

THE PAST, PRESENT, AND FUTURE OF MICROARRAY TECHNOLOGY, CLINICAL APPLICATION OF CDNA MICROARRAYS IN TUMOURS OF HAEMATOPOIETIC AND LYMPHOID TISSUES

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Abstract: An electron microscope is a type of microscope that produces an electronically-magnified image of a specimen for detailed observation. An ideal custom DNA microarray platform should allow a user complete freedom in defining which genes are assayed using the array. The microarray platform should also be compatible with computer-based probe design software and molecular methods so that measurements can be performed with high sensitivity and specificity, thereby making independent validation of the assay unnecessary.

WHILE DNA MICROARRAYS HAVE THE POTENTIAL OF DEVELOPING INTO POWERFUL MOLECULAR DIAGNOSTIC TOOLS, MORE WORK NEEDS TO BE DONE BEFORE THIS POTENTIAL IS REACHED.

Molecular diagnostic tests typically analyze key protein, DNA, or RNA markers to characterize diseases. Immunohistochemistry, Western blotting, DNA sequencing, polymerase chain reaction (PCR), Southern blotting, Northern blotting, and quantitative reverse-transcription PCR (qRT-PCR) are molecular techniques that have been effectively used in clinical diagnostic laboratories, where broadly trained technologists perform the tests rather than researchers with specific methodological expertise. However, more-parallel molecular tests that involve simultaneous analysis of tens to hundreds of markers within a clinical sample will soon be required to allow maximum translation of postgenomic research to patient care.

Many basic research projects have focused on methods to measure the behavior of the approximately 30,000 genes in the human genome. This research has been done at different levels within the gene, measuring both genotypic and phenotypic changes. For example, parallel analysis has been applied at the DNA level to analyze sequence polymorphisms that distinguish closely related human alleles or microbial genes; at the level of DNA methylation patterns; at the mRNA level to analyze gene expression patterns; and at the protein level.

With the development of microarray-based tests for diagnosing a wide range of diseases, the ability to gain more information from a limited clinical sample by using highly parallel expression-analysis techniques has emerged. This technology's greatest potential exists for those diseases in which histological differentiation between disease entities is difficult and the consequences of inappropriate treatment can be serious. In the research environment, analyzing sets of gene products with microarrays, rather than individual proteins or RNAs, has been shown to provide effective diagnoses. However, improving high-throughput diagnostic methods will require the continued development of new second-generation custom microarrays. This article describes the challenges in developing this technology and the custom microarray techniques that are currently available.

MICROARRAYS AS TRANSCRIPTION ANALYSIS TOOLS

High-density DNA (HD DNA) microarrays have been useful in the semi-quantitative, or comparative, analysis of thousands of individual messenger RNA (mRNA) species. Experiments using HD DNA microarrays suggest that new tests measuring the expression of tens to hundreds of genes will be better able to characterize disease states than is

currently possible using histopathology and other existing molecular methods. As stated above, clinical application of this DNA microarray-based research will require further development of sophisticated new second-generation custom DNA microarray platforms that are more suitable for routine clinical laboratory testing (see Figure 1).

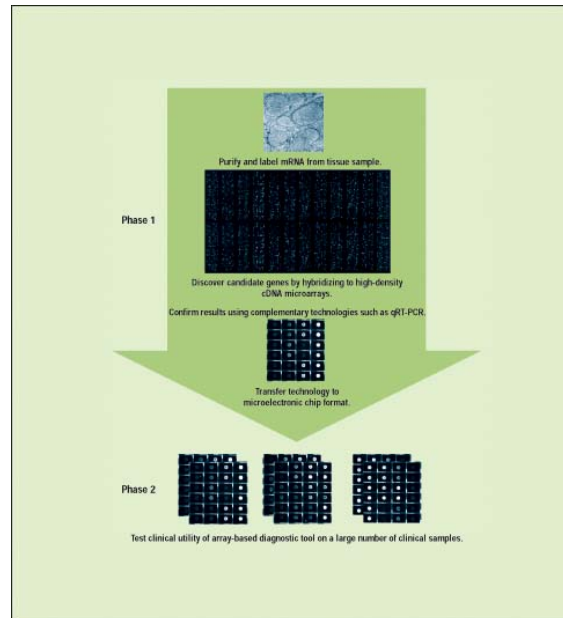


Figure 1. The workflow in developing a prototype gene expression-based diagnostic array (Phase 1) for testing in prospective clinical trials (Phase 2).

