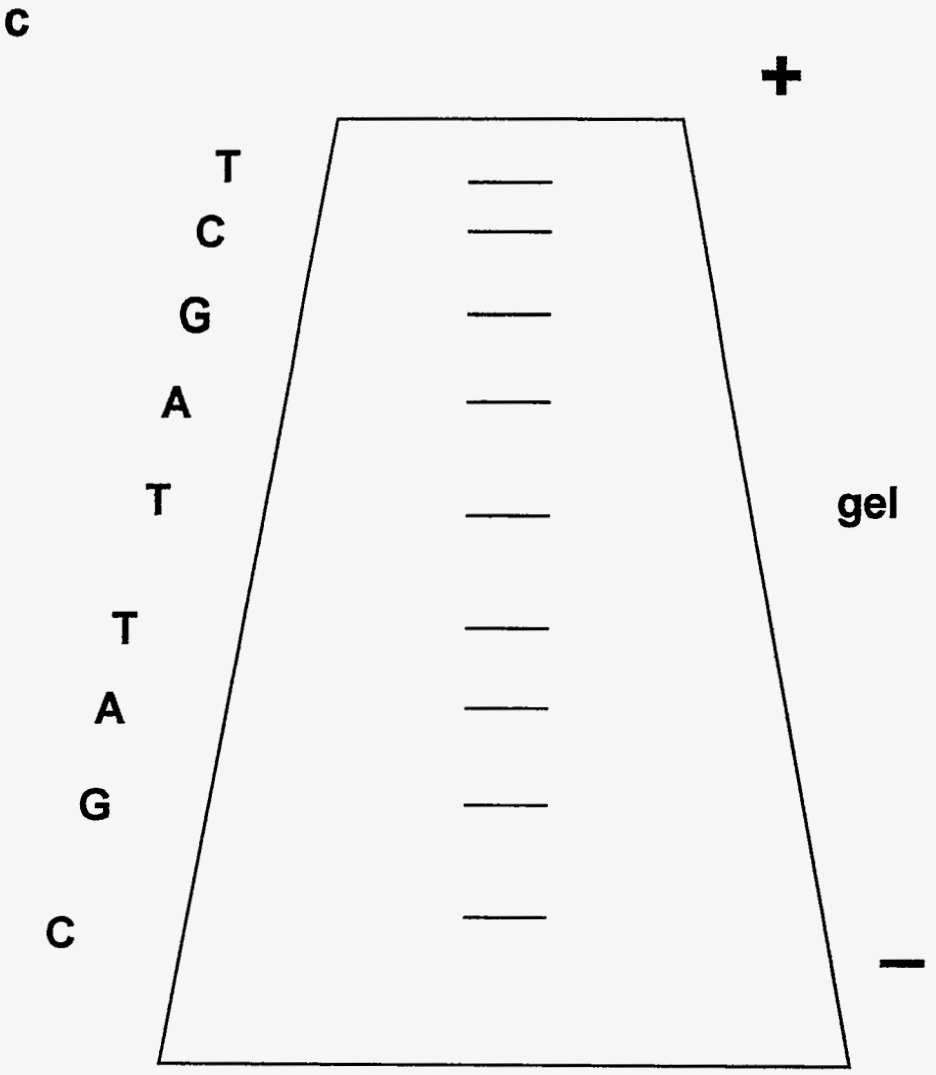


Figure 1.1 (Continued)

The generation of a Sanger ladder is done enzymatically. The DNA fragment to be separated (the template) is usually cloned into a plasmid or a vector. A mixture of primer, dNTP's ddNTP's and polymerase is then added to the plasmid. The ddNTP's differ from the dNTP's at the 3' position, having an -H instead of an -OH. Before the enzymatic reaction, the primer which is complimentary to part of the template binds to the single-stranded template. During the reaction, polymerase (the enzyme) elongates the template chain by incorporating one ddNTP or dNTP to the 3' end. If it incorporates a dNTP, the elongation goes on. However, if it incorporates a ddNTP, the reaction stops due to the lack of an -OH at the 3' position. A series of DNA fragments with different lengths is generated in this fashion. Traditionally, four such reactions for each base (G, C, A, T) are carried out separately. Then the Sanger ladder is separated according to size by slab-gel electrophoresis. The smaller fragments come out before the larger fragments come out later. The sample from each reaction is loaded into a separate lane of the slab gel. Detection of the fragments is done by autoradiography. Either the primer or the dNTP's are labeled with a radioactive isotope, typically, ^{35}P. The sequence of the template can then be determined by comparing the locations of the peaks.

This sequencing method is time-consuming (12 hours or more is usually required), labor-intensive and requires the use of radioactive compounds. It proves to be too slow for large scale sequencing like the human genome. In order to push the separation speed further, development of new schemes is one of the primary goals of the Human Genome Project (HGP).

New schemes

Multiplexing

Due to the inadequacy of slab-gel electrophorectic-based separations and autoradiographic detection for fast DNA sequencing, much research has focused on developing automatic and non-isotopic methods. Smith *et al.*[5,6] demonstrated in 1986 the use of fluorescence detection in automatic DNA sequencing. In this

method, a different fluorophore is used to label each terminator or primer specific for each of the four bases. The four reactions are mixed and co-electrophoresed. The labels are sorted out spectroscopically, thus, one lane is sufficient to provide full sequence information. In addition, the base-calling can be done automatically by a computer. This opens up the possibilities of running multiple samples on the same gel and thereby scaling up the rate of separation (and sequencing). Other systems based on fluorescence detection have been demonstrated.[7] There are numerous applications of this detection method.[8-11] Besides separating single-stranded DNA fragments, the same method can be applied to separate double-stranded DNA fragments. In this case, the samples are tagged with different intercalating dyes.[12, 13]

As mentioned before, the use of a fluorescence detection scheme allows sufficient sequence information to be collected in one lane, and hence, four samples can be run on a 4-lane gel. Even so, the speed of DNA sequencing would still be limited unless many sequencers are running at the same time. Much work has been done on scaling up these systems. The most straightforward idea is multiplexing, i.e. running multiple samples simultaneously. With the development of two-dimensional array detectors such as the Charge-Coupled Device (CCD), up to 48 lanes can be detected simultaneously. A commercially available ABI 377 sequencer with a maximum of 48 lanes is built based on the use of a CCD. This maximum can be further pushed to 100 lanes with the use of capillary electrophoresis since the capillaries have smaller outer diameters than a lane on a slab gel. Ueno and Yeung demonstrated the use of a 100-capillary array for DNA sequencing with a CCD.[14] Mathies *et al.* also demonstrated the use of capillary array electrophoresis (CAE) for DNA sequencing.[15] Other groups[16-17] demonstrated the use of sheath flow for high sensitivity array detection.

High-speed DNA separations

Electrophoretic methods

In the past few decades, slab gels have been used to separate biomolecules such as proteins[18], DNA[1-2] and RNA.[19] Slab gels offer the advantages of single base resolution, long read length and low cost. However, the running time is usually over 12 hours, and in some cases, up to two or three days. The reason for this is the slow dissipation of Joule heat which prevents the use of high voltage (typically, 100 V is used). In the past decade, much work has been done to increase the separation speed. This includes applying capillary electrophoresis (CE), ultrathin slab gels and microchips to DNA separations. High electric field strength were adopted for all these techniques because of the efficient heat dissipation possible because of the large area-to-volume ratio of the separation channels. Among these methods, capillary electrophoresis has received the most attention.

Capillary electrophoresis In 1983, Jorgensen *et al.*[20] demonstrated the efficient separation of proteins using capillary tubing and high electric field strengths. Since then, capillary electrophoresis has undergone very rapid development and it is gaining popularity in industrial R&D. CE offers the advantages of fast separations with high separation efficiency. In addition, it requires minute amounts of sample (usually several μl) which makes it ideal for biological samples. The typical inner diameter of a capillary is between 20 to 150 μm while the outer diameter is usually between 150 to 360 μm. Due to the large surface-to-area ratio of the capillary tubing, Joule heating is minimized, and thus, a high potential difference of 10-30 kV can be applied. There are several common detection methods: electrochemistry, fluorescence, absorption and mass spectrometry. The method of choice depends on the analytes and nature of the information needed (e.g. structural information).

In free solution electrophoresis, for example, capillary zone electrophoresis, the separation is based on the different mass-to-charge ratios (m/z) of the analytes.

In other words, the analytes must be charged. Besides capillary zone electrophoresis, there are different modes of capillary electrophoresis for separating different analytes: capillary isoelectrofocusing (cIEF),[21] capillary electrokinetic chromatography (CEC),[22] micellar electrokinetic chromatography (MEKC)[23] and capillary gel electrophoresis (CGE).[24]

Since all DNA molecules have the same mass-to-charge ratios except those smaller than 10 bp, they cannot be separated in free solution in the absence of electroosmotic flow. A sieving matrix must be used to achieve size based separations.[25] Therefore, CGE is commonly used to separate DNA molecules. Linear polyacrylamide (LPA),[26] poly(ethylene oxide) (PEO),[27-28] hydroxyethylcellose (HEC),[29] Poly(N-acryloylaminoethoxyethanol),[30] end-capped Poly(ethylene glycol) (PEG)[31] and mixtures of these polymers are some of the most commonly used sieving matrices.

Microchip Microfabrication has become a new player in the field of DNA analysis. Miniaturization, multiplexing, high separating speed and the possibility of making inexpensive, disposable chips are some of the advantages of microchips. By etching separation channels on a piece of quartz plate, Mathies *et al.* demonstrated the separation of 200 bp in less than 5 min.[32] Aside from etching separating channels on the chip, Mathies *et al.*[33] also demonstrated successfully the coupling of PCR reaction with the high speed DNA separations on the same chip. Another advantage of applying microfabrication to DNA analysis is that it is not limited to an electrophoretic method. In fact, one of the most actively engaged areas in microchip research is separation by hybridization which will be discussed later.

Ultrathin slab gel Although CE has significant potential in separating DNA, it has a number of problems such as cost and complexity. Thus, some groups are working on improving the speed of DNA separations on slab gels. The use of ultrathin slab gels in DNA sequencing was first reported by Ansorge *et al.* in 1991.[75]

With a gel thickness of less than 200 mm, a higher electric field (80 V/cm) was applied to achieve fast separations. Smith *et al.* further decreased the thickness to 25 mm and increased the field strength to 250 V/cm.[35-36] In all these cases, sample loading is a problem especially with multiple samples. In order to overcome the problem, Ewing *et al.*[37] demonstrated that easy loading of multiple samples can be accomplished by a motor-controlled movable capillary. Ultrathin slab gel offers the advantages of compatibility with the currently available slab gel based commercial sequencers, and they are relatively cheap. However, this method still suffers from the disadvantages associated with cross-linked polyacrylamide, namely bubble formation and gel shrinkage.

Non-electrophoretic methods

Although there are a number of advantages to separate DNA by electrophoresis, the efficiency and resolution are conformation dependent. In other words, identical fragments with different conformations can have different migration velocities. A good example is the compression band for G-C rich regions in DNA sequencing.[38] In order to overcome this barrier, much work has been done on developing non-electrophoretic-based DNA separation methods. Some of the methods to be discussed are flow cytometry, mass spectrometry (MS) and sequencing by hybridization (SBH).

Flow cytometry Keller *et al.*[39] demonstrated sizing of megabase DNA fragments. The DNA fragments were tagged by intercalating dyes, and then the stream (flow) of DNA solution was allowed to pass through a focused laser beam. Laser induced fluorescence of the DNA-dye complex was recorded. The smallest size measured was approximately 200 kbp. In addition, they found that the fluorescence intensity is independent of the conformation of the DNA fragments. The resolution is limited by the ability to differentiate fluorescence intensities of different fragments.

Mass spectrometry (MS) There are a number of advantages in using mass spectrometry. These include accurate mass determination (and hence, no separation is needed prior to mass determination), structural information and fast data acquisition. In addition, it is not necessary to label the DNA fragments with dyes which eliminates a lot of sample preparative work. Another advantage is that more than one sample can be analyzed at the same time. The sequence is read the same way as in optical methods. All that necessary is to sort out the fragments after data acquisition. Because of these potential advantages, many groups have worked on applying MS to DNA separations.[40-41]

In the case of MS, the ionization method plays a crucial role. Matrix-assisted laser desorption and ionization (MALDI) and electrospray ionization (ESI) are the most common methods used to ionize biomolecules such as DNA and proteins. MALDI is a soft ionization method in which the parent ions are usually preserved and a relatively small amount of fragmentation is observed. It provides significant information on the molecular weight of the parent ions. The major problem with DNA fragments is that the larger fragments (for example, >80 bp) tend to undergo fragmentation. Much work has been done to overcome this problem including choosing the right matrix to optimize the laser coupling efficiency and fragmentation and modifying the DNA fragments.[42-43]

ESI often produces multiply-charged ions which effectively lower the mass to charge (m/z) ratio of the ions so that they do not exceed the upper mass range of the instrument. However, it poses a stricter requirement on the mass resolution of the instrument. In the case of DNA fragments, relatively gentle ionization conditions are used to avoid excessive fragmentation. Smith *et al.* demonstrated the ionization and detection of a double-stranded PCR product by ESI-MS.[44] The general problem of ionizing DNA fragments with ESI is the detrimental effect of the high salt concentration of the DNA samples. Smith *et al.* demonstrated improved spectral quality by removing the salt from the sample by microdialysis prior to mass

spectrometric analysis.[45] Like MALDI, fragmentation of large fragments is problematic.

So far, MS has had limited success in DNA sequencing. Only 80 bp can be sequenced due to fragmentation and the high mass resolution required for large DNA fragments. However, MS has potential in PCR products analysis since in these cases moderate resolution is sufficient while short analysis time is strongly desired. Another interesting area is the non-covalent interaction of DNA adducts.[46]

Sequencing by hybridization (SBH) SBH is another active avenue of DNA analysis. It was made possible because of the maturity of microfabrication technology. The basic idea behind SBH is that longer sequences can be obtained by the unique overlap of their constituent oligonucleotides. [47] For example, three nanomers with the sequences of:

AGCTCCTGG

GCTCCTGGC

CTCCTGGCA

uniquely define the sequence of AGCTCCTGGCA.

There are two modes of SBH: (a) the unknown DNA is immobilized on the support and tagged oligonucleotides are then added to hybridize with the bound DNA, (b) oligonucleotides are immobilized on the support and tagged unknown DNA is added to hybridize with the bound oligonucleotides. Excess tagged DNA is then washed away, and the fluorescence intensity is measured. The sequence of the sample can be determined.[48] Theoretically, the sequence can be determined in a short period of time, since the time taken for hybridization and washing is less than an hour. In addition, a high density of DNA probes can be immobilized on the chip, enormous amount of information can be obtained in a limited space.

Currently several groups[49-50] are actively engaged in developing fast medical diagnostic methods combining microfabrication and hybridization.

In summary, much has been done to improve the speed of DNA analysis. Each technique discussed above has its own advantages and disadvantages. The method of choice depends on the particular needs of the application.

DNA separations with capillary electrophoresis

In our group, we are working on a multiplexed DNA sequencer to increase the separation speed and throughput. Capillary gel electrophoresis was chosen as the separation method because it acts favorably in those regards. Other goals of this work are to achieve optimum resolution for accurate base calling and lower the cost. The first part of this section is intended to provide some background on CZE and CGE and the application of CGE to the separation of DNA.

CZE

Other than electrophoretic mobilities (which governed by the analytes' mass-to-charge ratios) (Eqn. 1), one important factor in capillary electrophoresis is the electroosmotic flow (EOF).

$$n_{ep} = m_{ep}E \propto m/z \qquad (1)$$

where n_{ep} is the electrophoretic velocity,

m_{ep} is the electrophoretic mobility,

m is the mass,

z is the charge,

and E is the external electric field strength.

EOF is unique to CE. It is generally believed that EOF is generated by the movement of the ions within the surface of the double layer under the influence of an external electric field. EOF is illustrated in Figure 1.2. Since the pKa of the surface silanol groups is around 3-4, at pH above 2, these silanol groups are partially or even fully ionized, leaving the surface negatively charged. A layer of

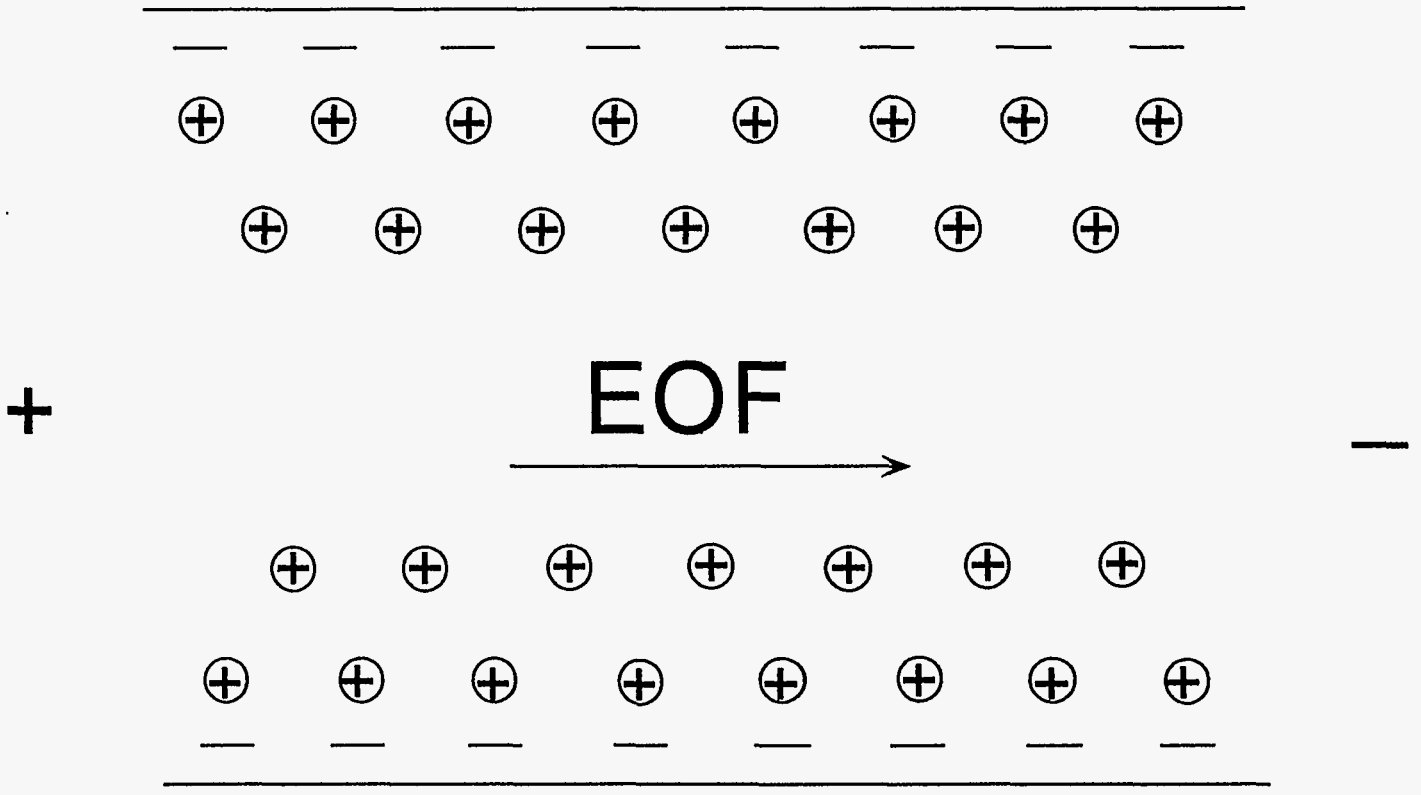


Fig. 1.2 A diagram to illustrate electroosmotic flow (EOF) in capillary electrophoresis.

positive ions is then absorbed on the surface. Under the influence of an external electric field, the ions in the loosely absorbed layer move from the anode to the cathode (Eqn 2).

$$n_{eo} = m_{eo}E \qquad (2)$$

where n_{eo} is the electroosmotic velocity,

m_{eo} is the electroosmotic mobility.

