

## Chapter 2

# Electrochemical Detection on Microarrays

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### 2.1 Introduction

Microarray detection methods have long been based upon optical methods: visible detection, fluorescence, luminescence, and surface plasmon resonance (1–6). In the case of the visible detection method an enzyme produces a substrate that forms an insoluble precipitate at the spot site, which becomes visible to the naked eye. Surface plasmon resonance also uses visible light, but in this case the energy (and reflected angle) of the light absorption on the metal surface is altered; the researcher finds the absorption minimum using an array detector. This method utilizes more expensive equipment in the process of detecting the material on the chip/slide (4).

More sensitive methods such as fluorescence and luminescence have also been employed for the detection of material on the chip/slide surface. The fluorescence-based system requires a laser, so the beam can be rastered over the chip area. Or conversely, the chip can be illuminated with light and then a signal detected with a CCD camera using the appropriate filters (3). Similarly, luminescence detection utilizes a CCD camera after an enzyme (such as alkaline phosphatase) has generated a product that luminesces (1).

Both these methods require expensive optics and equipment that may cost \$60,000 to \$500,000. Moreover, the footprint of the larger and more expensive systems requires a benchtop area on one side of the room.

A less expensive, mobile, and more sensitive platform for microarray detection purposes is highly desirable. To this endeavor, many researchers in the field have instituted programs for electrochemical detection on microarrays (6–20). In order to do this, one must have active electrodes on the chip or glass surface. Thus many of the spotted slides out in the marketplace contain surface areas where the spots are not electroactive and do not qualify for electrochemical detection.

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Even in the case of electrochemical detection, there are a host of methods that can be employed. They range from inductive measurements to simple redox tags, and to compounds that are wedged into the grooves of a DNA duplex to enzyme amplification (6–20). Even the latter may be performed by various methods: product redox shuttle (between two electrodes) or a method whereby the product is reduced/oxidized at the single electrode. In the former case the current output is monitored while voltage to the electrodes is changed in sign (cycled). In the latter case, the signal is stored on a capacitor on the chip and is accessed every so often (usually once). The charge on the capacitor is then converted to amperage and hence the amount of enzyme at the surface of the electrode. Electrochemical methods of detection are discussed in detail below.

## 2.2 Electrochemical Detection

There are numerous advantages of electrochemical detection (ECD) over conventional fluorescence.

1. *Superior Performance.* In optical detection schemes, the signal-to-noise ratio is often limited by the amount of stray light (often from the incident beam) that gets into the detector channel. With ECD there is no incident background. The only background that exists comes from the inherent background currents in the measurement systems and the capacitive charging currents at the chip. Both of these are relatively small, thus a much higher signal-to-noise ratio is achieved in the ECD mode. As a result greater sensitivity (perhaps several orders of magnitude) can be achieved.
2. *Fewer Components.* For ECD there is no need for optical components such as the light source, mirrors, filters, detectors, their support mechanics, or the movement mechanics for chip scanning. Consequently, the system is simpler and less expensive. Also, the fewer component requirements enable smaller footprints and weight.
3. *Portability.* Because the instrument is small and relatively light, and the power can be supplied via batteries, ECD systems can potentially be portable. This characteristic alone will enable significant applications in the IVD marketplace where small, inexpensive, and portable systems are necessary.

ECD systems for microarray analysis are portable and low cost, and coupled with their superior performance will revolutionize the use of microarrays not only for IVD applications but also for conventional R&D studies. One can imagine a situation where array readers can be purchased for a few hundred dollars (as opposed to the hundreds of thousands for the top-of-the-line optical scanners) and can be taken anywhere.

We discuss four ECD methods that have been described in the literature for microarray applications (6–20). All of these methods have a spatial array of electrodes, which are hardwired to be individually addressable. Scanning is performed

