GigaMatrix™: An Ultra High-Throughput Tool for Accessing Biodiversity

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Keywords:
ultra
high-throughput
screening,
vision-guided
robotics,
drug discovery,
enzyme discovery,
protein
optimization,
biodiversity,
microarrays

Recombinant approaches for tapping into the biodiversity present in nature for the discovery of novel enzymes and biosynthetic pathways can result in large gene libraries. Likewise, laboratory evolution techniques can result in large but potentially valuable libraries. Thorough screening of these libraries requires ultra high-throughput methods. The GigaMatrix™ screening platform addresses this opportunity using reusable high-density plates with 100,000 to 1,000,000 through-hole wells in a microplate footprint. In addition to throughputs of over 10^7 wells per day, the platform offers a significant reduction in reagent use and waste, has fully integrated automated "cherry picking," and uses no complicated dispensing equipment. Wells containing putative hits from targeted fluorescent liquid phase assays are revealed by a fluorescent imaging system. Vision-guided robotics are utilized to recover hits by accessing individual 200 μm and smaller wells with a disposable sterile needle. The GigaMatrix platform has proven to be an effective and efficient tool for screening gene libraries for both discovery and evolution applications. (JALA 2004;9:200–8)

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1535-5535/$30.00

doi:10.1016/j.jala.2004.03.005

Introduction

Naturally occurring biomolecules are increasingly being harnessed as powerful tools in the chemical, pharmaceutical, agricultural, and other industries. Most of these industrially useful biomolecules are naturally produced by microbial organisms. Molecular analyses suggest that the vast majority of microorganisms present within the natural environment remain uncharacterized[1,2] and are not amenable to cultivation. One of our primary objectives is to discover unique and commercially valuable biomolecules by sampling from the enormous biodiversity present on our planet. The primary strategy for accomplishing this task involves using recombinant DNA techniques that obviate the vicissitudes of culturing unknown microbes in the laboratory.[3,4] In this recombinant approach, small samples (about 200 g) of soil, water, insect guts, plants, etc. are collected from both extreme and targeted ecosystems worldwide. Collection sites include deep sea hydrothermal vents, active volcanoes, hazardous waste sites, polar ice caps, tropical rain forests, coral reefs, and deserts (Fig. 1). The samples are legally collected via agreements with local governments in accordance with the Convention on Biological Diversity. To date, Diversa Corporation has established biodiversity collaborations with Alaska, Antarctica, Australia, Bermuda, Costa Rica, Ghana, Hawaii, Iceland, Indonesia, Kenya, Mexico, Meadowlands, Russia, South Africa, and Yellowstone National Park. In return for access, the governments may receive research funding, product royalties, supplies, and technology transfer in the form of training, equipment, and data.