The net velocity of the analyte is the sum of its electrophoretic velocity and its electroosmotic velocity

Capillary gel electrophoresis

Since all DNA molecules have essentially the same mass-to-charge ratios (except those smaller than 10 bp), they cannot be separated in free solution in the absence of electroosmotic flow. A sieving matrix must be used to achieve size based separations.[51] The application of CGE to DNA separations was first demonstrated by Karger[52] in 1988. In that work, a mixture of oligonucleotides (20-mers) were separated in less than 20 minutes. Since then, a lot of work has been done on applying CGE to different areas of DNA analysis such as sequencing,[53-58] gene mapping and PCR reaction products analysis[8, 59-61] and genotyping.[62] The primary focus of these works was on the sieving matrices and on the capillary wall coating.

Figure 1.3 shows the fundamental setup for capillary (gel) electrophoresis. Both ends of the capillary are immersed in buffer vials. High voltage is applied between the two electrodes.

Laser induced fluorescence (LIF) is the most commonly used detection method due to its high sensitivity. DNA fragments are tagged with fluorescent labels, for example, fluorescein or rhodamine derivatives either on the primers or the terminators. Fluorescence from the DNA dye labels can be collected on-line easily from the detection window without any post-column modification or processing.

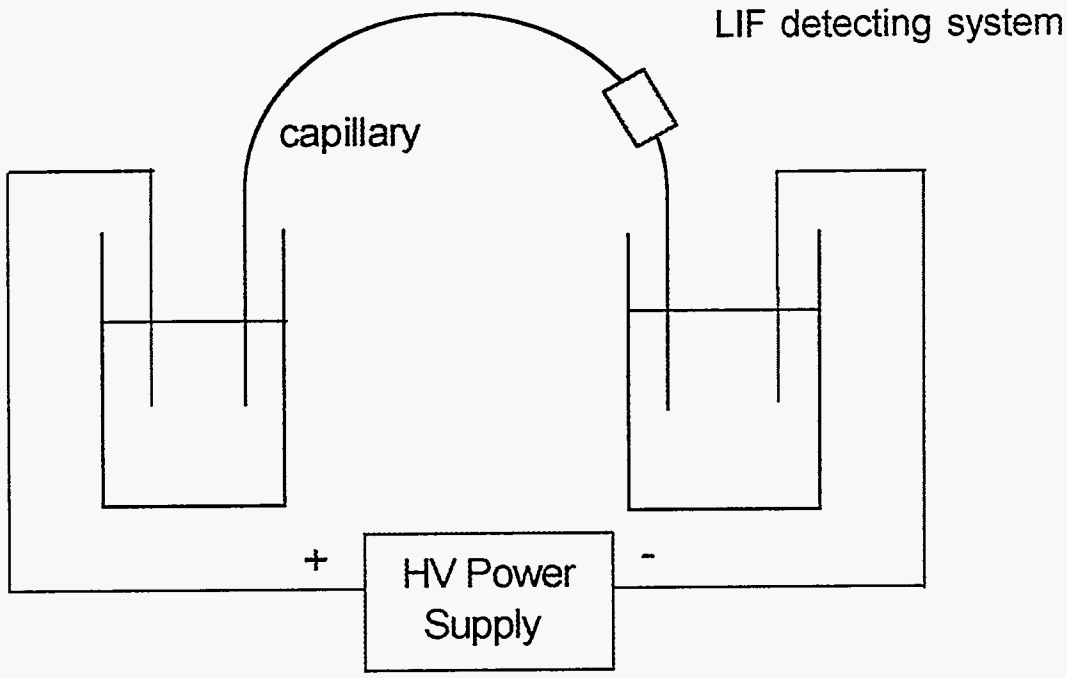


Fig. 1.3 A fundamental setup for capillary electrophoresis.

Theory

There are three concentration regimes for polymer solutions: dilute, semi-dilute and concentrated. For dilute solutions, the concentration of the polymer is so dilute that no intermolecular entanglement is possible. The polymer chain is hydrodynamically isolated from chains and thus each act as individual chains. Soane and Barron[63] successfully demonstrated separation of DNA fragments with dilute HEC solutions and proposed that transient interaction between the polymer chain and the DNA chain is responsible for the separations. Their hypothesis is supported by experiments from other groups.[64-65]

For semi-dilute and concentrated polymer solutions, the concentration of the polymers is above the entanglement threshold, so the polymer chains are entangled with one another, forming a transient network of obstacles. DNA fragments of different sizes reptate under the influence of an electric field through the different size pores formed by the polymer chains.[66-67] This forms the basis of DNA size separations.

A number of models have been proposed to account for the behavior of the DNA fragments at different electric field strengths, polymer concentrations and polymer molecular weights. The details of these models are beyond the scope of this thesis. The most popular model is the Ogston model.[68-69] In this model, DNA fragments are viewed as hard spheres which reptate through a random array of fibers (the polymer chains). A linear relationship exists between the logarithm of the electrophoretic mobility and the polymer concentration. This observation holds true for low to moderate electric fields and low molecular weight DNA fragments.

The electrophoretic mobility of DNA fragments under a low external electric field is inversely proportional to the length of the DNA fragments. This observation is accounted by the biased reptation model.[70-71] In this model, DNA fragments are viewed as having wormlike motion, and the sieving matrix is viewed as a tube that envelops the chain and restricts its motion along the curvilinear contour of the tube.

The equation in its simplified form is

$$m = (Q/3x)(1/N_t + e^2) \quad (3)$$

where Q is the total effective charge on the chain,

x is the total friction coefficient,

N_t is the total number of tube segments,

and e is the electric field strength.

However, at high electric fields, e becomes greater than $1/N_t$. In other words, the mobility is independent of size and separation fails. The exact value of the electric field depends on the size of DNA fragment.

This upper separation limit, which decreases with increasing electric field can be counteracted by a constant change in the direction of the electric field. This is achieved experimentally with a pulsed-field.[72] In this way, the reorientation time of the DNA fragments is dependent on size. DNA fragments of the size of several mega basepairs can be separated with pulsed fields.[73-74]

Yeung and Kim[75] demonstrated the use of pulsed field to separate DNA Sanger ladder fragments. They found that the pulsed field has no effect on small DNA fragments (smaller than 500 bp) while it has some effect on fragments close to 1000 bp.

Applications

There are three areas of focus: DNA sequencing; restriction enzyme mapping or PCR reaction product analysis; and, forensic applications.

DNA sequencing is the most competitive area. LPA and PEO are the most commonly used sieving matrices. Since the introduction of LPA by Karger, much work has been done on optimizing the viscosity, running time, read length and resolution. However, the polymerization process varies from batch-to batch and only a few laboratories have success in preparing the polymer solutions. This prompted Yeung and other laboratories to develop other polymer solutions. Among them, PEO solutions provides the best resolution for DNA sequencing.

There has been much focus on optimizing the separation efficiency and resolution. It is generally agreed that using a low field strength (for example, 150 V/cm) and/or longer capillary length increases the read length and resolution while sacrificing time.[54, 76-78] Also, higher molecular weight polymers enhance the resolution of high molecular weight DNA fragments while lower molecular weight polymers enhance the resolution of low molecular weight DNA fragments.[27, 78] The particular combination of field strength, polymer composition and capillary length depends on the read length, time and resolution desired.

In restrictive enzyme mapping or PCR reaction products analysis, a resolution of 10 bp is usually sufficient in most cases. The DNA fragments to be analyzed are double-stranded and are tagged with intercalating dyes. Ethidium bromide, TOTO, YOYO and POPO are commonly used.[74-75] Intercalating dyes may also play a role in the separation efficiency.[12] HEC is the most commonly used sieving matrix for restrictive enzyme mapping because of its low viscosity and simple preparation. There are two sizes of DNA fragments to be analyzed, small to medium ones with a length of 0.1 kbp to 50 kbp and large ones with a length of 100 kbp to 2000 kbp. DC-field is usually employed to separate the small to medium sized fragments[59-61] while pulsed field is required to separate the mega sized fragments.[74-75]

Similar to DNA sequencing, the separation efficiency and resolution are affected by polymer concentration, composition and capillary length.[76-77]

Recently, Yeung[80] , Oefner[81] and Heller[83] demonstrated separately that the restrictive DNA fragments can be separated in free solution, in other words, without any sieving matrices. However, these methods have limited dynamic ranges.

Much has been done to apply CGE to forensic science. The fundamental concept of DNA separations is the same as DNA sequencing and PCR products analysis[83-84] since the DNA fragments to be separated can be single-stranded or double-stranded. Therefore, the sieving matrix used, its concentration, its composition and capillary length depend on the particular set of information desired.

Zhang and Yeung demonstrated the use of PEO solutions in genotyping the DS180 loci.[62] Isenberg and McCord demonstrated the use of HEC solutions for the separation of PCR-amplified products.[85] In the case of forensic science, statistical validation of the data is important. Several forensic laboratories are actively engaged in this area.[86-87]

Our goal

Due to the importance of the sieving matrix in CGE, a lot of work has been done on the development of the sieving matrices in the past decade. Besides this, special attention has been given to the development of the capillary coating in order to prolong the lifetime of the capillaries and lower the cost of the sequencers.

Sieving matrices

Cross-linked polyacrylamide is the most commonly used sieving matrix in biological separations, followed by agarose. It is not a surprise that cross-linked polyacrylamide was also the sieving matrix of choice for CGE in its earliest days.[24] However, it suffers from the disadvantages of irreproducibility in polymerization, instability and non-replaceable nature. It would be impractical to use cross-linked polyacrylamide as the sieving matrix in a multiplexed automatic sequencer. Clearly, cross-linked polyacrylamide is far from the ideal sieving matrix for high-speed DNA separations.

Due to the potential drawbacks of cross-linked polyacrylamide, much has been done to develop better sieving matrices. The ideal sieving matrix must fulfill the following requirements:

1. High separation speed: this is especially important in the development of high-throughput sequencing instruments to support the Human Genome Project.

2. Long read length: simplifies sequencing an unknown piece of DNA by reducing the computational effort required to assemble a finished sequence from a randomly cloned template.
3. Replaceable operation: the sieving matrix must have low enough viscosity to be easily injected and withdrawn from the capillary so that every run has a fresh matrix.

Low to medium viscosity entangled polymer solutions are developed to match specific requirements. We have concentrated our effort on the development of dilute Poly(ethylene oxide) solutions (PEO). The results are described in Chapter 2.

Coating

Electroosmotic flow (EOF) can be detrimental to CGE. The EOF tends to move the sieving matrices out of the capillary which is disastrous for the separation. This is especially important in DNA sequencing where single-base resolution is necessary. Since the EOF is caused by the ionized surface silanol groups, deactivating the surface silanol groups should minimize the EOF. This is usually achieved by derivatizing the surface silanol groups with a bi-functional silane and then coating the surface with linear polyacrylamide (LPA).[88] This is by far the most common coating procedure used in DNA sequencing. However, the Si-O bond of the coating is not stable especially if the running pH is above 8.0. Novotny *et al.* achieved a more stable bonding by replacing the Si-O-C-Si bond with a Si-C-C-Si bond.[89] Even then, coating stability is still problematic. In addition, the frequent replacement of capillaries for multiplexed sequencers can be costly and impractical. We have focused on the development of alternative coating methods[90] and this work will be discussed in Chapter 2.

In 1995, Barron and Soane[91] demonstrated that the presence of EOF benefited the separation of a mixture of λ-DNA/Hind III and λ-DNA/ Hae III fragments. This is the only documented case in which EOF was beneficial to DNA separation.

Temperature

The running temperature also plays an important role in the resolution and efficiency. Running the separation at elevated temperatures (for example, 60°C) increases the resolution of G-C rich regions.[92] In these studies, it was found that running the separation at elevated temperatures gives longer read lengths as predicted in the original Ogston theory. This is due to the fact that the DNA fragment can have higher internal randomness (higher kinetic energy) to overcome the effect of the external electric fields at elevated temperatures. While urea is always added to the sieving medium to keep the single-stranded DNA fragments from renaturing, it unfortunately increases the viscosity of the solutions and causes problems in regenerating the performance of the capillaries. We have concentrated on developing a non-denaturing matrix so as to increase the read length and resolution. The results will be discussed in Chapter 3.

CHAPTER FOUR. CONCLUSIONS

Capillary electrophoresis has gained acceptance over the years within the industrial and academic analytical chemistry community. Since the successful demonstration of separating DNA fragments by CGE in 1987, much work has been done on further pushing the ultimate limit of fast and accurate DNA separations.

This work has demonstrated the use of mixed PEO solution as a sieving matrix in DNA sequencing by capillary electrophoresis. PEO offers fast separations, excellent resolution and is easily replaceable. In addition, DNA sequencing in *uncoated* capillaries is made possible with the use of PEO solutions. The separation efficiency can be maintained by rinsing the capillary with hydrochloric acid between runs.

Furthermore, both the read length and separation speed can be increased by running the separations at elevated temperatures. We have also demonstrated that urea can be eliminated from the sieving matrix without losing resolution in the elevated temperature condition. This may be useful to couple with techniques that are not compatible with high concentration of organic constituents.

When the Human Genome Project (HGP) is completed in a few years, the need to sequence will become greater. Thousands and thousands of genes will be sequenced routinely for various medical and research purposes. The development of high-throughput DNA sequencers remains one of the primary goals of the HGP. In fact, the separation speed of DNA by CE with the use of PEO solution can be further increased by running multiple capillaries at the same time. The separation methods we demonstrated in this work can be readily incorporated into a multiplexed sequencer.

The future trend in developing DNA sequencers is in further pushing the present limits on separation speed and accuracy.

PART 2. SINGLE CELL ANALYSIS BY LASER VAPORIZATION / IONIZATION MASS SPECTROMETRY

CHAPTER FIVE. INTRODUCTION

Cells are the fundamental units of living organisms. Complex organisms like humans have different types of cells in different organs. Each type of cell has its own particular shape, composition and function. Each cell has a complex mixture of biomolecules such as proteins and DNA. The direct analysis of individual cells and compartments within individual cells is rapidly becoming a powerful tool with which to investigate questions related to cellular metabolism, signal transduction and differentiation.[92-93] Any malfunction in these cellular functions can have detrimental effects on the organism. Therefore, the techniques developed for single cell analysis can have tremendous impact in medicine.

The techniques that are used must be very sensitive since there is a minute and limited amount of analytes present in a cell. In addition, the ability to provide fast analysis is an added asset. Nanoscale techniques such as flow cytometry and capillary electrophoresis are well-known and effective tools for analysis of biomolecules.[94-102] These techniques offer the advantages of fast separation and the use of small sample volumes (μL). Mass spectrometry (MS) can provide information on the identity and structure of biomolecules.[103-106] Coupling these nanoscale separation techniques with mass spectrometry seems feasible for analysis of biomolecules.[107-109] This hybrid may prove to be an effective disease screening tool. In addition, the possibility of automation and quantitation can significantly reduce labor requirements.

Flow cytometry

Flow cytometry is a very powerful research tool in cell biology. It has been applied to detect DNA, RNA and proteins. This section is intended to provide a brief introduction to flow cytometry. Interested readers are referred to some excellent reviews on flow cytometry.[110-112]

Flow cytometry is a process in which individual cells, or other biological particles are made to pass in single file, within a fluid stream past sensors which measure physical or chemical characteristics of the cells or particles. In principle, any detection method can be coupled with flow cytometry. However, in practice, optical methods are the sole methods used. Interesting analytes such as proteins, DNA, RNA are usually labeled with fluorescent dyes. Both the scattering (forward or backward) and fluorescence of the cells are measured. A schematic of a typical flow cytometer is illustrated in Figure 5.1.

The sample is injected into the flow cell body. The sample volume is surrounded and supplemented by a buffering sheath fluid prior to leaving the flow cell nozzle. The laser is focused to an ellipse at right angles to the flow stream. As a particle (or cell) passes through the laser beam, the fluorescence is detected at right angles to the laser beam through a lens which focuses the collected light through a small 'pinhole'. This reduces the amount of light from sources other than the cells and increases the signal to noise ratio.

Optics are in place to collect the laser light scattered at right angles. This 90° light scatter has been correlated with increasing cytoplasmic granularity. A 45 degree dichroic mirror is designed to reflect the scattered light to a detector, while longer wavelengths of fluorescence pass through. Fluorescence separation of up to three colors (blue, red, green) is achieved with additional filters specific for each color detector. Five dyes have been extensively used as immunofluorescence probes; fluorescein (FITC), phycoerythrin (PE), rhodamine (RITC), Texas red and allophycocyanin (AP). Photomultiplier tubes (PMT) are popular optical detectors. Pulses of optical signals are digitalized by analog-digital convertors (ADC) and the digital signals are then stored in a computer. The flow rate can be as fast as 10 m/s. Thus, tens of thousands of cells can be analyzed within minutes.

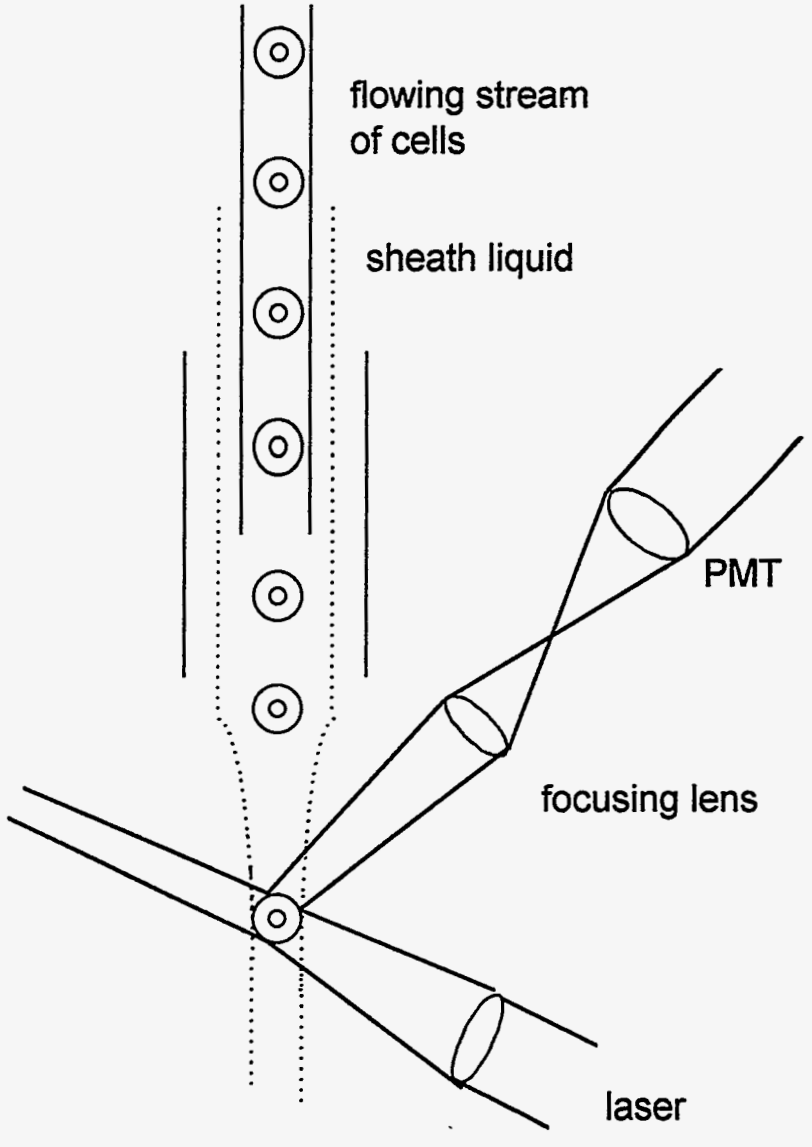


Figure 5-1 Basic principle of fluorescence flow cytometer using orthogonal axes of cell flow, illumination (laser) and detection (PMT). Extensions include fluorescence spectroscopy with more than one detector, light scatter detectors and polarization measurement.