HD DNA microarrays are small devices that hold tens of thousands of gene capture probes at known positions on a solid surface. One of the most widely used platforms is the arrays by Affymetrix Inc. (Santa Clara, CA), in which DNA oligonucleotides are chemically synthesized in situ on the array surface using masked photolithography. Another widely used platform is the cDNA array, in which PCR products are generated separately and placed on the array surface using a robotic arrayer.^{1,2} Thousands of laboratories have used these two platforms to catalog the expression of large numbers of genes in normal and diseased cells and tissues.

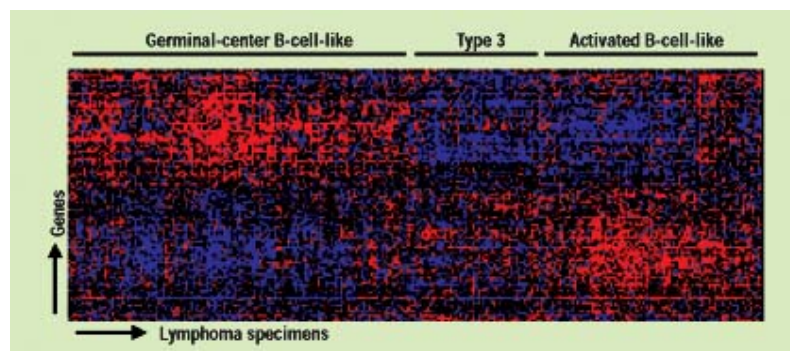


Figure 2. Subgroups of diffuse large B-cell lymphoma (DLBCL) that are identified using high-density microarrays. Researchers used cDNA microarrays to analyze the expression of 100 selected genes (represented by rows) in 240 clinical DLBCL specimens (represented by columns).

When transitioning from a normal to a diseased state, cells and tissues undergo a variety of histological and biochemical changes. These changes reflect in large part the dysregulation in the expression of genes. The quantities and types of proteins that are expressed from their respective genes determine the normal and abnormal functions of

cells. When genes are expressed, their DNA sequences are first copied into temporary molecules, or mRNAs. These mRNAs are then translated into proteins that perform the functions and generate the structures that allow cells to conduct their biological activities. Researchers can take advantage of this flow of genetic information from DNA to mRNAs to proteins by purifying populations of all mRNAs expressed in specimens, synthesizing fluorescently labeled cDNA copies of the mRNAs in vitro, and hybridizing the labeled cDNAs to a microarray. The intensity of the fluorescent signal at each gene-specific probe on the microarray reflects the level of mRNA expressed from that gene in the specimen. By measuring the types and quantities of tens of thousands of individual mRNAs expressed in samples, researchers can infer information regarding the state of gene expression, the approximate quantities and types of proteins present, and the biological behavior of the sample.

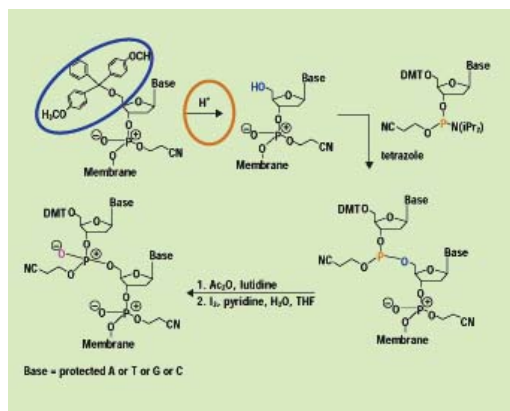


Figure 3. The synthesis of oligonucleotides for microarray experiments. Classical phosphoramidite synthesis relies on the acid catalyzed (red circle) removal of the DMT protecting group (blue circle) from an activated nucleotide, which is used to regulate the addition of the next nucleotide.