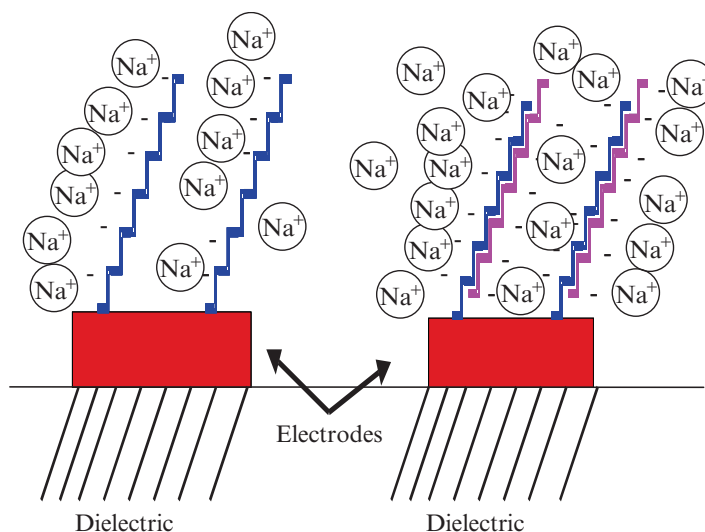
by measuring an electrical property, independently, at each electrode, in either a serial or parallel manner.

### 2.2.1 Capacitance Measurements

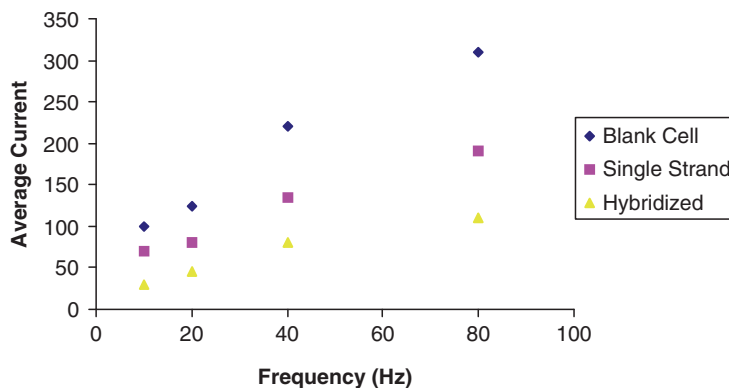
Capacitance is determined by charged ions at the surface of a layer covering the electrode (20). These counter-ions and water dipoles lie at the surface of the bound material. There is also a more remote layer that consists of diffuse counter-ions known as the Guoy–Chapman layer (20). The inner layer, tightly captured, changes the nature of the electrode capacitance. This surface transductance results in a capacitance change.

There are numerous traditional and new methods for detecting capacitance change at the electrode surface. These may include chronoamperometry and impedance spectroscopy. Both are difficult to measure and require multiple electrodes in any given electrical cell. Additional sample conditions will play a great role, which may include salts found within the sample.

In the case of DNA analysis, the capture probe must be placed upon the chip surface. This in itself changes the capacitance of the electrode and introduces a sodium ion countercharge (to offset the negatively charged phosphate groups). Duplex formation (hybridization with the complementary strand) introduces additional charges to the outer layer (more sodium ions). See Fig. 2.1 for details.



**Fig. 2.1** Detection of DNA via capacitance measurements. Hybridization allows the accumulation of charge near the electrode due to the phosphate groups on each strand. One strand of DNA is chemically attached to the electrode. The complementary strand is then hybridized to the stationary strand



**Fig. 2.2** Results from a capacitance measurement based upon single-strand and hybridized DNA. Data was obtained from Table 2.1

**Table 2.1** Frequency Measurement of Average Current Flowing Through the Cell

F(Hz)	Cell	Single Strand	Hybridized
10	100	70	30
20	125	80	45
40	220	135	80
80	310	190	110

How can this be measured in a simpler manner? If the voltage applied to the capacitive cell is AC in nature, then the ion double layer moves with the frequency of the applied voltage. From a static system to a dynamic system the movement of ions can be mathematically described as

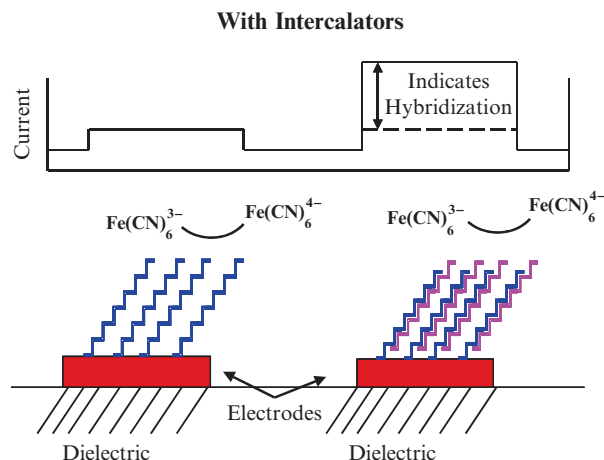
$$I_{\text{avg}} = I_{\text{DC}} + C(\Delta V / 2)f.$$

Then the current on average can be described as based upon the DC current followed by a cell constant and the frequency of the applied AC voltage;  $\Delta V/2$  is periodic, the pulse applied to the electrode to allow the capacitor to fully charge and discharge (20).