Mass spectrometry

Different modes of mass spectrometers have been employed to couple with nanoscale separation techniques to analyze biomolecules. These modes are summarized in Table 5.1.

Detailed description of each mode is beyond the scope of this thesis, interested readers are referred to some excellent reviews.[113-115] However, some interesting points are briefly mentioned here. Time-of-flight (TOF) is ideal to couple with flow cytometry for analyzing biomolecules due to its unlimited mass limit and fast data acquisition. Fourier transform ion cyclotron resonance (FT-ICR) is ideal for mass and structural determination due to its high resolution.

Ionization of biomolecules

Since ions are the only constituents that can be analyzed by mass spectrometers, the vaporization and ionization of analytes are crucial steps in

Table 5.1 Performance characteristics for different mass spectrometers

Mass Spectrometer	Ion utilization efficiency	Spectra/s	Resolution
Quadrupole	10^3 - 10^4	1	10^5
Orthogonal Time-of-flight	0.03 - 0.8	10^4	200 - 1000
ITMS	0.1 - 0.5	1 - 5	10
FTICR	0.03 - 0.3	0.05 - 0.5	10^4 - 10^5

coupling nanoscale separation techniques and MS. Many ionization methods have been developed. Among them, Fast-atom Bombardment (FAB),[116] Electrospray Ionization (ESI)[117] and Matrix-assisted Laser Desorption/Ionization (MALDI)[118] are the most commonly employed methods for ionizing biomolecules.

ESI and MALDI are the most common methods to couple CE-MS for the analysis of biomolecules. They are considered as 'soft' ionization methods since fragmentation is in general not severe. In ESI, the analytes are evaporated and vaporized by the application of an electric field to a sharp spray tip at atmospheric pressure. A number of theoretical models on the ion production process have been proposed.[119] Mass spectra of ESI interfaces are characterized by multiply charged ions and adduct peaks (usually H^+ adducts) . This is advantageous since these multiply charged ions significantly reduce the m/z ratios of the large biomolecules. However, it poses a stricter requirement on the resolution and the detection limit of the mass spectrometer. Electrospray ionization (ESI) is ideal to work with Fourier transform ion cyclotron resonance (FT-ICR) and quadruple mass spectrometers due to their inherent good resolution and low mass limit.[120] Also, the low flow rate and atmospheric spray condition make it a good candidate for an interface to couple with CE.[121-122]

In MALDI, the analyte is usually mixed with a light-absorbing compound (matrix). The matrix to analyte ratio ranges from 10 to 10,000. Lasers are used as source of energy for vaporization and ionization. Two major groups of lasers are commonly employed, UV with wavelengths around 250 nm, for example, Kr-F excimer (248 nm) and frequency quadrupled Nd:YAG (266 nm) lasers; and wavelengths above 300 nm, for example, nitrogen (337 nm), frequency-tripled Nd-YAG (355 nm), Xe-Cl excimer (308 nm) and Xe-F excimer (351 nm) lasers. There is a large variety of matrices. They can be roughly classified into two groups, solid and liquid matrices. Table 5.2 summarized these two groups.

Table 5.2 Examples of some of the most commonly used matrices in MALDI

	Matrix	Reference
Solid	Nicotinic acid	118
	Vanilla acid	123
	Cinnamic acid derivatives	124-125
	Benzoic acid derivatives	126-128
	3-hydroxypicolinic acid	129-130
	ice	131
	Phenol derivatives	132
Liquid	ultrafine metal powder in glycerol	133
	3-nitrobenzyl alcohol	114

The MALDI process is not clear but is generally thought to be absorption of energy by the matrix and followed by vaporization of the matrix and analytes. Ionization of the analytes is believed to be due to direct photoionization and photofragmentation by the desorption laser, protonation and deprotonation reactions of excited and ground state molecules and decay of clusters. In MALDI, the excess matrix may form adducts or serve as a source of protons for the analytes.[134-139] Matrix molecules are likely to play an important role in ionization by acting as intermediate radical molecular ions. The choice of matrix is crucial for successful ionization of the analyte.[140] The development of matrices has been one of the primary focuses in MALDI-MS.

Interfacing nanoscale separation techniques and MS

Since the introduction of the first CE-MS interface by Olivares *et al.*,[141] a number of interfaces have been developed.[142-146] The key function of a CE-MS interface is to convert an aqueous solution of analyte molecules into gas phase ion and then ionize these molecules while maintaining electric contact. Unlike traditional CE, the distal end of the capillary is inside the mass spectrometer and therefore no buffer vials can be used. Thus, the conventional method of maintaining electrical contact is not possible here. The main challenge in designing the CE-MS interface is to achieve this goal without sacrificing the resolution and efficiency of the separation. There are two main approaches to this problem: sheath-flow and sheathless flow.

Sheath flow

In the sheath-flow geometry, a make-up solvent is added to supplement the low flow rate of the CE so as to maintain a stable spray as in the case of electrospray ionization or to add the buffer for ionization such as the matrix in the case of MALDI and continuous flow fast atom bombardment (CF-FAB). There are two versions of sheath-flow interfaces, coaxial liquid flow[141,145] and liquid junction.[146] In coaxial liquid flow, the make-up solvent is added coaxially by the use of concentric capillaries as illustrated in Fig. 5.2. In liquid junction flow, the make-up solvent is added by the way of a tee as illustrated in Fig. 5.2. However, in these two cases, a make-up solvent is required which may cause a loss of resolution and efficiency, sample dilution (which results in poor sensitivity) and increase in background chemical noise.

Sheathless flow

Unlike sheath flow interfaces, no make-up solvent is required for sheathless flow interfaces. There are several common methods to maintain electrical contact

Figure 5.2 A diagram to illustrate CE/electrospray interfaces.

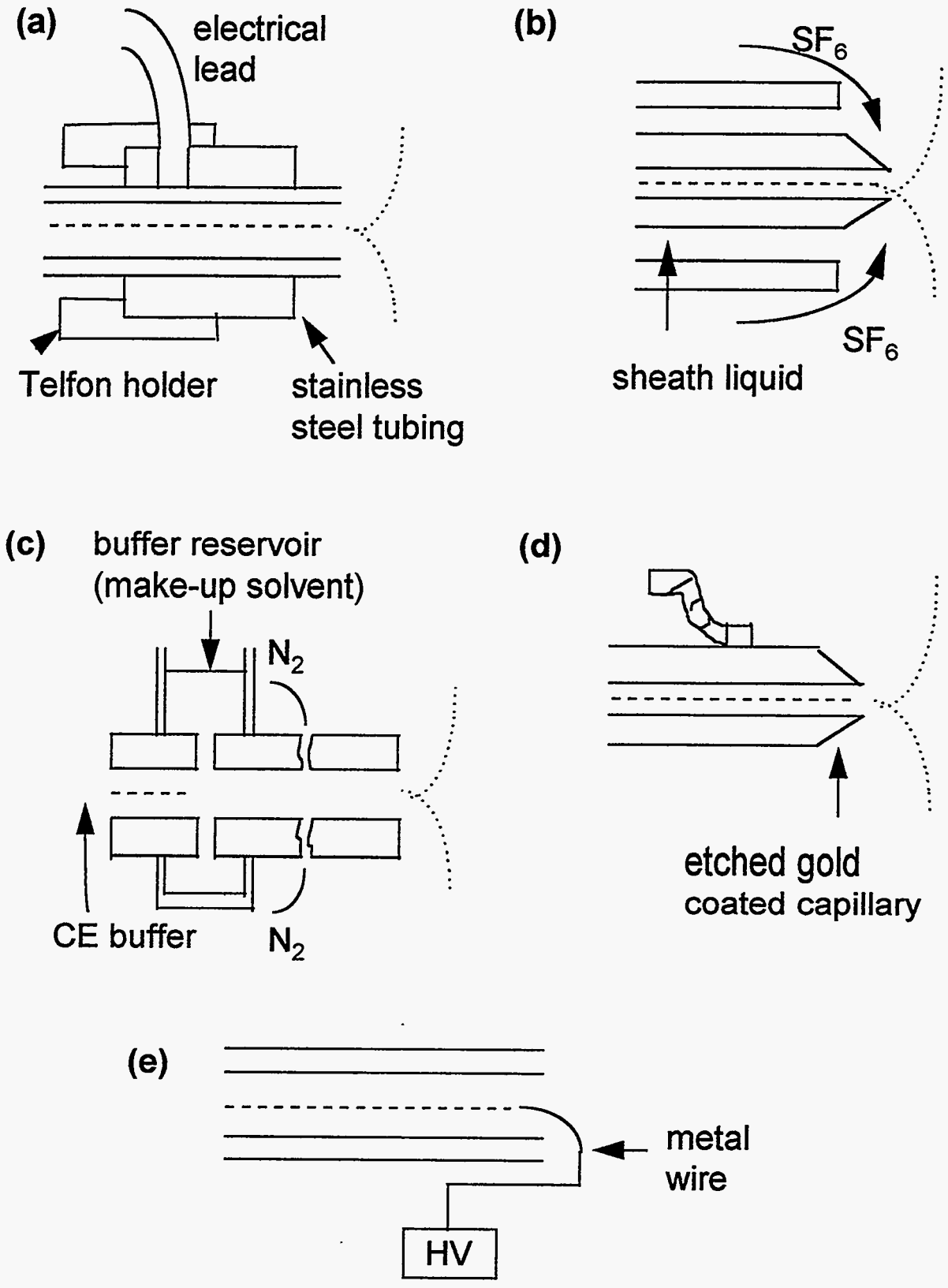
(a) The original design by Smith *et. al*[141], the capillary tip was coated with metal;

(b) a coaxial sheath flow;

(c) a liquid junction interface;

(d) sheathless flow interface;

(e) a metal wire was inserted into the capillary.


(a)
electrical lead
Telfon holder
stainless steel tubing
(b)
SF_6
SF_6
sheath liquid
(c)
buffer reservoir (make-up solvent)
N_2
CE buffer
N_2
(d)
etched gold coated capillary
(e)
metal wire
HV

and achieve stable ionization including: gold-coated capillary tip;[147-150] and insertion of wire into the capillary. [143,151]

The coated capillary tip method is most commonly employed in sheathless flow ESI. In this mode, the capillary tip is usually etched to around 2-20 μm o.d. and a thin layer of gold coating is applied to the etched tip to help maintain electrical contact. A stable electrospray flow is maintained by the extremely low flow rate, in the low nl/min. range. This is commonly known as nanospray ionization. The i.d. of the capillary is usually less than 20 μm. Due to the fact that an extremely low flow rate is used, this mode can be used to couple microchips with MS. Ramsey and Karger have demonstrated separately the coupling of microchip and MS.

Since a make-up solvent is not required in this case, the contribution of the electrospray ion current from the analyte becomes more significant, which results in an increase in sensitivity.[148]

Another way to maintain electrical contact is to insert a wire in the capillary. Zare et. al has demonstrated a stable electrospray flow by inserting a gold wire into a 100 μm i.d. capillary.

Even though MALDI has been widely recognized as a valuable method to ionize biomolecules, a limited number of papers have been published on coupling a separation technique such as LC to MALDI-MS.[152-153] In most of these works, the coupling is done off-line. Yeung and Chang[151] demonstrated by inserting a 15 μm tungsten wire into a 75 μm i.d. capillary and adding $CuCl_2$ as both light-absorbing matrix and running buffer, CE and MS can be coupled on-line with MALDI.

Applications

CE-MS and LC-MS have been used in analysis of a number of biomolecules such as proteins, peptides, oligonucleotides, pesticides and anitbiotics. They are summarized in Table 5.3. Peptides are commonly adopted as the standard for characterizing the CE-MS systems due to their easy ionization. Recently, CE-MS and LC-MS have found a major application in the analysis of combinatorial libraries.

Table 5.3 Examples of biomolecules analyzed by CE-MS or LC-MS

Compound Class	*CF-FAB*	*ESI/Ionspray*	*MALDI/LVI (off-line, on-line)*
Acid pesticides		122	
Anthracyclines		154-155	
Benzodiazepines		156-158	
Quaternary ammonium cpds		159-160	
Deoxynucleotide-PAH adducts	161	162-164	
Macrolide antibiotics	165	166	
Peptides	167-171	146, 172-176	
Proteins		177-182	153,183
Sulphonated compounds		184-186	

Combinatorial chemistry is widely used to produce a large number of structurally similar compounds in a short period of time. Since only one of these compounds may have pharmaceutical value, a sensitive, fast and high-resolution method is needed to screen the library synthesized. CE-MS and LC-MS with their fast and high resolving power are highly suited for such screening.

Our goal

One of the major challenges in coupling nanoscale separation techniques with MS is to develop a suitable buffer system. Traditionally, single cell experiments

are performed in buffer solutions with more than 100 mM concentration salt. However, this large amount of salt is detrimental to mass spectrometry due to clogging at the tip of the capillary, cation adduction, and reduced ionization efficiency. One way to overcome this problem is to mix a sheath liquid with the analyte after the CE separation. This sheath liquid can act as a make-up solvent to supplement the low flow rate of CE. However, this may cause an increase in background noise. In addition, some of the most commonly used buffer solutions, especially in electrospray, contain a high proportion of organic solvent such as methanol and operate at pH 4 to 5. Unfortunately, these conditions are not compatible with cells which are very sensitive to the pH of the environment.

Yeung and Chang developed a CE-MS interface with a laser vaporization / ionization mechanism.[151] In this interface, a totally aqueous buffer system is used. $CuCl_2$ is added to couple the laser energy to the vaporization process and act as an electrolyte for the CE part. However, the ionic strength and pH (around 5) of this buffer system is still too low for biological cells. Cell lysis due to osmotic shock can occur in a matter of seconds. In this work, we have slightly modified the interface for flow cytometry. In addition, we developed a suitable buffer solution that can prolong the lifetime of the cells while keeping the resolution and detection limit of the system. The results will be discussed in Chapter 5.

CHAPTER SEVEN. CONCULSIONS

We have demonstrated the analysis of single rat peritoneal mast cells by coupling flow cytometry and mass spectrometry. The mass detection limit of the system was 20 amol of serotonin with one laser pulse. An aqueous buffer at physiological pH was used to ensure compatibility with cells. Rat peritoneal mast cells (RPMC) were dispensed into the mass spectrometer in single file confined within a 20 μm i.d. capillary. By using the mass spectrometer as a detector, no pre-column staining or derivatization is required. Determination of serotonin and histamine in individual cells was demonstrated. With this method, hundreds of cells can be analyzed within a few minutes. The average amounts of histamine and serotonin per RPMC were found to be 0.75 ± 0.33 fmole and 0.11 ± 0.06 fmole respectively. No correlation was found between the amount of the two amines.

GENERAL CONCLUSIONS

Analysis of two different types of biomolecules were described. In the first application, DNA Sanger fragments were separated using a mixed poly(ethylene oxide) solution in uncoated capillaries. This sieving matrix offers fast separation, excellent resolution and is easily replaceable. The separation rate was 20 bp/min which is an order of magnitude improvement over the conventional methods. Furthermore, with the use of a temperature programming scheme, the rate was increased to 30 bp/min. In addition, we decribed the use of *uncoated* capillaries in separation of DNA Sanger fragments.

In the second application, histamine and serotonin from single rat peritoneal mast cells were determined simultaneously by combining flow cytometry and mass spectrometry. A buffer solution of 2 mM NH_4Cl at pH 7.3 was used to keep the cells alive without sacrifying the resolution and detection limit of the system. With this method, information on both cell population and individual cells can be obtained.

APPENDIX. DATA ACQUISITION PROGRAMS

The ion current from the MCP is digitized by a LeCroy 9350AM oscilloscope. The signal is then transferred to a PC computer for storage through a GPIB interface card. A number of programs were written to control the parameters of the data acquisition and waveform transfer and to decode the waveforms transferred. These programs were written with LabVIEW 4.0.1 for Windows 3.1. This appendix contains these programs with detailed explanations.

Each vi (Virtual Instrument, LabVIEW terminology for programs) consists of a front panel (with gray color display) and a block diagram (with color display). The user can control some of the data acquisition and waveform transfer parameters from the front panel. The waveforms decoded are also plotted on the front panel. The block diagram is essentially the code of the program.

The programs in this appendix can be grouped into three groups:

1. data acquisition with single acquisition mode;
2. data acquisition with sequence mode; and
3. data acquisition through memory card.

The 'data acquisition with single acquisition mode' group consists of *DMA.vi*, *LC9350 get new waveform.vi*, *9410 decode single waveform.vi and splits and displays.vi*. The *DMA.vi* initializes the GPIB, *LC9350 get new waveform.vi* controls the waveform transfer, the raw data are then decoded and plotted with the *splits and displays.vi* and the *9410 decode single waveform.vi.*

The 'data acquisition with sequence mode' group consists of *collect segmented data.vi*, *segments splits.vi*, *decipher segmented data w/o time.vi* and *decipher segment data w/o time movie.vi*. The *collect segmented data.vi* controls the waveform transfer, the *decipher segmented data w/o time.vi* decodes the raw data and plots them. The data can also be displayed in movie mode with the *decipher segmented data w/o time movie.vi.*

The 'data acquisition through memory card' group consists of *display data from .prn files.vi* and *display data from memory card.vi*. These vi's decodes raw data acquired by the memory card option.

A more detailed explanation of each vi can be found on the corresponding front panel.