For several decades, alternative methods have been available for identifying genes whose expression patterns are altered in disease. However, HD DNA microarrays have increased research scientists productivity in their gene- hunting efforts. As little as five years ago, identifying even a few differentially expressed genes may have taken several years and cost tens of thousands of dollars. Today, HD DNA microarrays can identify 10 times that number of genes in a few months and at a tenth of the cost.³

ADVANCES IN LYMPHOMA DIAGNOSTICS

Recent studies on the biology of non-Hodgkin lymphomas (NHL) illustrate some of the diagnostic advances made possible by HD DNA microarrays. In 2000, it is estimated that 54,900 patients were newly diagnosed with NHLs and 22,553 died of these diseases.⁴ Although there are many NHL subtypes, the diffuse large B-cell lymphoma (DLBCL) subtype is the most common, comprising 30% of all cases.⁵

Despite the complexity of this classification system, it is increasingly recognized that the NHL categories themselves encompass multiple disease subtypes with distinct molecular defects and clinical outcomes. For example, while 35-40% of patients diagnosed with DLBCL can be cured with chemotherapy, the remaining 60-65% die of this disease.⁶ Numerous attempts to distinguish patients who are likely to respond to chemotherapy from those unlikely to respond have been unsuccessful.⁷ In addition, these DLBCL tumors are indistinguishable using classical histopathology and currently available molecular markers.⁷

Researchers have approached this problem by using HD cDNA microarrays to profile gene expressions in DLBCL.⁸ These researchers identified genes that classified DLBCL

specimens into distinct subclasses (see Figure 2). One subclass, germinal-center B-cell-like DLBCL, showed an expression pattern similar to mature germinal-center B-cells and portended a more favorable prognosis with a 60% five-year survival rate. On the contrary, two other DLBCL subclasses, activated B-cell-like DLBCL and type-3 DLBCL, showed much less favorable prognoses with a 35% five-year survival rate.⁸

USING MICROARRAYS FOR PERSONALIZED MEDICINE

Research studies have predicted that in the near future, it may be possible to determine which patients might benefit from more-aggressive therapies, such as bone marrow transplants or alternative chemotherapy regimens, when DLBCL is diagnosed. HD DNA microarray studies have also highlighted molecular pathways important in subclasses of cancer. For example, researchers have observed that several genes in the activated B-cell subtype of DLBCL were downstream targets of the NF κ B transcription factor.⁹ These investigators demonstrated that NF κ B activity is indeed higher in this DLBCL subtype, suggesting that drugs specifically targeting the NF κ B pathway may be effective in treating these tumors.⁹

Several recent papers have suggested that gene expression profiling of tumor specimens may be useful for preselecting patients who may benefit from drug treatment.¹⁰⁻¹³ It may also be useful to examine gene expression profiles of cancers following chemotherapy in order to determine whether the tumors are responding to treatment.¹⁴ The goal of this approach is personalized medicine, in which detailed patient-specific molecular information would be assayed to predict an effective therapy.

CLINICAL APPLICATION OF CDNA MICROARRAYS IN TUMOURS OF HAEMATOPOIETIC AND LYMPHOID TISSUES

An electron microscope is a type of microscope that produces an electronically-magnified image of a specimen for detailed observation. The electron microscope (EM) uses a particle beam of electrons to illuminate the specimen and create a magnified image of it. The microscope has a greater resolving power (magnification) than a light-powered optical microscope, because it uses electrons that have wavelengths about 100,000 times shorter than visible light (photons), and can achieve magnifications of up to 1,000,000x, whereas light microscopes are limited to 1000x magnification.

The electron microscope uses electrostatic and electromagnetic "lenses" to control the electron beam and focus it to form an image. These lens are analogous to, but different from the glass lenses of an optical microscope that form a magnified image by focusing light on or through the specimen.