Data provided above are graphed in Fig. 2.2. Note that upon duplex formation, the average current is decreased as the frequency applied increases. Of course a blank measurement is also provided and this is based upon the cell containing all components but the attached oligomer.

### 2.3 Faradaic Current Measurements Through DNA

DNA is a chain comprised of organic and inorganic molecules, which can act to inhibit current flow, and it is well known that duplex DNA is a poorer insulator than single-stranded DNA. By introducing a redox couple, such as ferricyanide/ferrocyanide and measuring the current at different electrodes with attached DNA,



**Fig. 2.3** Detection of DNA via ferrocyanide/ferricyanide couple. Differences can be observed between single-stranded and double-stranded DNA in the presence of ferrocyanide/ferricyanide. However, they are heavily dependent on solution conditions with the potential to oxidize the sample. A method that does show reproducible results is when intercalators such as methylene blue are present. These mediators can be oxidized by the ferricyanide and the electron is shuttled through the DNA base stacked network to the electrode. The choice of an appropriate intercalator is crucial

one can determine the existence of a duplex by noting an increase in current at a particular applied voltage (Fig. 2.3). Similar to the case above, this system is very sensitive to interference by solution components, so events occurring at the chip surface may be difficult to differentiate.

The system described in Fig. 2.3 shows the best results when coupled to an intercalator or groove binder, which themselves aid in mediating electron transfer. Intercalators bind to the DNA duplex but not single-stranded DNA. These intercalators or groove binders may or may not be sequence-specific. The intercalators may be an organic molecule, such as methylene blue or Hoechst 33258, or transition metal complexes composed of cobalt. The existence of the intercalator makes the duplex even more conductive resulting in greater current at electrodes where hybridization has occurred. This method has had some commercial success (9, 18). Some of these intercalators are themselves redox active and may act as signal generators when the voltage is ramped up, so as to induce a current flow. The main drawbacks are specificity and lack of signal amplification as the data are collected on a single acquisition cycle. Additionally, the intercalators bind to all DNA duplexes formed, but some may prefer certain nucleotides in a specific order.

### 2.3.1 Direct Oxidation of DNA (or Mediated Oxidation)

DNA can be electrochemically oxidized directly but the process is slow and the signal weak. There are also methods that utilize metals such as osmium or ruthenium,

which oxidize the sample as the reading occurs (9, 18). Guanine is oxidized using a  $\text{Ru}(\text{bpy})_3$  complex. The oxidized  $\text{Ru}(\text{bpy})_3$  is reduced at the electrode. The signal generated would be amplified to a small degree based upon the number of guanine residues present. By design, the capture probe would contain a limited (or no) G in the sequence making single-stranded DNA and hybridized, double-stranded DNA distinguishable by this electrochemical technique. An alternative nucleotide is used, so as not to interfere.

## 2.4 Signal Transduction Using Various Labelless Systems

A new concept has evolved called labelless detection, somewhat incorrectly as one does need a redox species to provide the signal and that redox species does need to be attached (bound) to the DNA duplex. In most cases it is hybridized to the duplex extension or binds to one of the grooves of the duplex. It is not thought to be part of the sample modification as the amplicon contains no external modifiers (11, 9, 18).

We discuss two cases in some detail. Case one is used by a company called Osmetech ([www.osmetech.com](http://www.osmetech.com); the technology is discussed in a later chapter). The redox signal generator is a modified piece of DNA (containing ferrocene molecules) that is complementary in sequence to a captured amplicon which has a long overhang from the capture probe. The ferrocene moiety readily undergoes a transition from the 2+/3+ iron state when the voltage is ramped higher. It is the amperage that is monitored and can only exist if a duplex has formed with the correct complementary pairs.