LC9350 new get waveform.vi

wave to read

expand A
expand B
memory C
memory D
Trace A
Trace B
channel 1
channel 2

sparsing

10

number of points to read

0

number of waveform to acquire copy

1

data type

word
byte

full wf?

yes
no

File path

c:\nkfung\data

GPIB address

4

error in (no error)

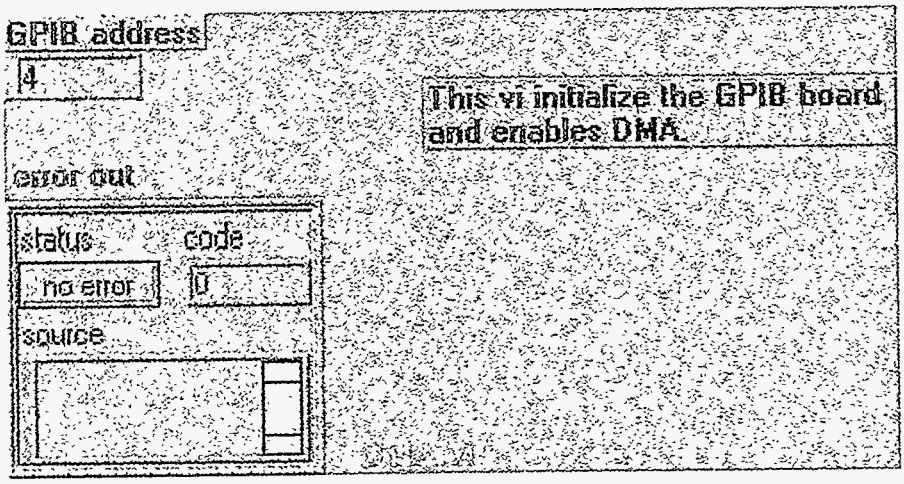
status
1 no error
code
0
source
number of waveform to acquire copy
1

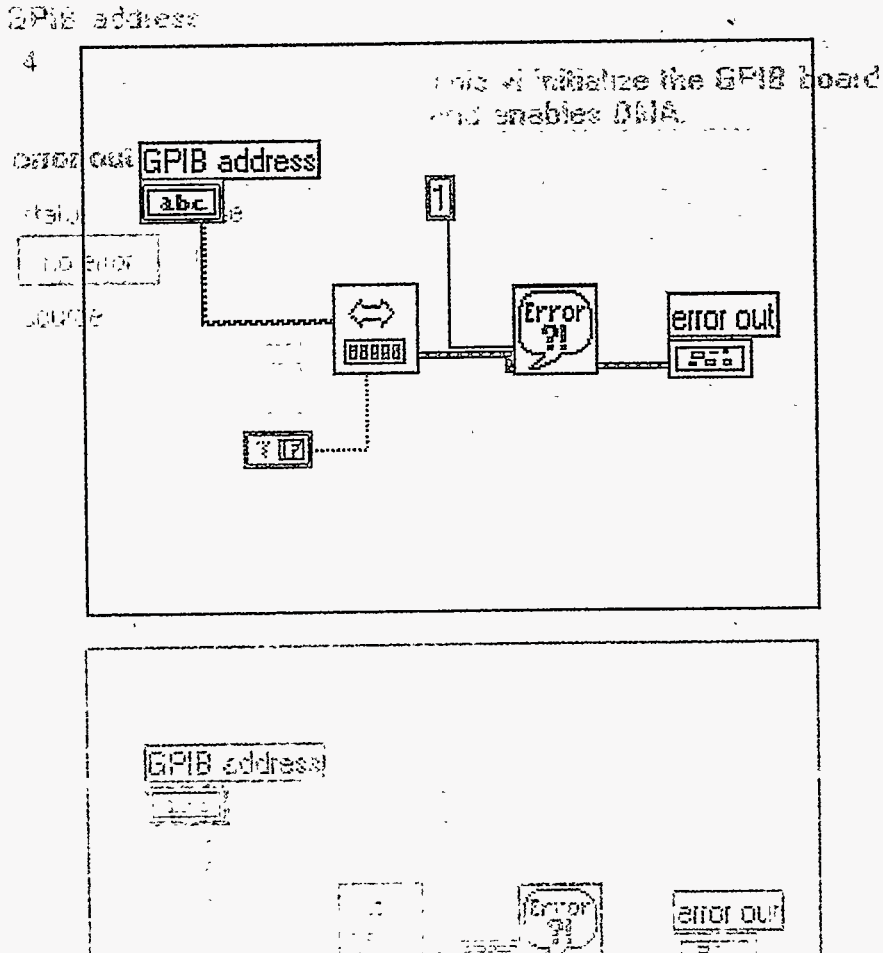
error out

status
no error
code
0
source
data type
full
word
yes
no

This vi acquire data from LeCroy 9350/9410 through GPIB.
The oscilloscope will be operated in single acquisition mode.

DMA.vi


GPIB address
4
This vi initialize the GPIB board and enables DMA
error out
status
code
no error
0
source


GPIB address
4
error out
GPIB address
abc
1
Error
error out
GPIB address
Error
error out

9410 decode single waveform.vi

This vi decodes individual waveforms collected with the 9410 new get waveform vi, 9350 new get waveform vi and displays it. Just fill in the name of the input file.

Input file
c:\nkfung\data

Vertical gain
0.00E+0

Vertical offset
0.00E+0

byte

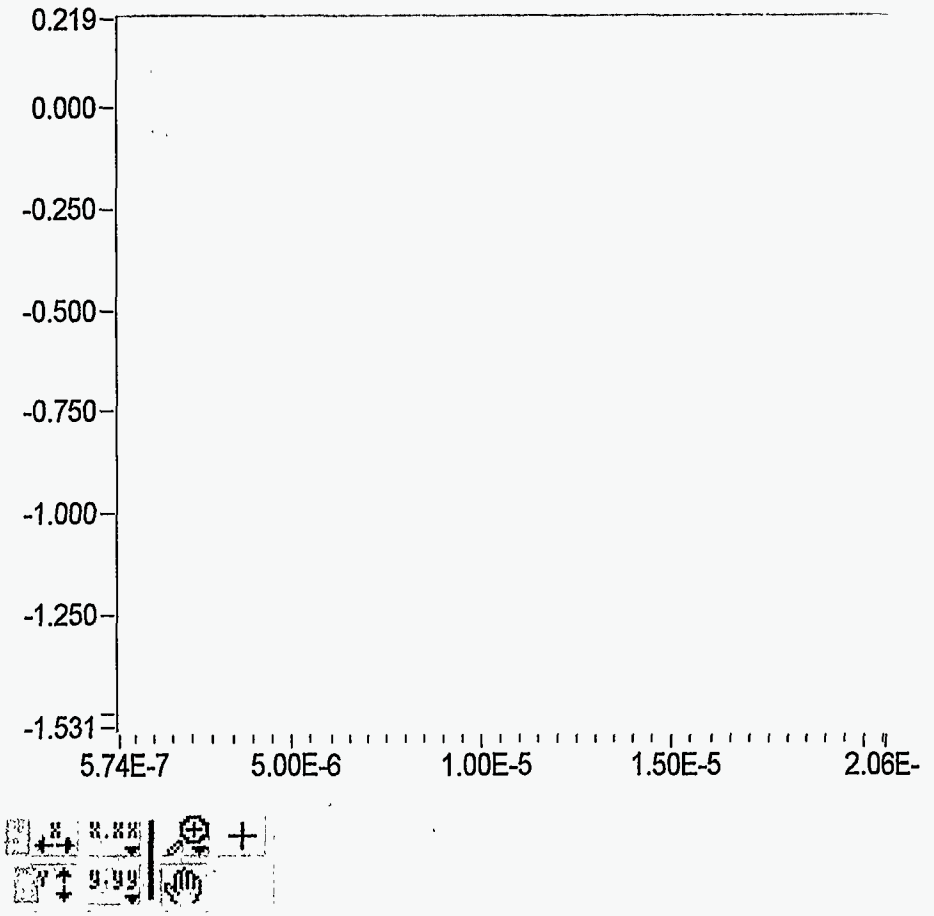
word

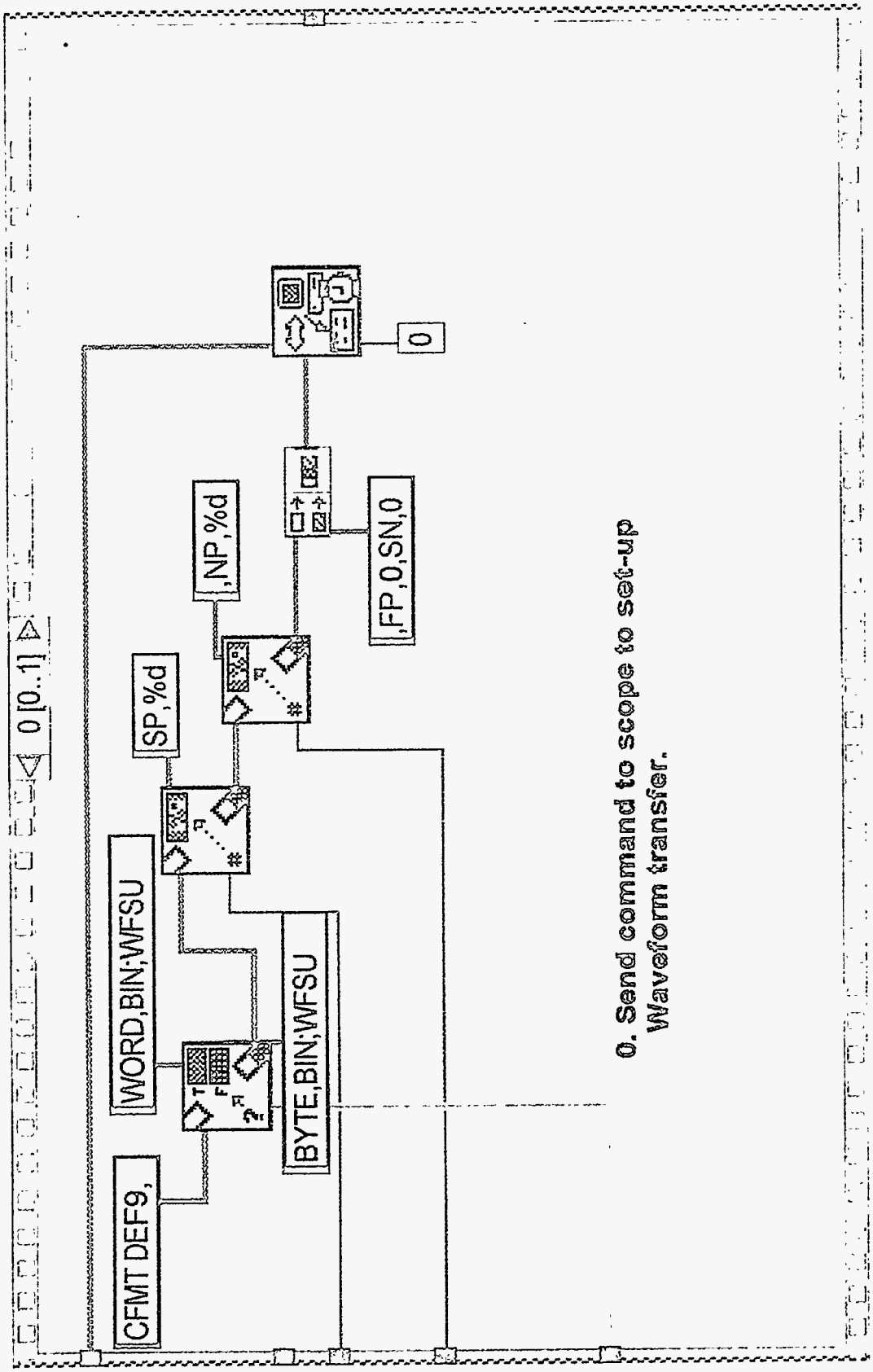
Horiz interval
0.00E+0

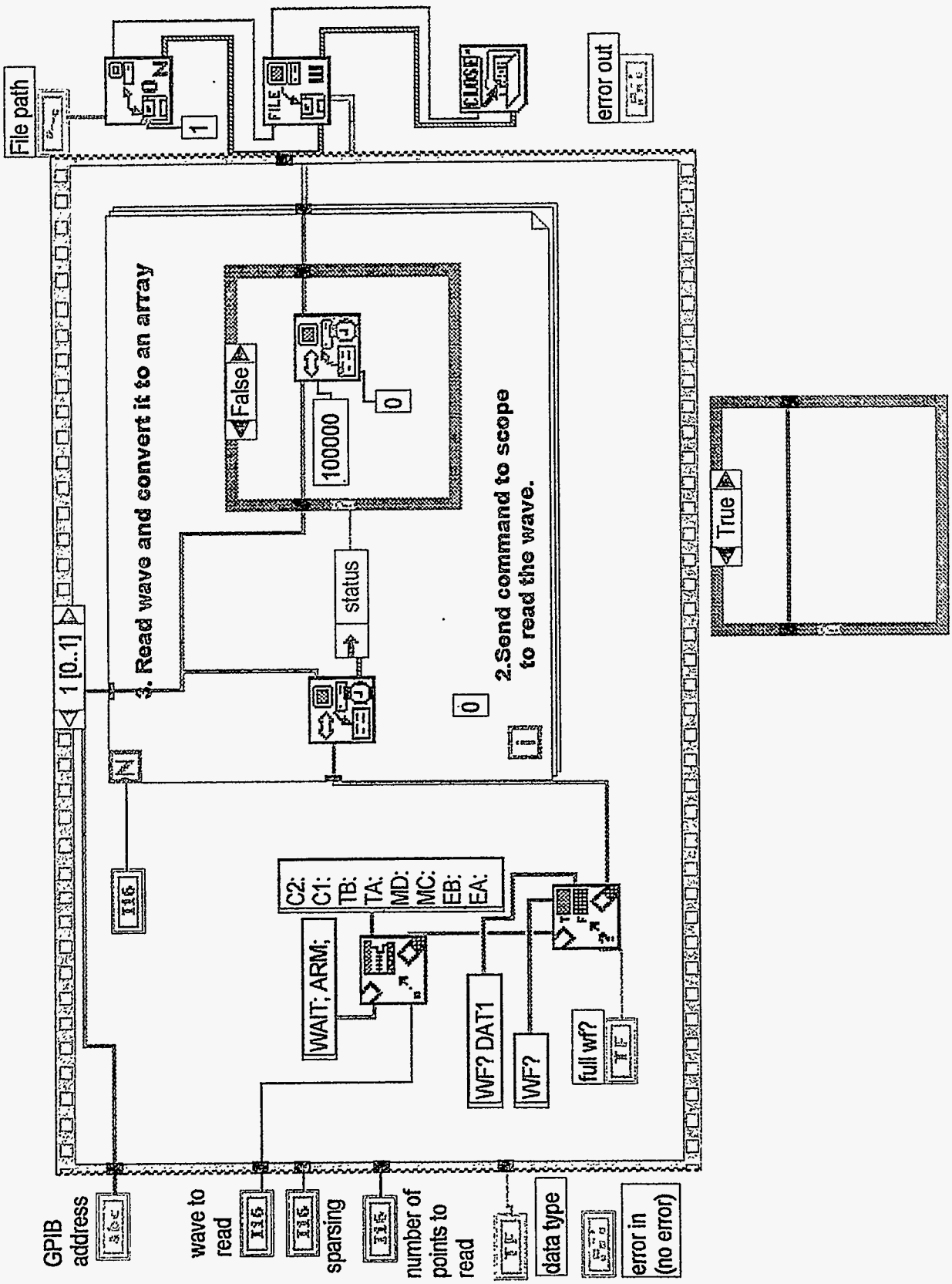
data
1000
0.00E+0

Horiz offset
0.00E+0

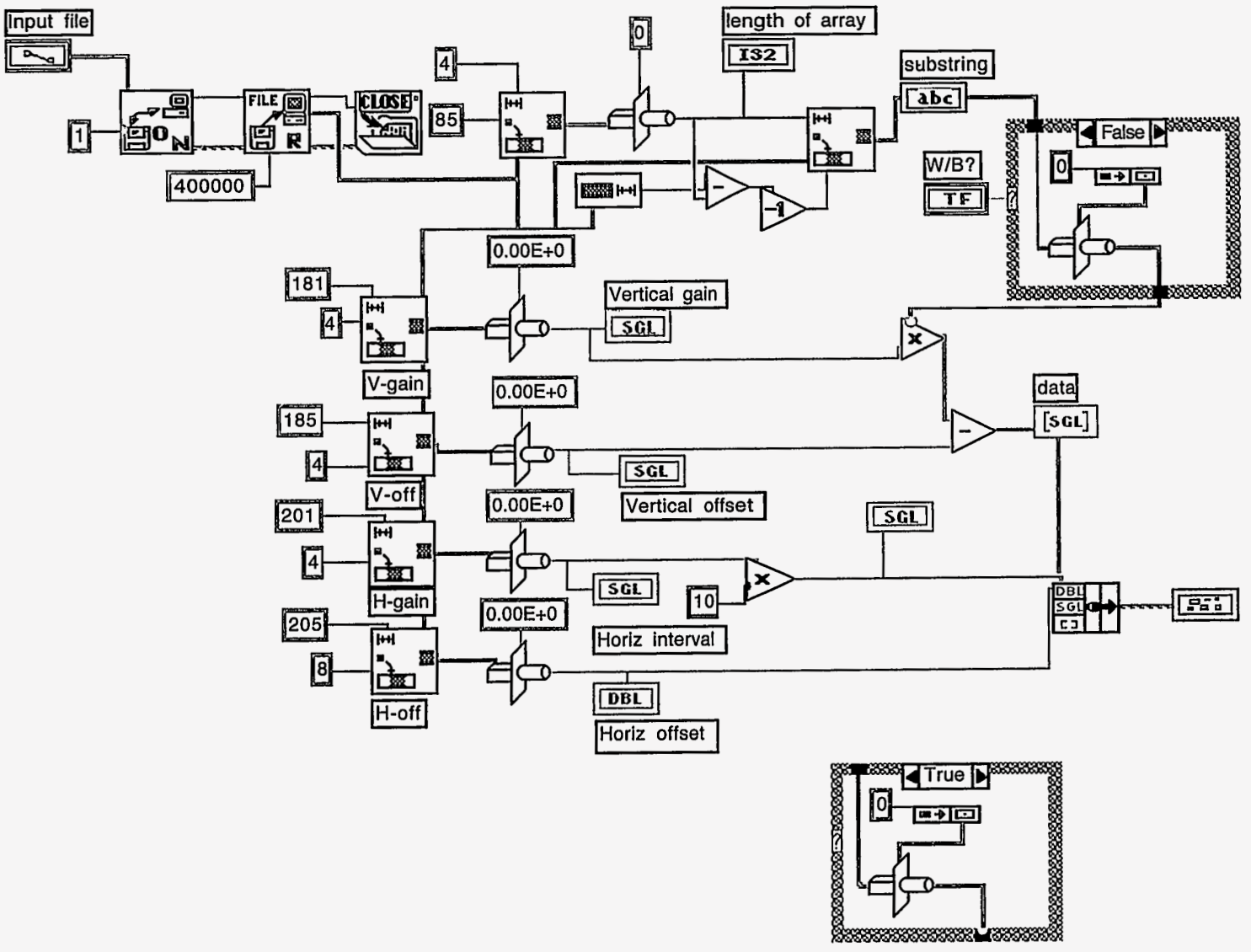
length of array
0




0. Send command to scope to set-up
Waveform transfer.
0 [0..1]
CFMT DEF9,
WORD,BIN,WFSU
SP,%d
,NP,%d
,FP,0,SN,0
BYTE,BIN,WFSU
0


File path
error out
1
FILE
CLOSE
1 [0..1]
3. Read wave and convert it to an array
False
100000
0
status
2.Send command to scope
to read the wave.
0
1
True
I16
C2:
C1:
TB:
TA:
MD:
MC:
EB:
EA:
WAIT; ARM;
WF? DAT1
WF?
full wf?
TF
GPIB address
wave to read
sparsing
number of points to read
data type
error in (no error)