Figure 4. Electron microscope (TEM)

TRANSMISSION ELECTRON MICROSCOPE (TEM)

The original form of electron microscope, the transmission electron microscope (TEM) uses a high voltage electron beam to create an image. The electrons are emitted by an electron gun, commonly fitted with a tungsten filament cathode as the electron source. The electron beam is accelerated by an anode typically at +100 keV (40 to 400 keV) with respect to the cathode, focused by electrostatic and electromagnetic lenses, and transmitted through the specimen that is in part transparent to electrons and in part scatters them out of the beam. When it emerges from the specimen, the electron beam carries information about the structure of the specimen that is magnified by the objective lens system of the microscope. The spatial variation in this information (the "image") is viewed by projecting the magnified electron image onto a fluorescent viewing screen coated with a phosphor or scintillator material such as zinc sulfide. The image can be photographically recorded by exposing a photographic film or plate directly to the electron beam, or a high-resolution phosphor may be coupled by means of a lens optical system or a fibre optic light-guide to the sensor of a CCD (charge-coupled device) camera. The image detected by the CCD may be displayed on a monitor or computer.

Resolution of the TEM is limited primarily by spherical aberration, but a new generation of aberration correctors have been able to partially overcome spherical aberration to increase resolution. Hardware correction of spherical aberration for the High Resolution TEM (HRTEM) has allowed the production of images with resolution below 0.5 Ångström (50 picometres)[6] at magnifications above 50 million times.[7] The ability to determine the positions of atoms within materials has made the HRTEM an important tool for nanotechnologies research and development.

SCANNING ELECTRON MICROSCOPE (SEM)

Unlike the TEM, where electrons of the high voltage beam carry the image of the specimen, the electron beam of the Scanning Electron Microscope (SEM)[9] does not at any time carry a complete image of the specimen. The SEM produces images by probing the specimen with a focused electron beam that is scanned across a rectangular area of the specimen (raster scanning). At each point on the specimen the incident electron beam loses some energy, and that lost energy is converted into other forms, such as heat, emission of low-energy secondary electrons, light emission (cathodoluminescence) or x-ray emission. The display of the SEM maps the varying intensity of any of these signals into the image in a position corresponding to the position of the beam on the specimen when the signal was generated. In the SEM image of an ant shown at right, the image was constructed from signals produced by a secondary electron detector, the normal or conventional imaging mode in most SEMs.

Generally, the image resolution of an SEM is about an order of magnitude poorer than that of a TEM. However, because the SEM image relies on surface processes rather than transmission, it is able to image bulk samples up to many centimetres in size and (depending on instrument design and settings) has a great depth of field, and so can produce images that are good representations of the three-dimensional shape of the sample.

REFLECTION ELECTRON MICROSCOPE (REM)

In the Reflection Electron Microscope (REM) as in the TEM, an electron beam is incident on a surface, but instead of using the transmission (TEM) or secondary electrons (SEM), the reflected beam of elastically scattered electrons is detected. This technique is typically coupled with Reflection High Energy Electron Diffraction (RHEED) and Reflection high-energy loss spectrum (RHELS). Another variation is Spin-Polarized Low-Energy Electron

Microscopy (SPLEEM), which is used for looking at the microstructure of magnetic domains.

SCANNING TRANSMISSION ELECTRON MICROSCOPE (STEM)

The STEM rasters a focused incident probe across a specimen that (as with the TEM) has been thinned to facilitate detection of electrons scattered through the specimen. The high resolution of the TEM is thus possible in STEM. The focusing action (and aberrations) occur before the electrons hit the specimen in the STEM, but afterward in the TEM. The STEMs use of SEM-like beam rastering simplifies annular dark-field imaging, and other analytical techniques, but also means that image data is acquired in serial rather than in parallel fashion.

LOW VOLTAGE ELECTRON MICROSCOPE (LVEM)

The low voltage electron microscope (LVEM) is a combination of SEM, TEM and STEM in one instrument, which operates at relatively low electron accelerating voltage of 5 kV. Low voltage increases image contrast which is especially important for biological specimens. This increase in contrast significantly reduces, or even eliminates the need to stain. Sectioned samples generally need to be thinner than they would be for conventional TEM (20-65nm). Resolutions of a few nm are possible in TEM, SEM and STEM modes.