Another method utilizes a redox dye that intercalates into a DNA duplex. The redox dye then undergoes an oxidation/reduction that is monitored. The most obvious dye is Hoescht 33258 used by Toshiba (11). It only binds to DNA duplexes and not single-strand DNA. Thus, if that electrode site does not contain a hybridized species, which can bind the redox active dye, no signal is observed. Excess dye is readily washed away, so background signal is minimal. In both systems, the temperature dependence of the signal is used to measure SNPs. The melting temperatures are different enough to afford a signal difference.

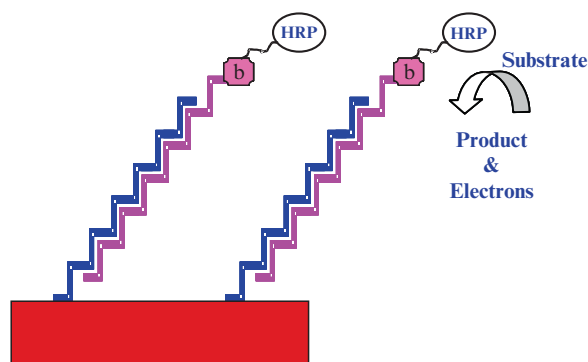
### 2.4.1 Redox Enzyme Mediated Measurements

A method which we feel is the most suitable for array applications is one that utilizes redox enzymes as the labels (9, 18) or enzymes that create products that are redox active. There are several approaches to this method. One uses a three-electrode system to each electrode and lets the enzyme (alkaline phosphatase) convert a redox inactive substrate to a product that can be oxidized and, conversely, reduced (called redox cycling). Thus with a three-electrode system, the enzyme

substrate can then be oxidized/reduced in a ping-pong-like manner by switching voltages. The greater the concentration of enzyme that is present, the greater the amperometric signal as more product can be oxidized/reduced. The downside to this and many other three-electrode systems is that one focuses on a single electrode at one time and the number of leads is large. Hence, the number of electrodes per array will remain low until the bottleneck can be released (time present per a single electrode).

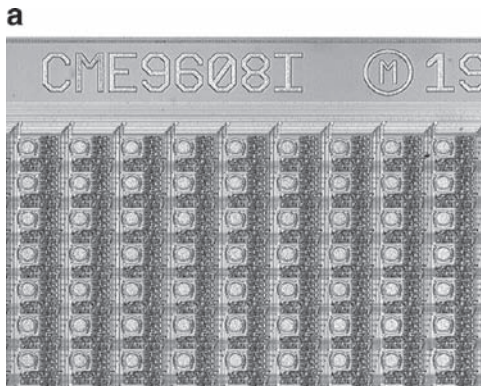
Another method is to use an electrode to monitor the output of a redox enzyme at the surface of the electrode. The redox enzyme functions to provide the signal indicating hybridization as well as to provide amplification comparable to optical methods (Fig. 2.4). Although this methodology has not been utilized for microarrays, it has been used for years in colorimetric enzyme-linked immunosorbent assays (ELISAs). In addition, redox enzymes have been used in very commercially successful glucose meters. This approach is simple, commercially successful, and technically superior to the methods discussed above. The signal is amplified by the action of the enzyme, and only minor modification to conventional gene expression protocols is necessary.

CombiMatrix has developed a commercial system that is based on this approach and unique semiconductor-based microelectrode arrays. The CombiMatrix system has the ability to address each electrode individually and measure the signal present at that electrode site. Fig. 2.5a shows a micrograph of one section of a chip that has roughly 1170 electrodes per sq. cm. Fig. 2.5b shows a photograph of the newer version 12 K chip (17,778 electrodes/sq. cm). On both chips, each electrode is individually addressable and can have unique oligomers synthesized at each site. Analysis of hybridization can be accomplished by fluorescence methods or by ECD.

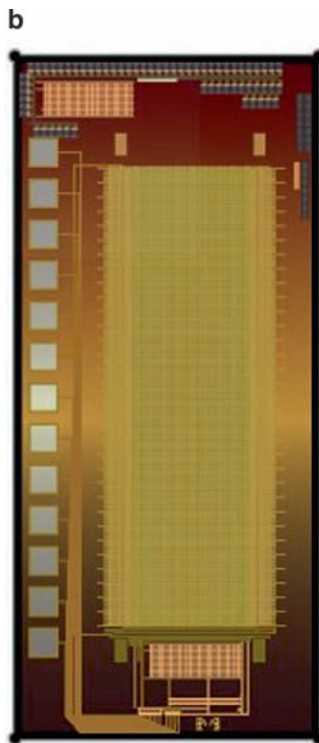


**Fig. 2.4** The CombiMatrix redox enzyme amplification system. A DNA capture probe is synthesized at the electrode. The complementary target is a PCR product containing a biotin molecule that may be attached at the end of the sequence or to bases within the sequence. Streptavidin-labeled horseradish peroxidase is then added to the sample, and HRP binds to biotin on the DNA strand. Addition of substrate allows HRP to produce a product and a current at the electrode