9410 decode single waveform.vi
Last modified on 12/11/97 at 2:45 PM



9410 split and display.vi

Byte
Word

Input file
c:\nkfung\data

Number of waveforms
0

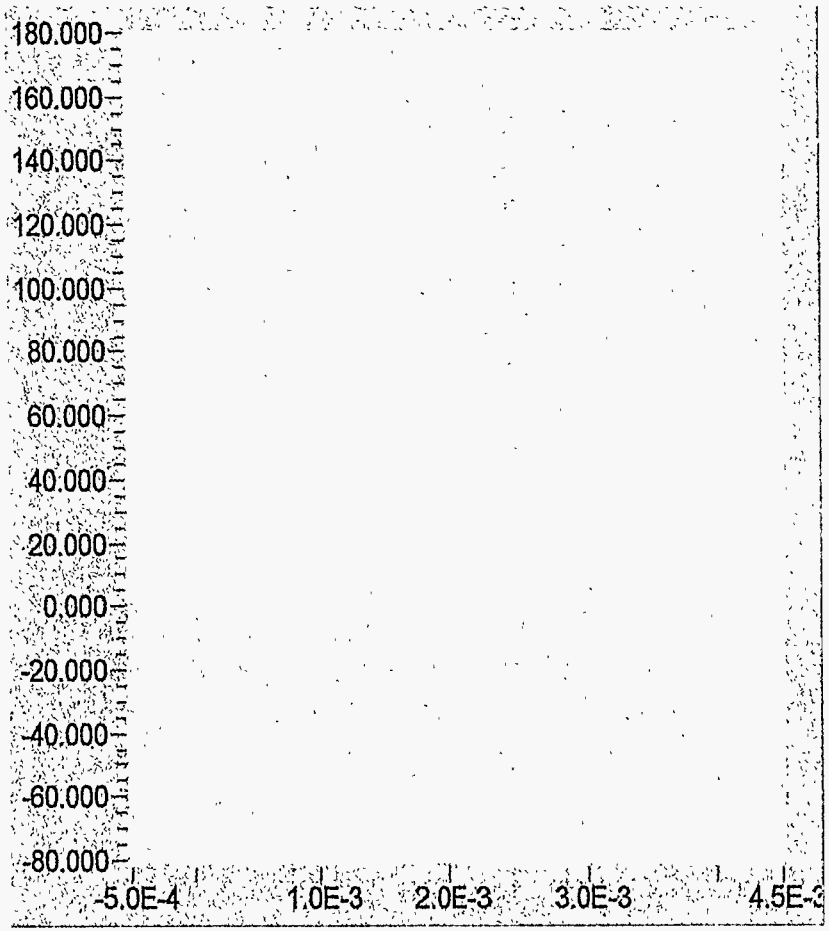
which waveform?
1

temp file
c:\nkfung\data\temp.dat

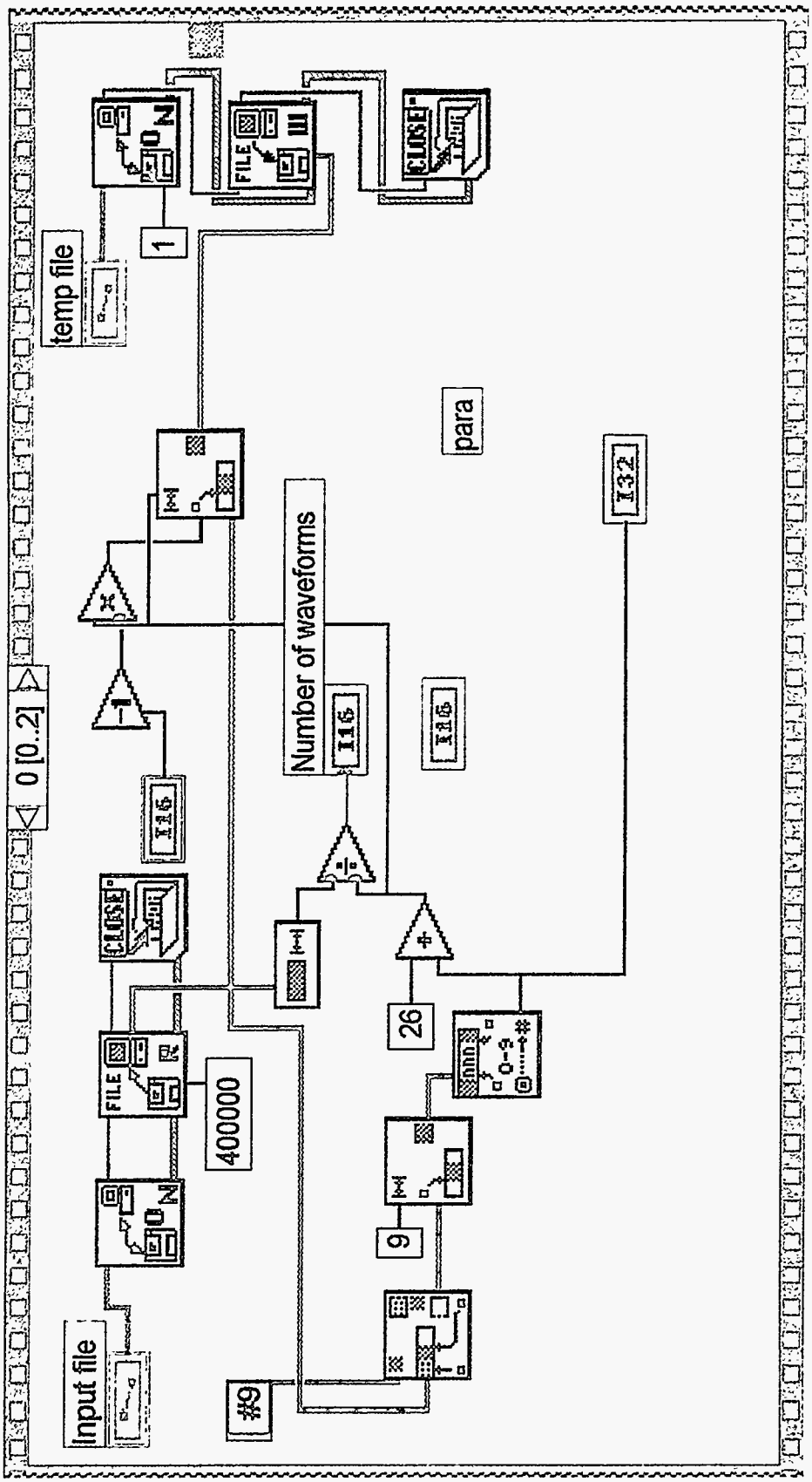
want to make a spreadsheet file?
no

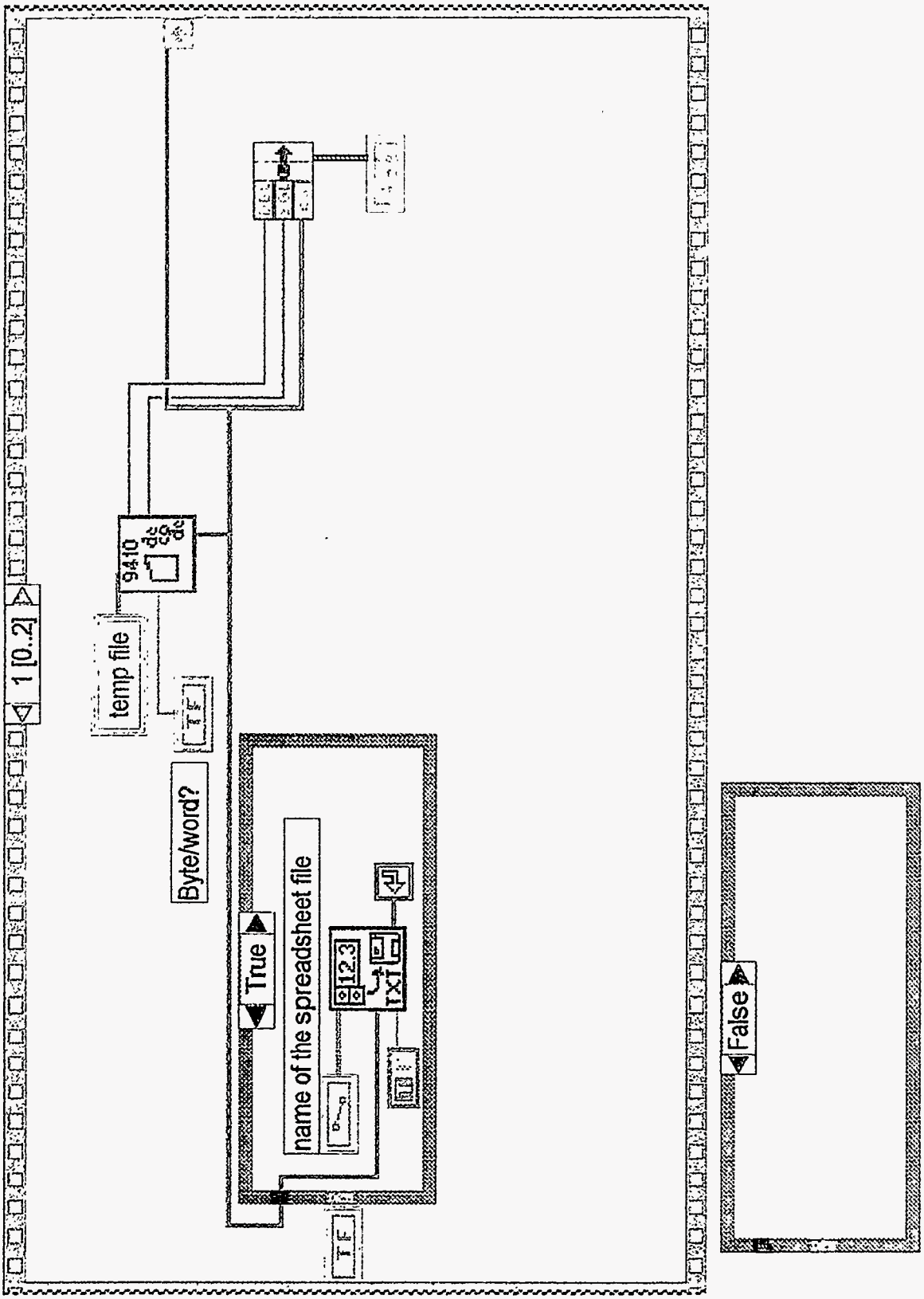
name of the spreadsheet file
c:\nkfung\data

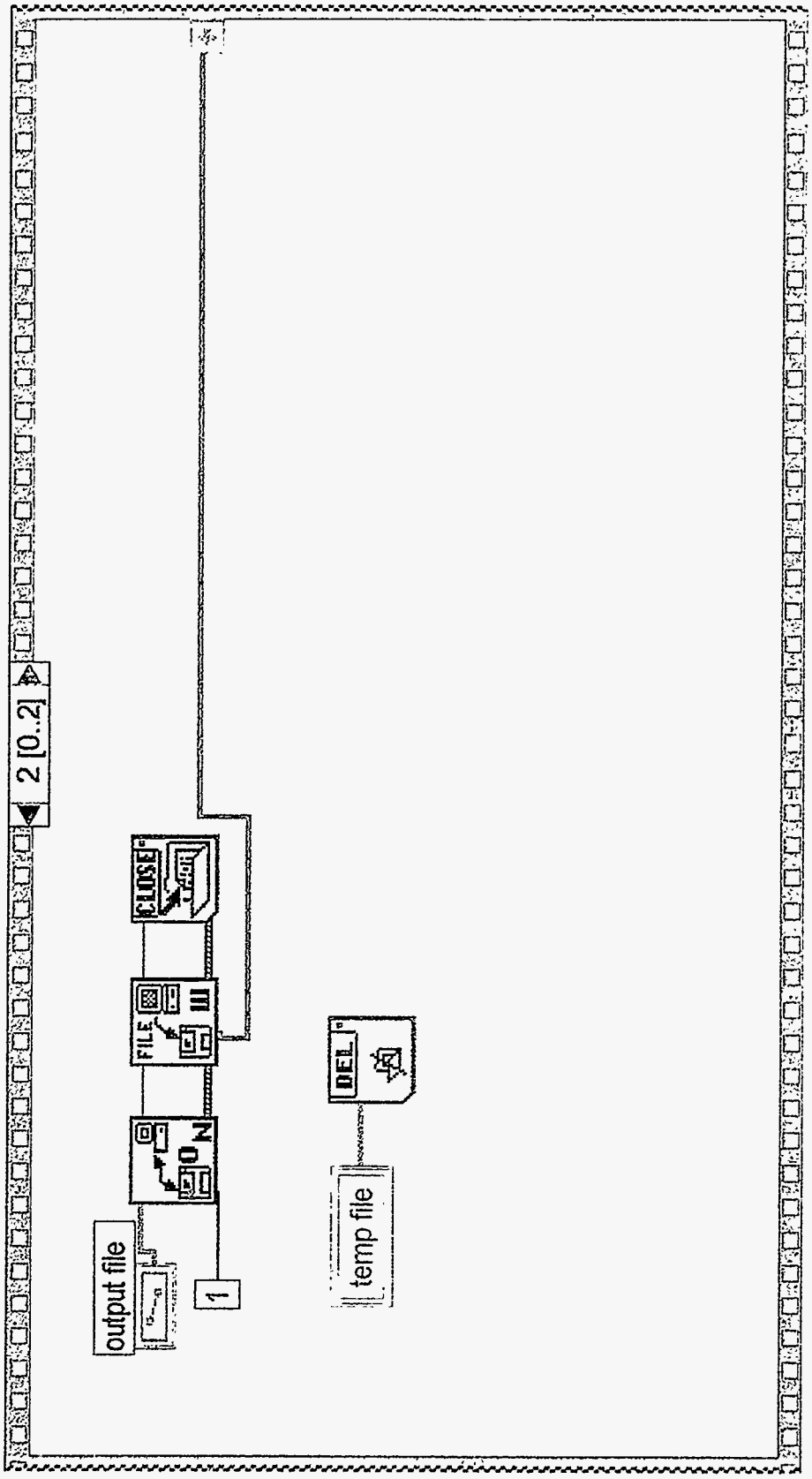
output file
c:\nkfung\data



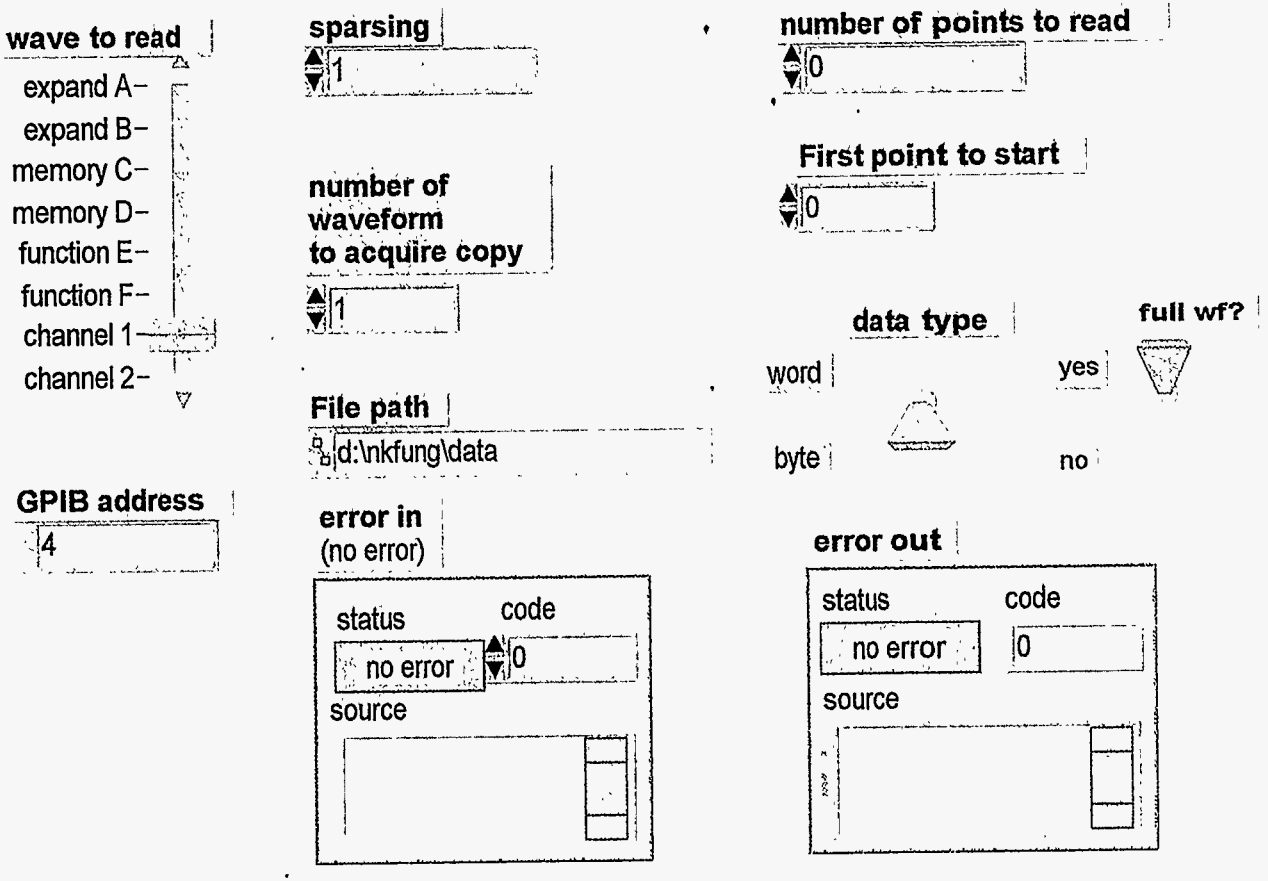
This vi splits the data files collected from the 9410, 9350 new get data.vi's and displays the individual waveforms. It can also generate a spreadsheet file for the waveforms if you check the box.


temp file
1
FILE
CLOSE
para
I32
Number of waveforms
I16
I16
0 [0..2]
-1
I16
CLOSE
FILE
400000
26
0-9
9
Input file
#9

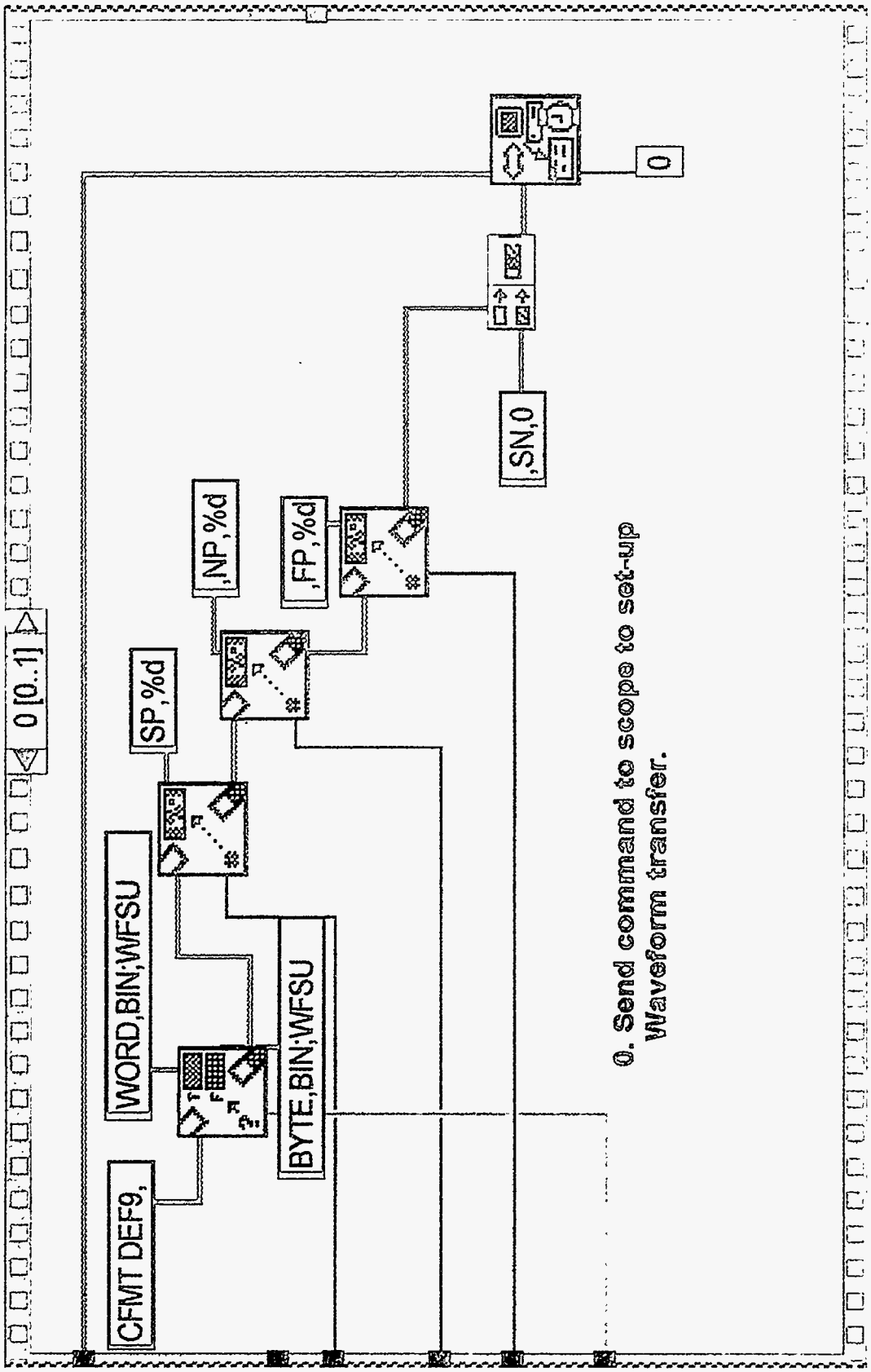

1 [0..2]
temp file
9410
Byte/word?
True
name of the spreadsheet file
12.3
TXT
False