DISADVANTAGES

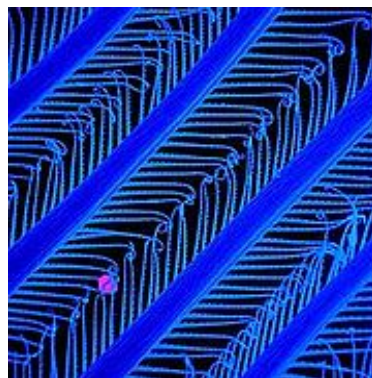


Figure 5. Image of the feeding basket of Antarctic krill

Pseudocolored SEM image of the feeding basket of Antarctic krill. Real electron microscope images do not carry any color information; they are greyscale. The first degree filter setae carry in v-form two rows of second degree setae, pointing towards the inside of the feeding basket. The purple ball is one micrometer in diameter. To display the total area of this structure one would have to tile this image 7500 times.

Electron microscopes are expensive to build and maintain, but the capital and running costs of confocal light microscope systems now overlaps with those of basic electron microscopes. They are dynamic rather than static in their operation, requiring extremely stable high-voltage supplies, extremely stable currents to each electromagnetic coil/lens, continuously-pumped high- or ultra-high-vacuum systems, and a cooling water supply circulation through the lenses and pumps. As they are very sensitive to vibration and external magnetic fields, microscopes designed to achieve high resolutions must be housed in stable buildings (sometimes underground) with special services such as magnetic field cancelling systems. Some desktop low voltage electron microscopes have TEM capabilities at very low voltages (around 5 kV) without stringent voltage supply, lens coil current, cooling water or vibration isolation requirements and as such are much less

expensive to buy and far easier to install and maintain, but do not have the same ultra-high (atomic scale) resolution capabilities as the larger instruments.

The samples largely have to be viewed in vacuum, as the molecules that make up air would scatter the electrons. One exception is the environmental scanning electron microscope, which allows hydrated samples to be viewed in a low-pressure (up to 20 Torr/2.7 kPa), wet environment.

Scanning electron microscopes usually image conductive or semi-conductive materials best. Non-conductive materials can be imaged by an environmental scanning electron microscope. A common preparation technique is to coat the sample with a several-nanometer layer of conductive material, such as gold, from a sputtering machine; however, this process has the potential to disturb delicate samples.

Small, stable specimens such as carbon nanotubes, diatom frustules and small mineral crystals (asbestos fibres, for example) require no special treatment before being examined in the electron microscope. Samples of hydrated materials, including almost all biological specimens have to be prepared in various ways to stabilize them, reduce their thickness (ultrathin sectioning) and increase their electron optical contrast (staining). These processes may result in artifacts, but these can usually be identified by comparing the results obtained by using radically different specimen preparation methods. It is generally believed by scientists working in the field that as results from various preparation techniques have been compared and that there is no reason that they should all produce similar artifacts, it is reasonable to believe that electron microscopy features correspond with those of living cells. In addition, higher-resolution work has been directly compared to results from X-ray crystallography, providing independent confirmation of the validity of this technique.[citation needed] Since the 1980s, analysis of cryofixed, vitrified specimens has also become increasingly used by scientists, further confirming the validity of this technique.

CONCLUSION

An ideal custom DNA microarray platform should allow a user complete freedom in defining which genes are assayed using the array. The microarray platform should also be compatible with computer-based probe design software and molecular methods so that measurements can be performed with high sensitivity and specificity, thereby making independent validation of the assay unnecessary.

The use of manufacturing techniques that allow miniaturization and massive scale-up and result in low costs and minimal biological sample requirements should contribute to a well-engineered microarray platform.²⁶ Because of these factors, there should be standardization among various clinical laboratories using microarrays, such that results from different laboratories can be directly compared.

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