**Fig. 2.5a** A white light photograph of a portion of the 902 chip. The electrode density is  $> 1000/\text{sq. cm}$

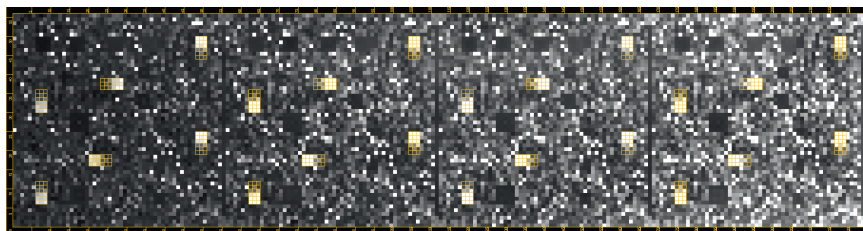


**Fig. 2.5b** Photograph of the commercial 12K chip

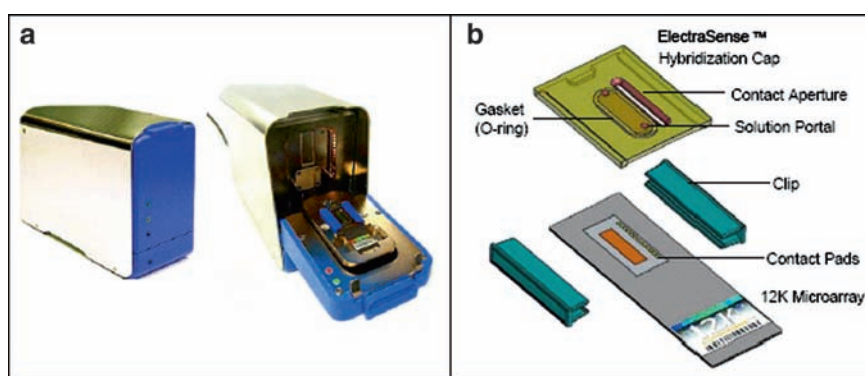


In the CombiMatrix signal amplification method, the attached reporter group is usually horseradish peroxidase. The enzyme oxidizes substrate to product in the presence of hydrogen peroxide. The product in return is reduced at the electrode under the appropriate conditions. Because the enzyme continues to create product at an extremely fast pace, we have an amplified signal that can be detected as current at the electrode. Fig. 2.6 depicts the ECD output of a gene expression





**Fig. 2.6** ECD output of a lambda spike experiment (0.375 pM, 0.75 pM, 1.5 pM, 3 pM, 6 pM, 12 pM) into a complex sample of biotinylated cRNA from a leukemic cell line. The boxed areas in yellow are where the various lambda spike-in DNA should bind. Other probes on the chip are complementary to specific sets of genes, which are either expressed or not expressed in this particular cell line. Twenty-four repeats of each concentration range were measured



**Fig. 2.7** ElectraSense™ Reader (a) and ElectraSense™ 12K microarray with hybridization cap (b)

experiment for a leukemic cell line containing lambda spike-in controls. The data were generated from a 12 K chip where given lambda capture probes were synthesized in specific regions of the chip (as indicated in the figure). Limit of detection was determined to be 0.750 pM of biotinylated lambda cRNA. Twenty-four replicates were taken for each lambda concentration with a given sequence.

Shown in Fig. 2.7 is the ElectraSense enzyme amplified electrochemical unit used in the CombiMatrix studies. The electroactive microarray is contained within a chamber that contains the appropriate buffers and enzyme substrate. It is then placed within a holder and contact is made with the reader through several pins. Each electrode is addressed individually and a measurement is taken via the charge buildup at a capacitor associated with each electrode. That charge release results in a nanoamp current flow.

In summary, the scope of this chapter is to provide some insights into new electrochemical methods of DNA and immunoassay detection that have the potential to revolutionize microarray application and hasten their use in IVD markets.

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