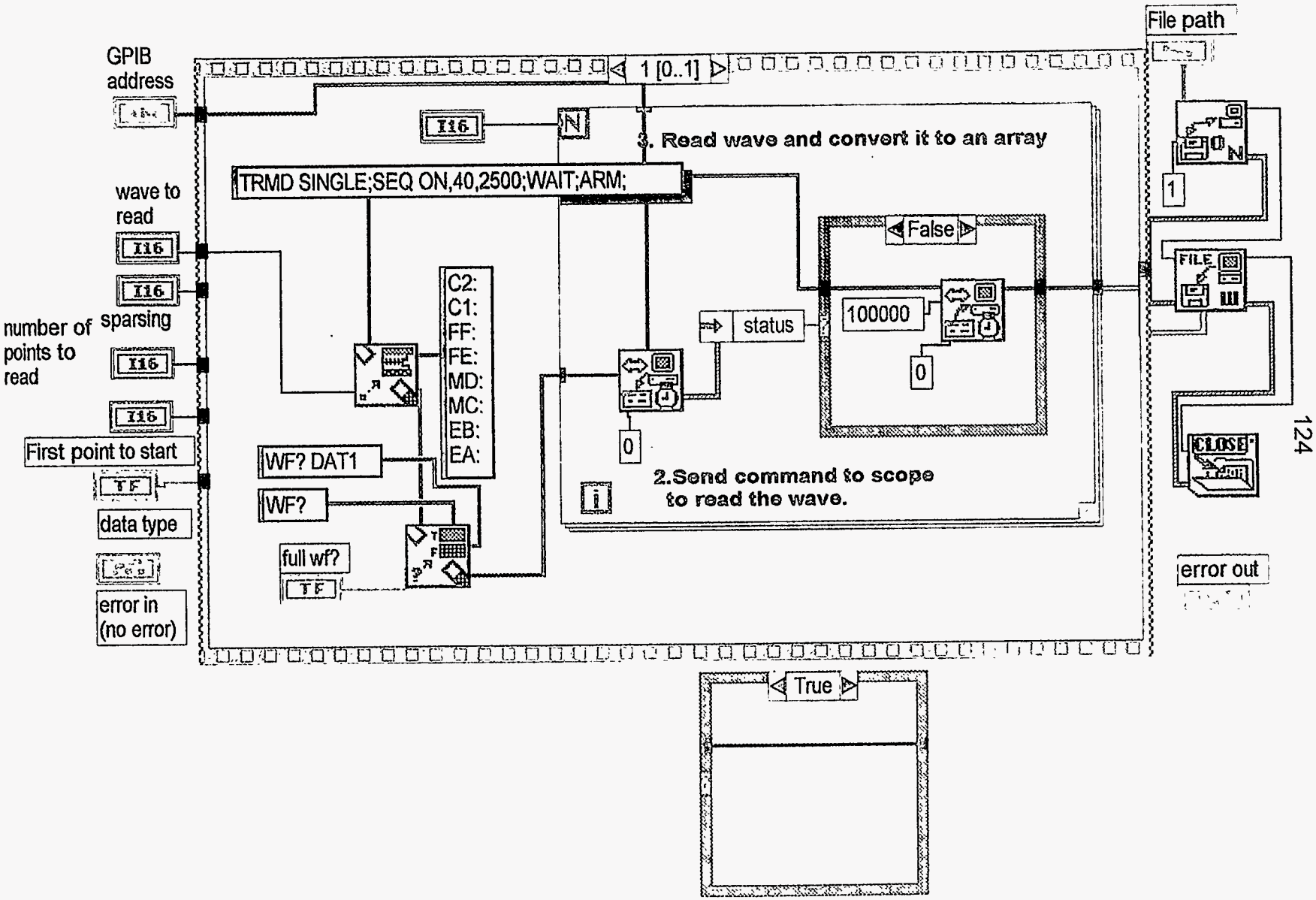

2 [0..2]
CLOSE
FILE
ON
output file
1
DEL
temp file

collect segmented data.vi

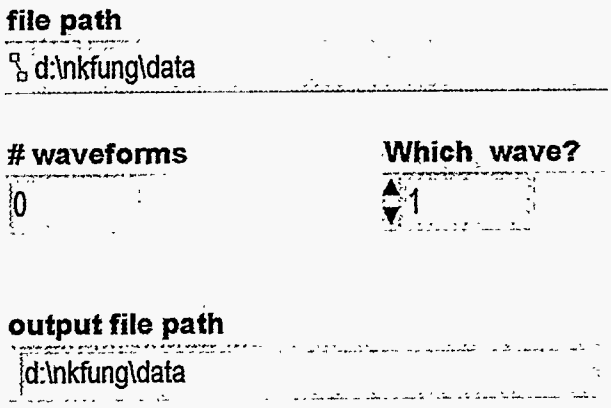


This vi collects data from LeCroy 9310 through GPIB. The oscilloscope will be operated in sequence mode, if you want to change any parameter, please modify the block diagram. For an explanation of the fields, please refer to the oscilloscope menu

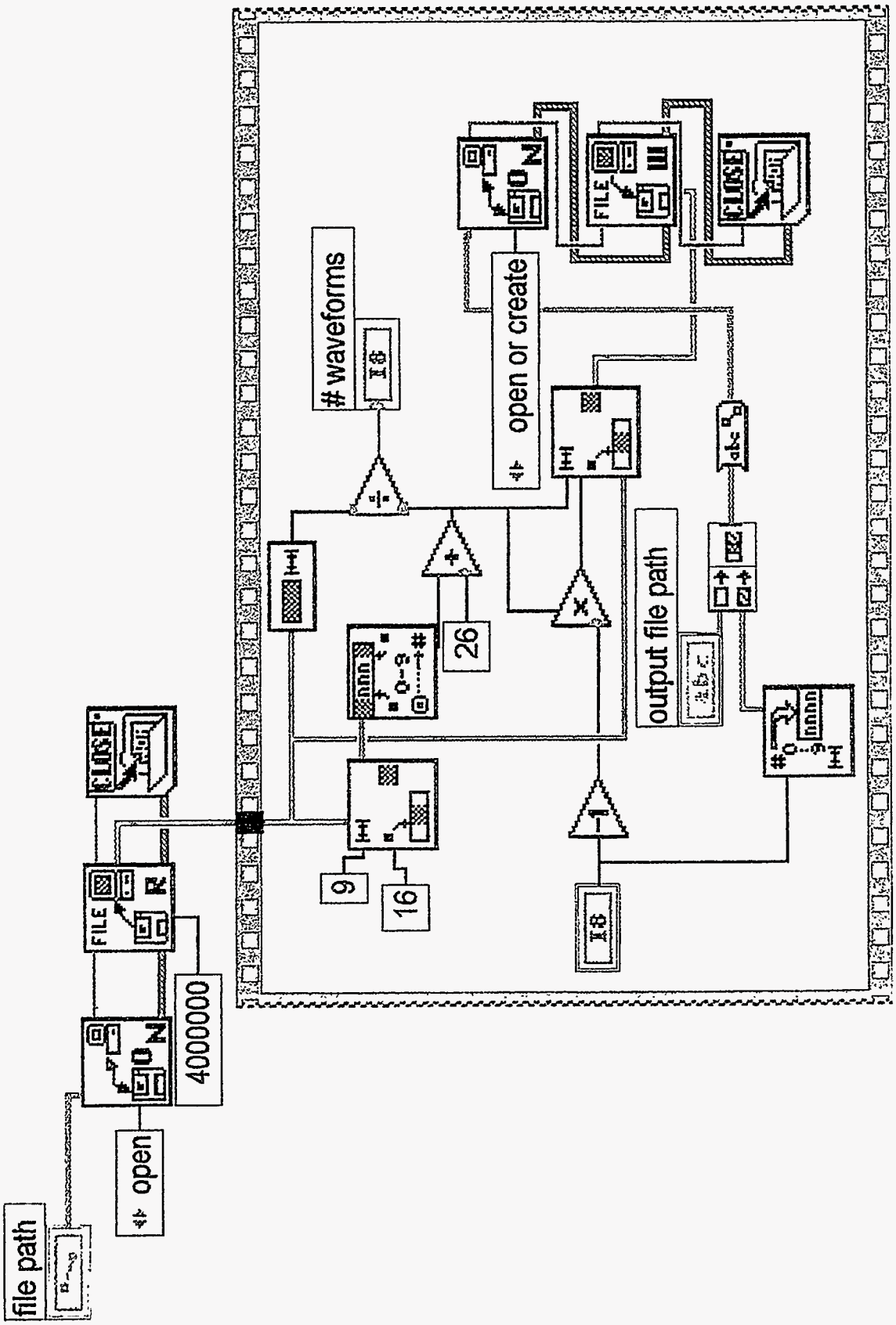

0. Send command to scope to set-up
Waveform transfer.
0
,SN,0
,NP,%d
,FP,%d
SP,%d
WORD,BIN;WFSU
BYTE,BIN;WFSU
CFMT DEF9,
0 [0..1]

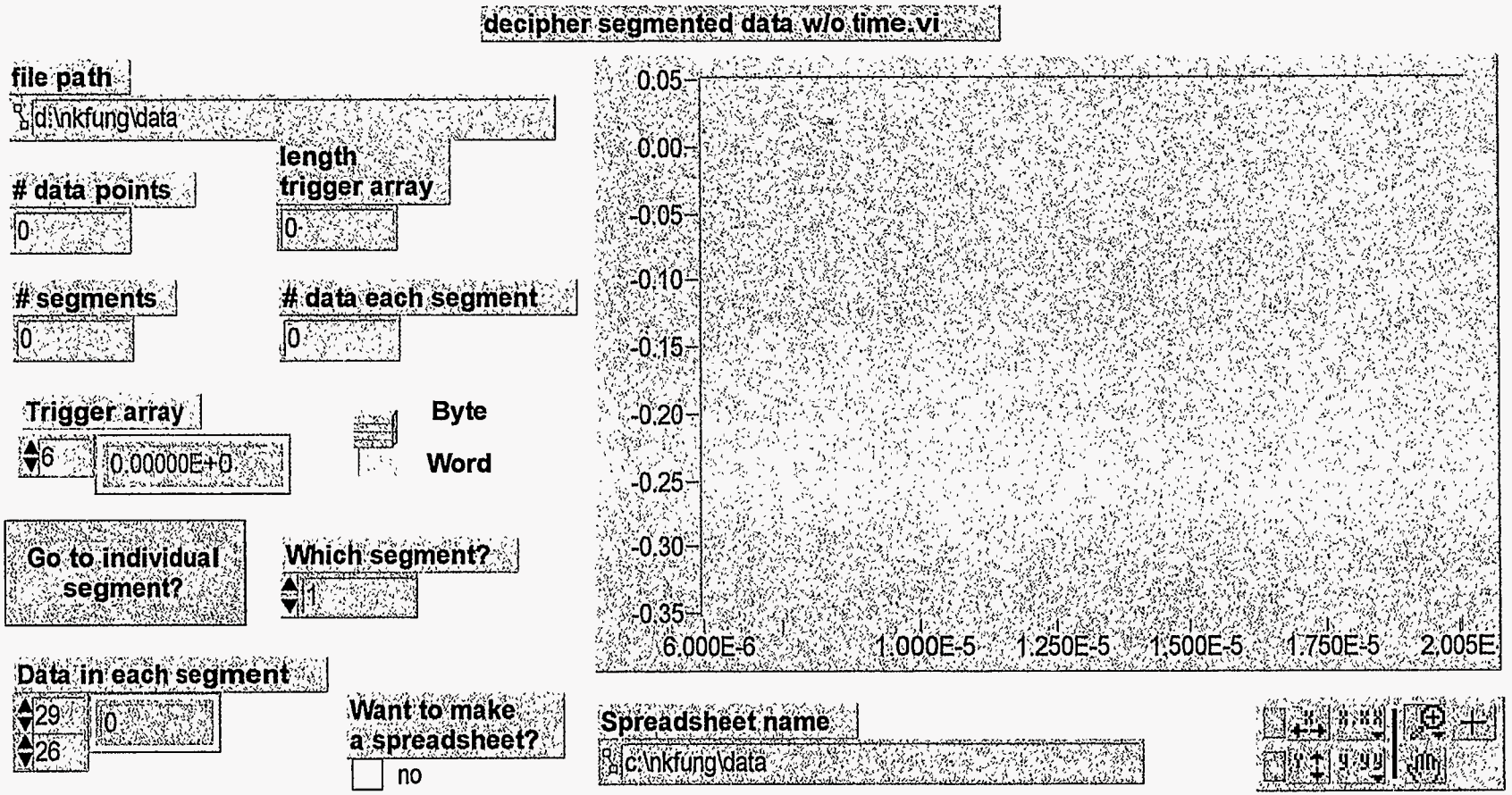

File path
GPIB address
1 [0..1]
I16
N
3. Read wave and convert it to an array
TRMD SINGLE;SEQ ON,40,2500;WAIT;ARM;
wave to read
I16
I16
number of points to read
sparsing
I16
I16
First point to start
TF
data type
error in (no error)
C2:
C1:
FF:
FE:
MD:
MC:
EB:
EA:
WF? DAT1
WF?
full wf?
TF
status
False
100000
0
0
2.Send command to scope to read the wave.
i
1
FILE
CLOSE
error out
True

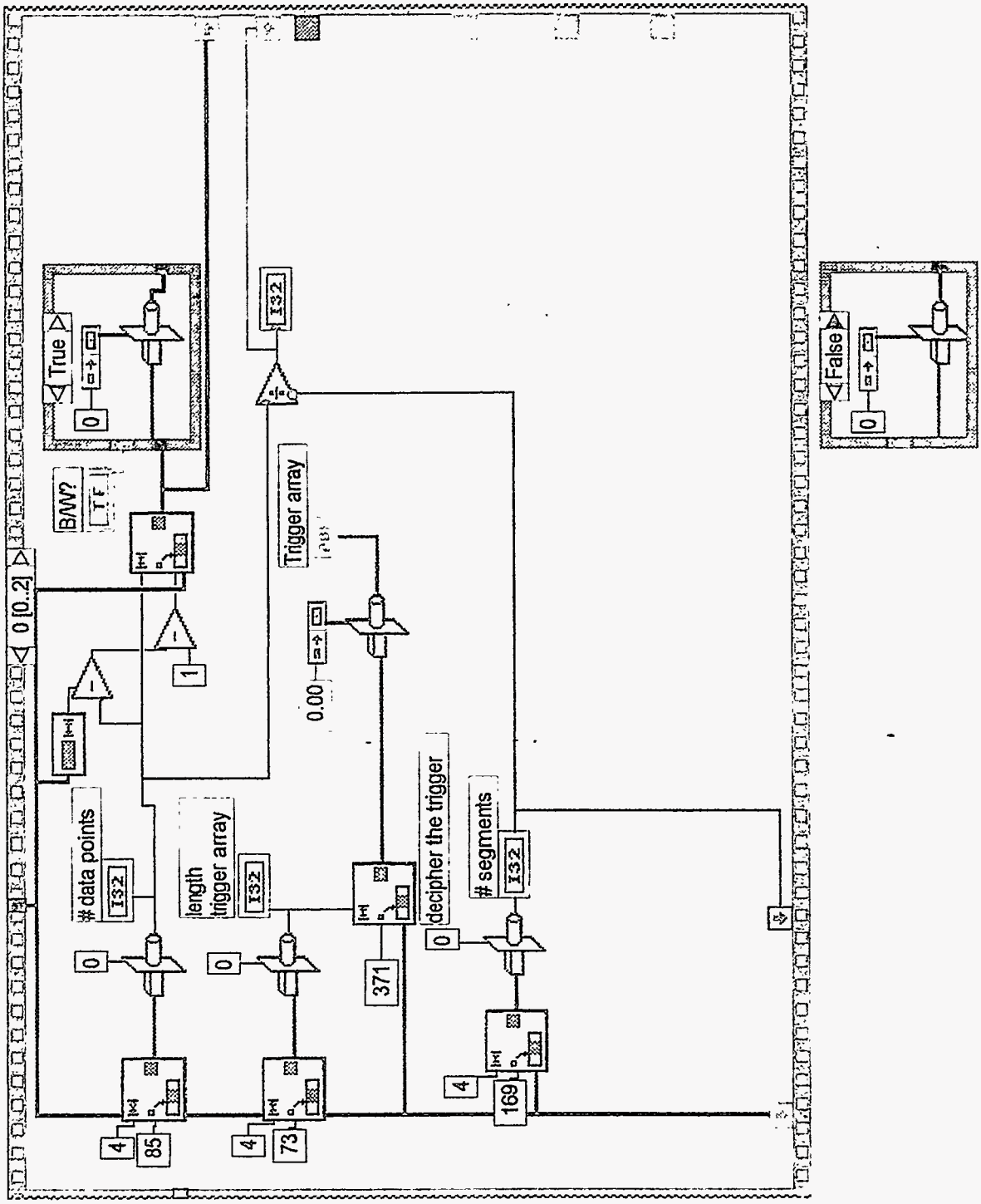
Segments splits.vi

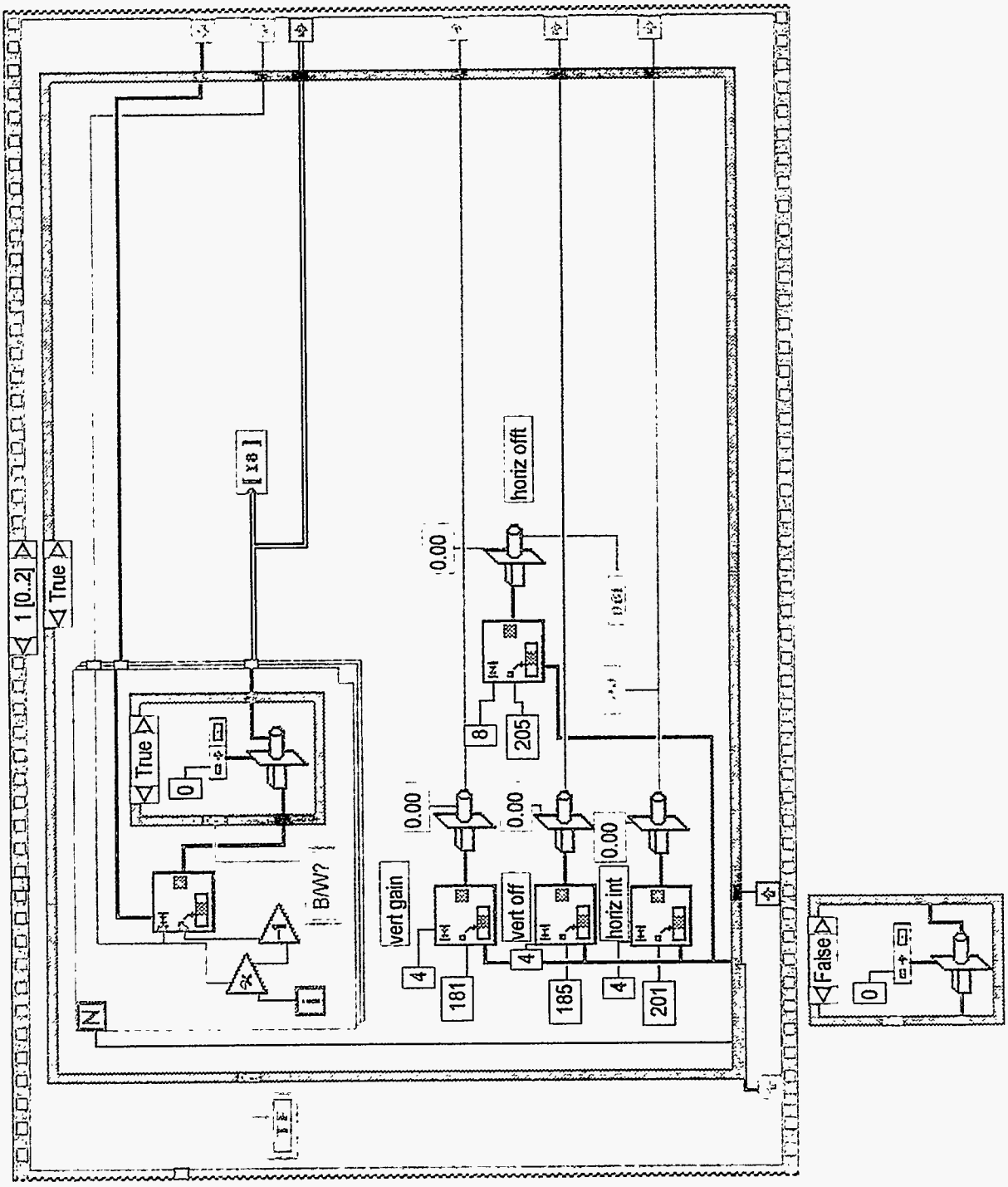


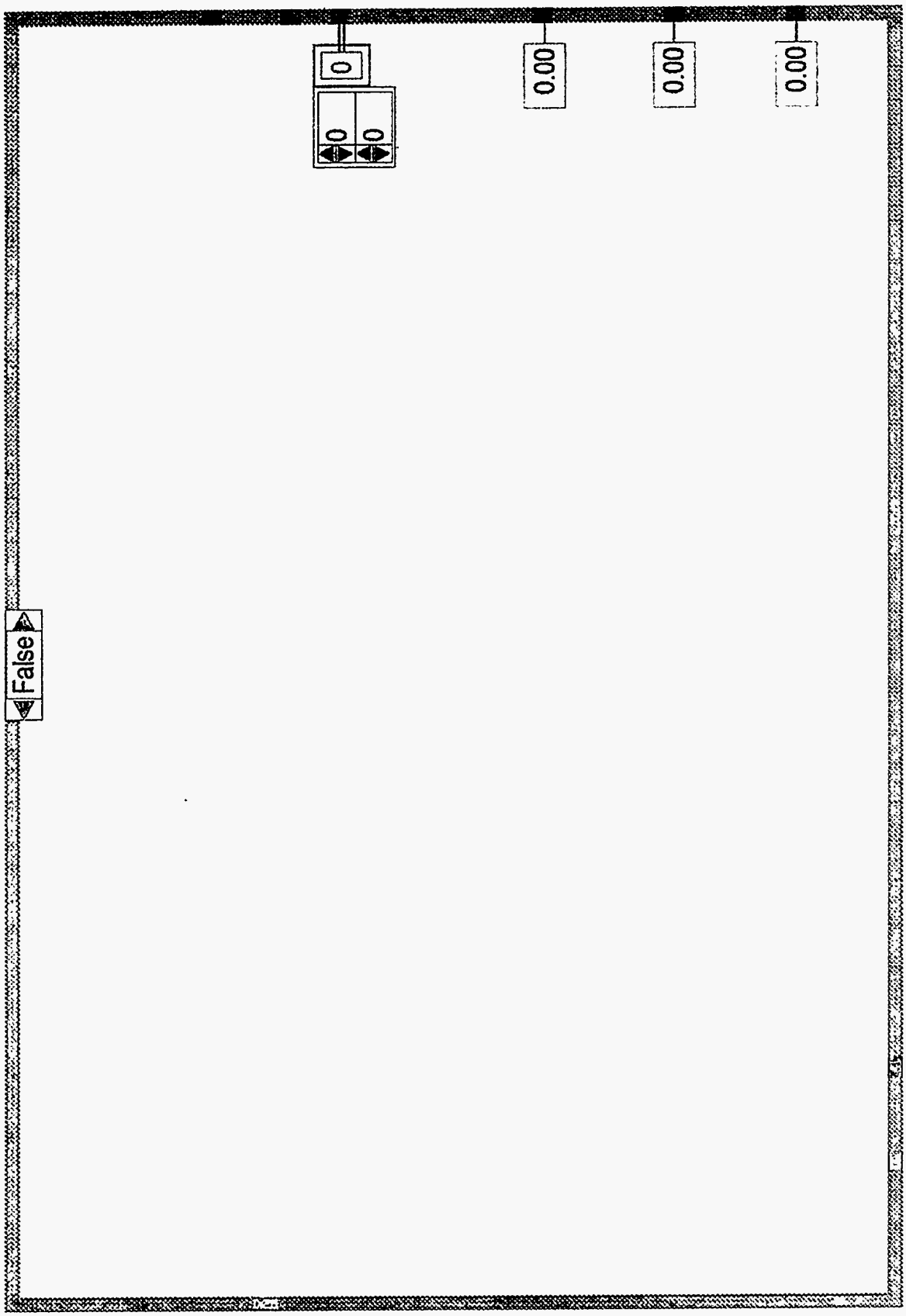
This vi splits the raw data collected by collect segmented data vi into individual acquisition. Each acquisition has specific number of segments specified in collect segmented data vi.

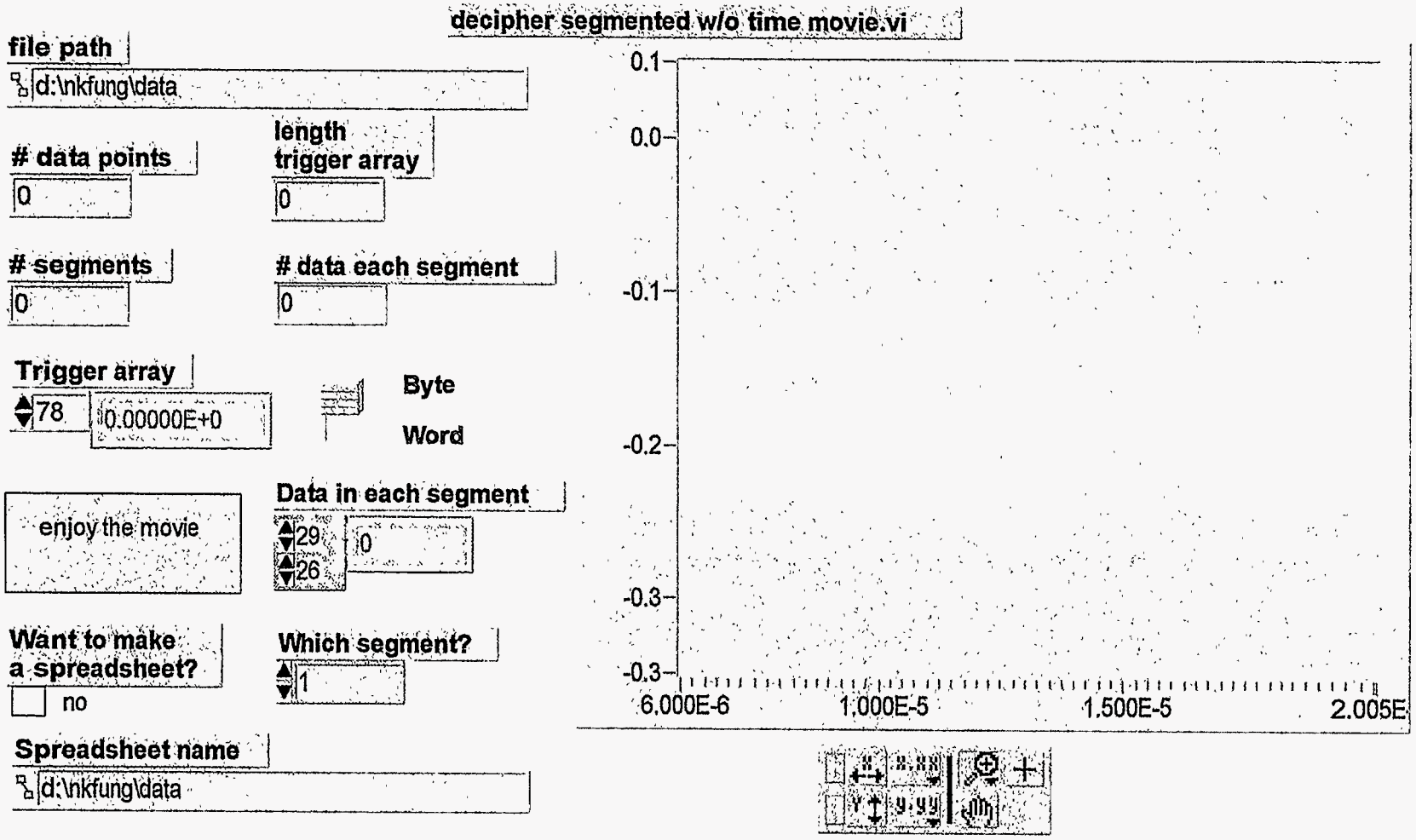

file path
open
4000000
FILE
R
CLOSE
waveforms
I8
open or create
FILE
CLOSE
output file path
26
9
16
I8


decipher segmented data w/o time.vi
file path
d:\nkfung\data
data points
0
length
trigger array
0
segments
0
data each segment
0
Trigger array
6
0.00000E+0
Byte
Word
Go to individual segment?
Which segment?
1
Data in each segment
29
26
0
Want to make a spreadsheet?
no
Spreadsheet name
c:\nkfung\data
0.05
0.00
-0.05
-0.10
-0.15
-0.20
-0.25
-0.30
-0.35
6.000E-6
1.000E-5
1.250E-5
1.500E-5
1.750E-5
2.005E
This vi deciphers waveforms collected by the collect segmented data.vi and segments split.vi
and displays the individual segment with the time with respect to the trigger of that segment.
The trigger time of each individual segment with respect to the very first trigger of the acquisition
can be read from the trigger array field.
You can also generate spreadsheet files for the individual waveform by checking the box.

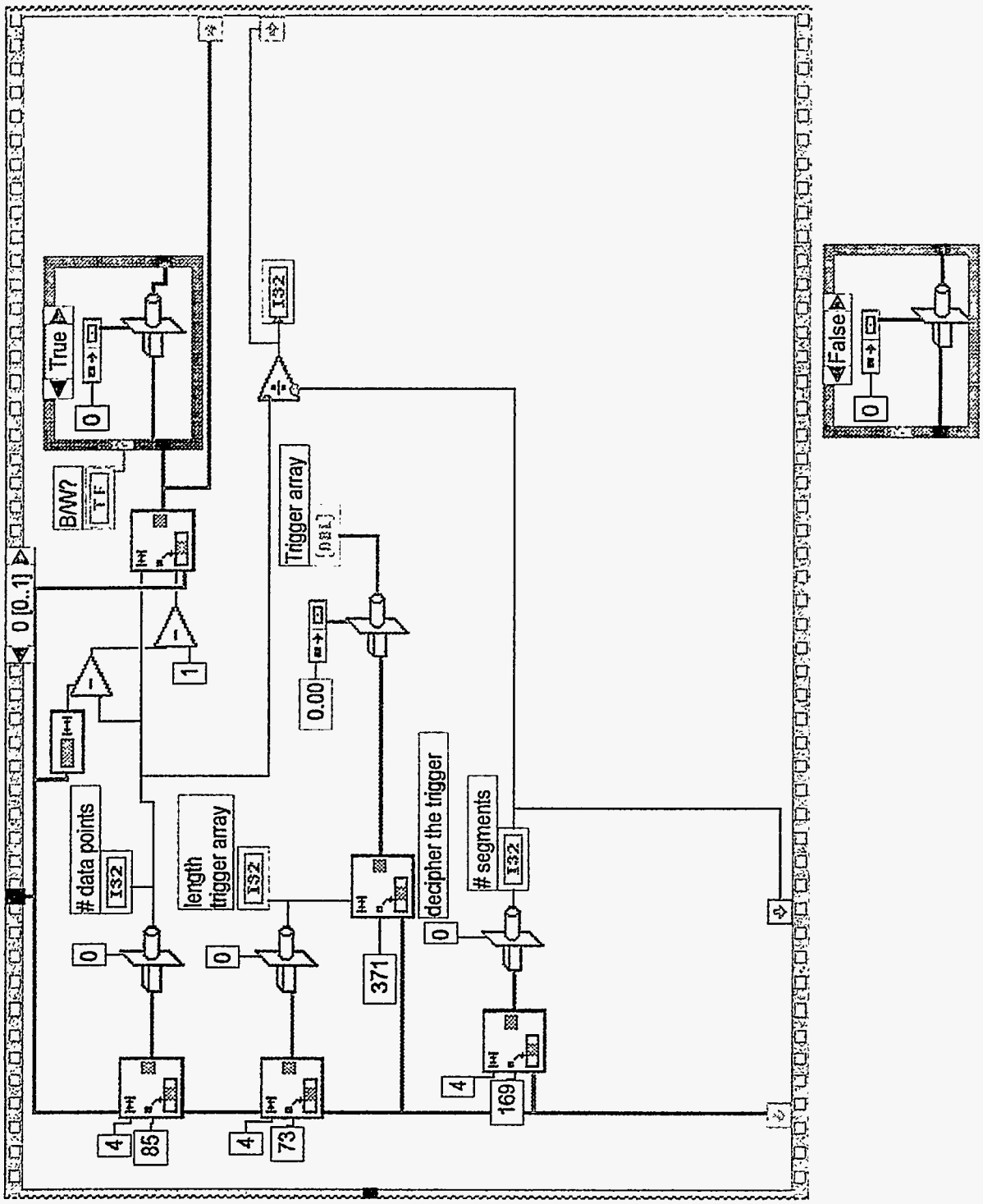

True
False
B/W?
TF
I32
Trigger array
0.00
data points
length trigger array
decipher the trigger
segments
371
169
85
73
0 [0..2]

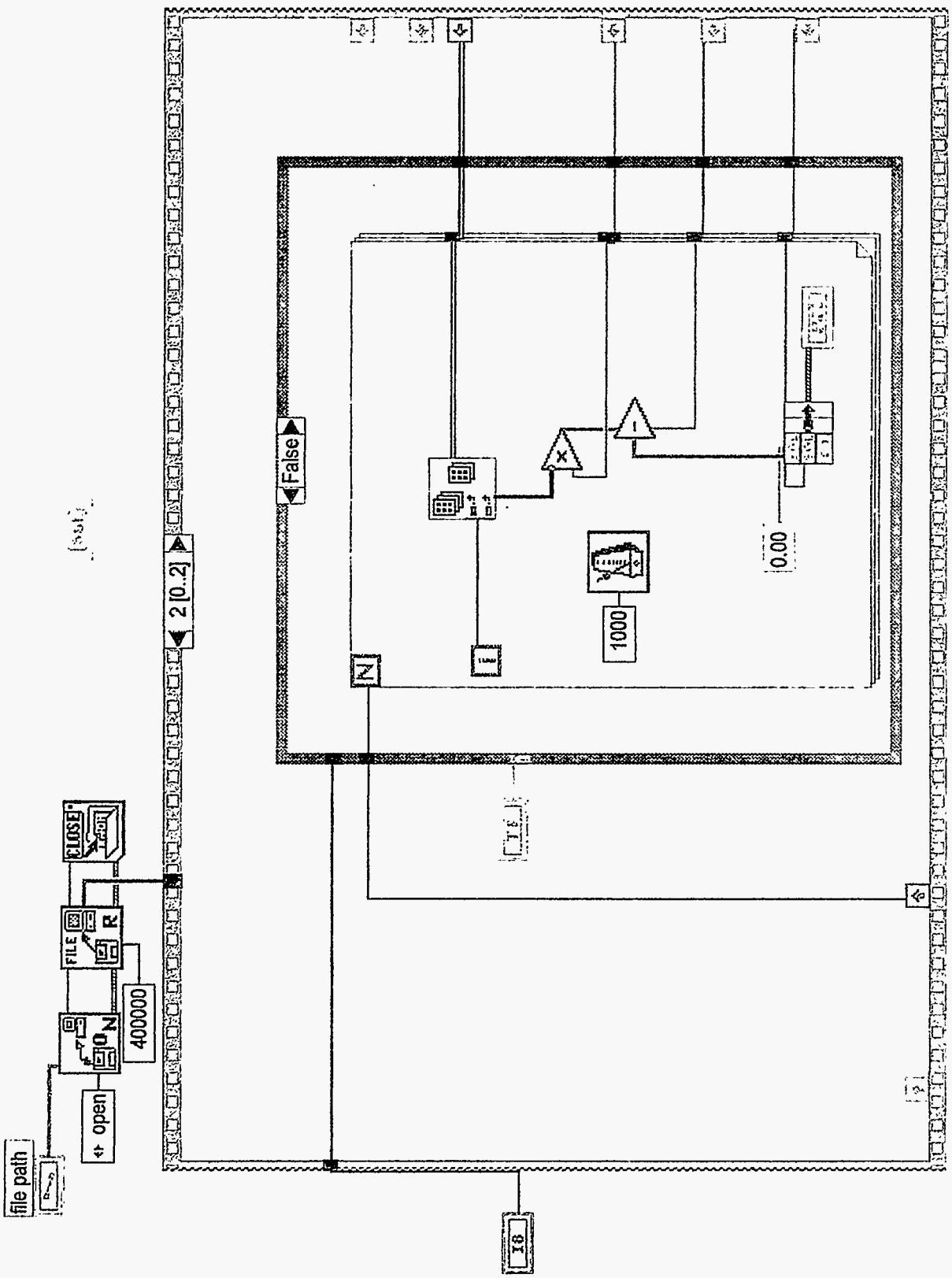

1 [0..2]
True
[x8]
True
0
B/W?
-1
i
N
0.00
horiz offt
8
205
0.00
0.00
0.00
vert gain
vert off
horiz int
4
181
4
185
4
201
False
0

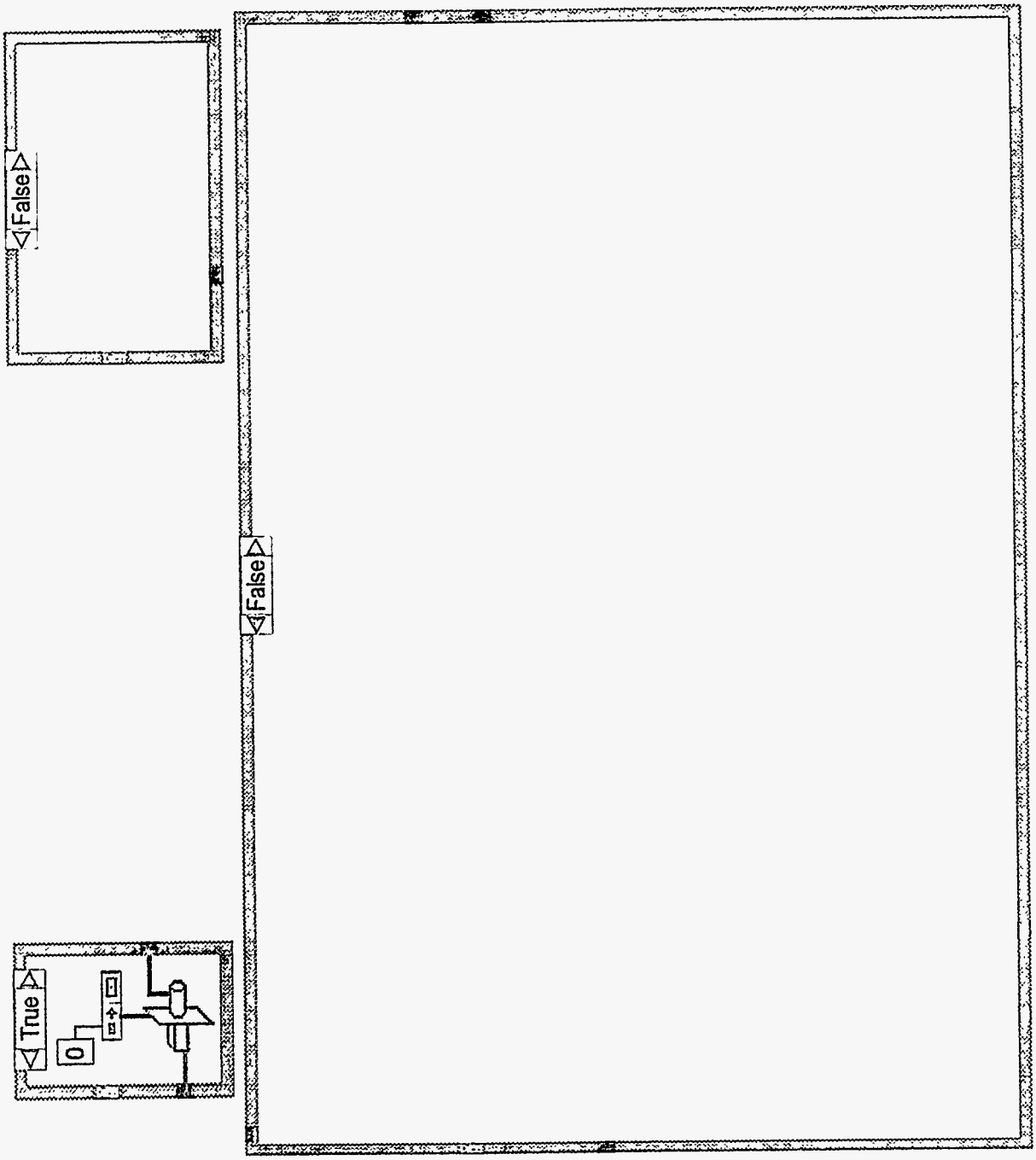

0
0
0
0.00
0.00
0.00
False

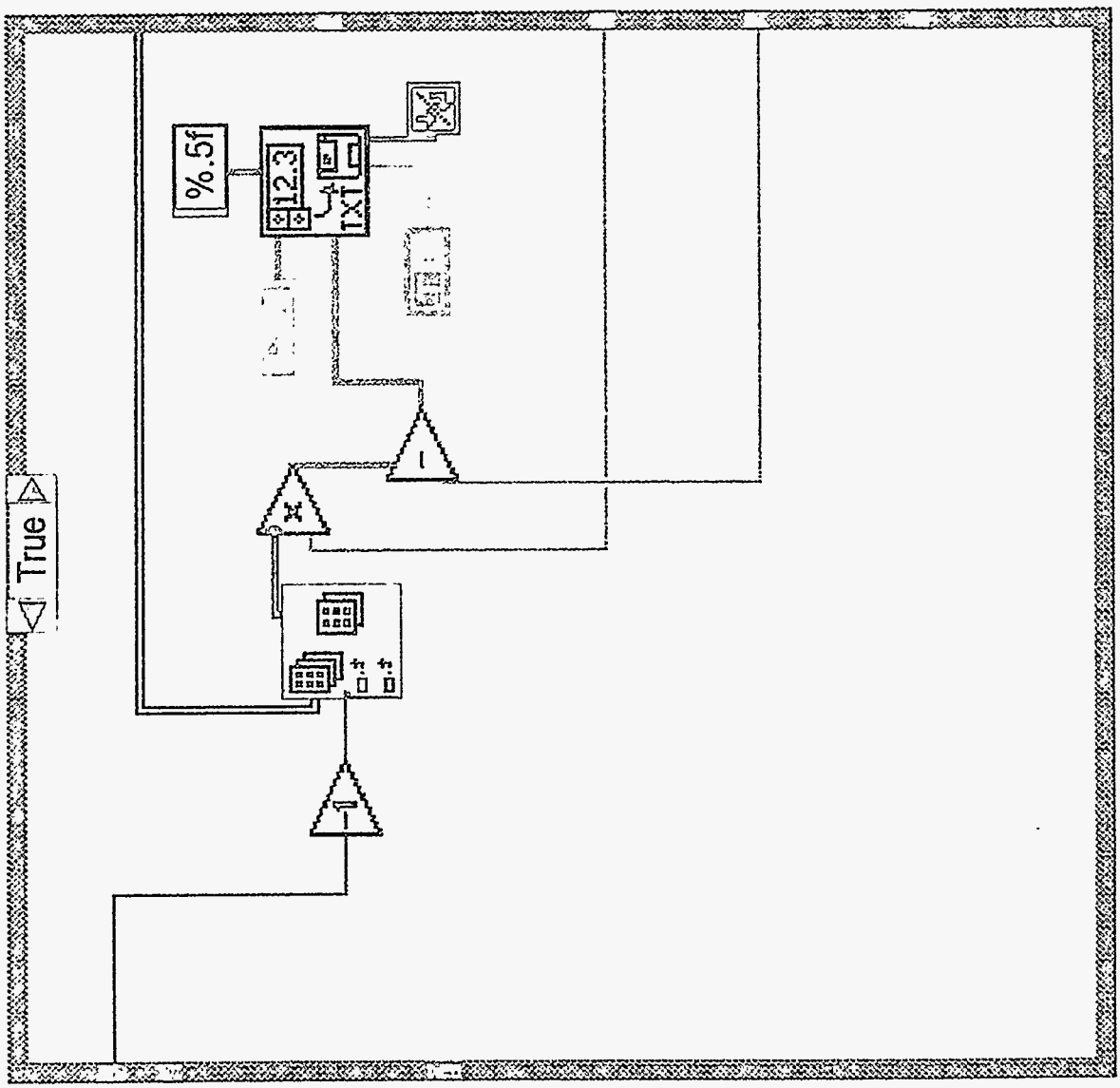


This vi decodes the waveforms collected with the collect segmented data.vi and segments splits.vi. It displays all the 40 segments in movie mode. If you want to make a spreadsheet file of an individual segment, just check the box.


True
False
B/W?
TF
I32
Trigger array
(DBL)
0.00
data points
length trigger array
decipher the trigger
segments
371
85
73
169
0 [0..1]


file path
open
400000
FILE
R
CLOSE
2 [0..2]
False
1000
0.00
i
N
X8


False
False
True
0


%.5f
12.3
TXT
True

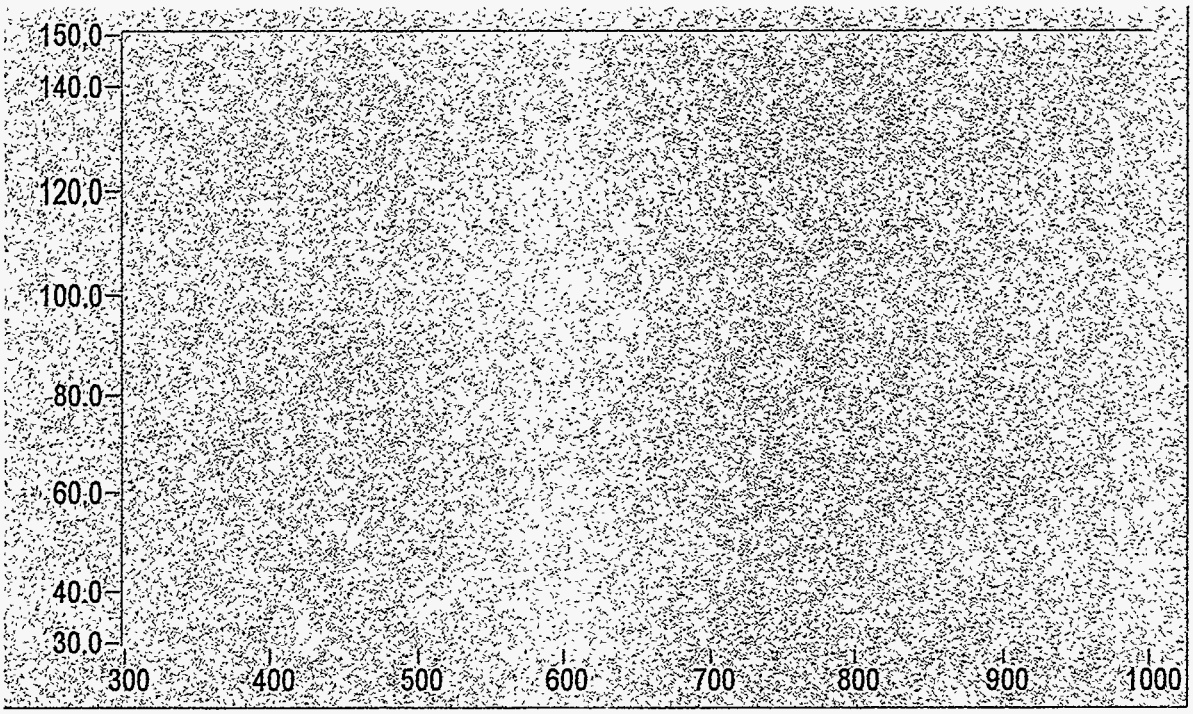
display daa from .prn files.vo

File path

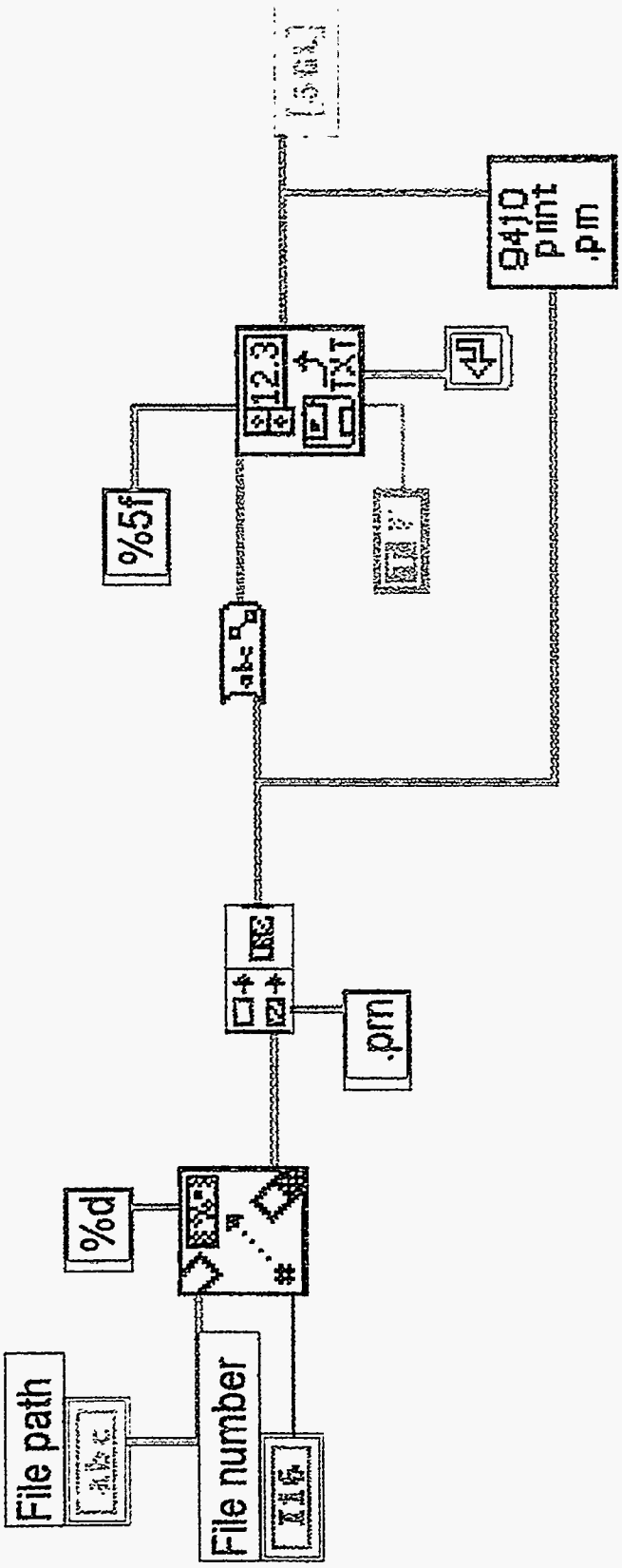
d:\nkfung\data

File number

1



This vi displays waveforms from .prn files.
The .prn files are generated from binas3 program, and acquired by the memory card option.
Just fill in the correct file path.


File path
File number
%d
.prn
%5f
9410 print .prn

display data from memory cards.vi

File path

d:\nkfung\data\

File number

0

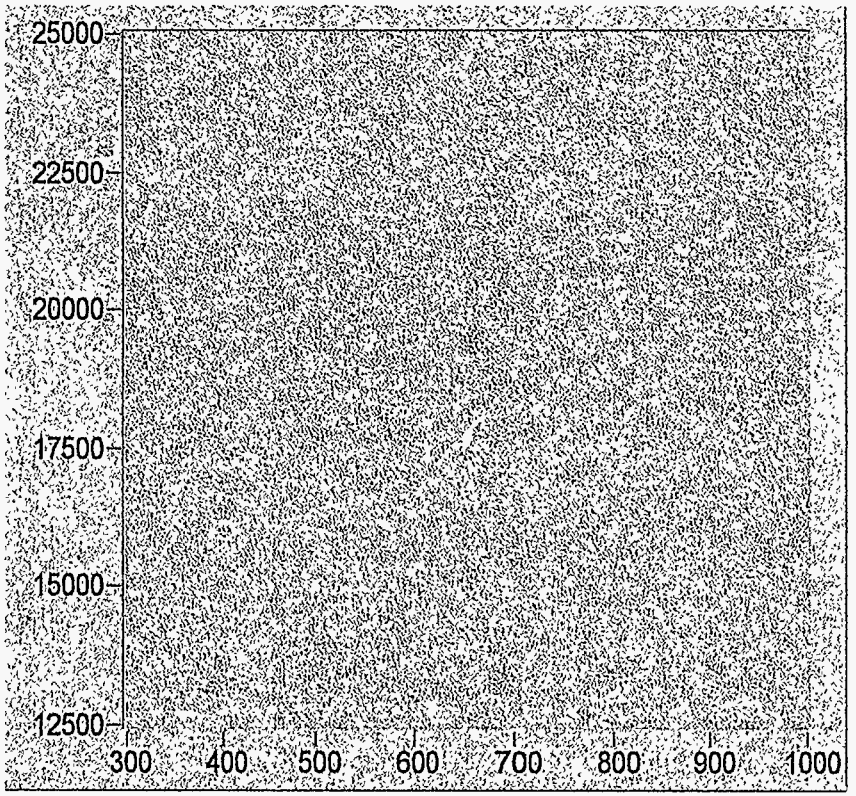
Want to create spreadsheet file?

no

Spreadsheet file path

d:\nkfung\data

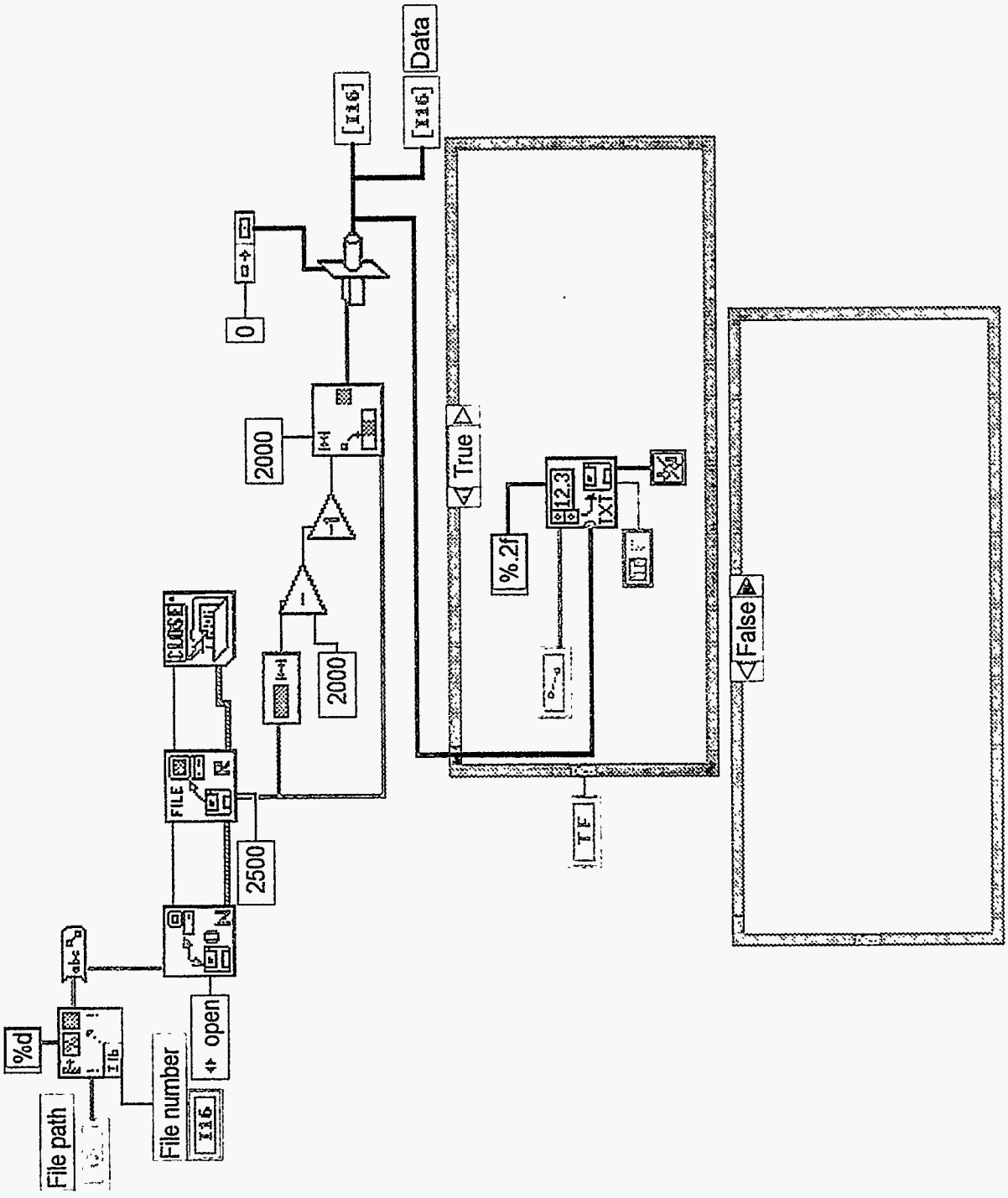
This vi displays waveforms collected by the memory card option.
The input file name must be in the form of [data][file number]
e.g. 12031, where the date is 1203 and the file number is 1.
It can also generate spreadsheet files by checking the box.



Data

0

0


Data
[I16]
[I16]
0
2000
2000
2500
True
False
%.2f
12.3
TF
FILE
CLOSE
open
%d
File path
File number
I16

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