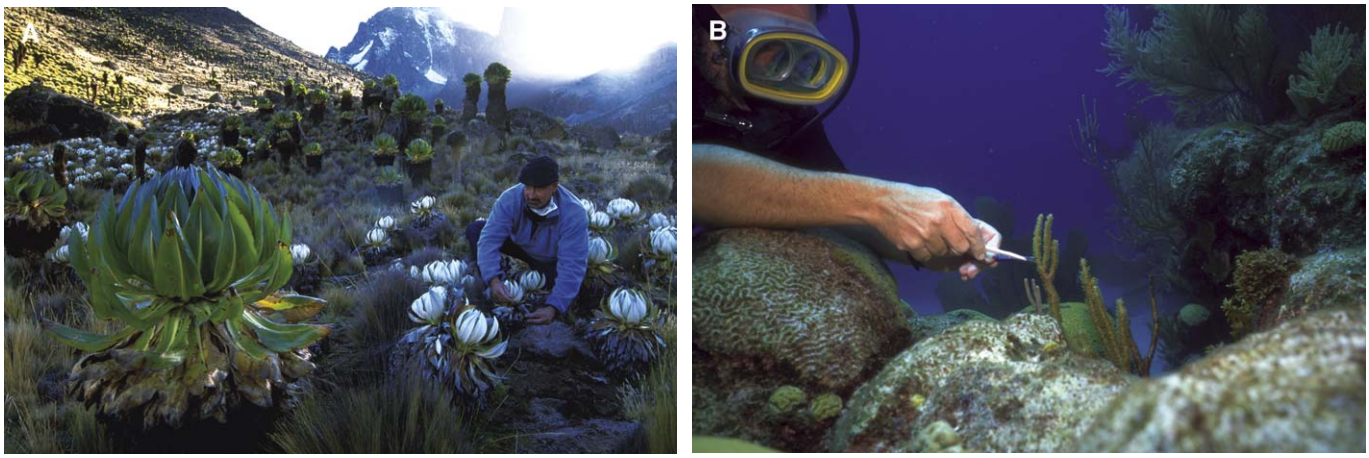

Figure 1. Sample collection sites. (A) Eric Mathur, head of Diversa's biodiversity access team, collecting samples on Mt. Kenya, Kenya and (B) Hank Trapido-Rosenthal, Ph.D., Bermuda Biological Station for Research, collecting marine samples from reefs surrounding Bermuda. Photos courtesy of Frans Lanting.

Gene libraries are then constructed from microbial DNA extracted from these samples. A single gene library may contain in excess of 5,000 different microbial genomes, and thus may contain more than 10^7 unique clones. These clones are then expressed in various host systems and subjected to high-throughput screens specific for a bioactivity of interest. Once a new biomolecule is discovered and identified as a lead candidate for use in a particular application, it can be enhanced using molecular evolution techniques to optimally function under commercial application conditions. Techniques such as Gene Site Saturation Mutagenesis (GSSM™) and GeneReassembly™ involve creating numerous mutants of a particular parental gene(s) in order to create phenotypic variants with improved properties.[5-11] Gene libraries created to evolve a biomolecule can be quite large, in some cases greater than 10^{10} members. Clearly, ultra high-throughput screening of these complex discovery and optimization libraries is key to the success of this strategy.

Current screening methods

A wide variety of screening methods for the discovery and optimization of proteins have recently been reviewed.[12,13] However, what is lacking is a method that provides both the assay flexibility and the throughput needed to fully exploit the opportunities afforded by our recombinant strategy.

The development of applicable automated laboratory equipment has been driven largely by the needs of the pharmaceutical industry to perform "traditional" chemical compound screening. Typically, in these screens, a known compound is located in a known well and screened against a single known target. Automated microplate-based equipment has been used successfully with this screening paradigm, however even state-of-the-art throughputs of 10^5 wells per day are insufficient to adequately screen a diverse environmental or optimization library. This throughput limitation is derived partially from the constraint that the location and identity of each sample in the compound library be known prior to screening. In contrast, each of our gene libraries is a heterogeneous mixture of library constituents in a single suspension. Hence, the identity of each sample in the suspension is unknown unless single genes are isolated and sequenced prior to screening. While this is currently impractical, there is no loss of information when samples are randomly screened as long as hits can be subsequently isolated and identified.

Scientists performing solid phase screening of bacterial colonies or phage plaques[14,15] have been randomly distributing their samples for years. Traditionally, libraries of bacterial colonies or phage plaques are randomly plated onto agar plates and screened by either DNA sequence hybridization or gene product assay techniques. Putative hits from the screen are then picked for subsequent characterization. Although broadly used and successful, these solid phase methods suffer from limitations in assay flexibility and throughput. Random distribution of clones in liquid-based formats is another useful and effective approach for screening libraries. These include growth selection,[16] in vitro expression cloning,[17] cell surface or phage display,[18] and FACS (fluorescence-activated cell sorter)-based strategies.[19] Although each of these has achieved a certain degree of success, they all have their limitations.

The GigaMatrix™ screening paradigm

The GigaMatrix platform is a patent pending screening technology that consists of special high-density plates, custom automated equipment, and biological methods, that was developed to address the need for ultra high-throughput protein discovery and optimization screening. The GigaMatrix screening paradigm combines many benefits of both liquid phase microplate screening and solid phase plate based

screening strategies to overcome throughput and functional limitations of these and other screening methodologies. GigaMatrix assays utilize biological and biochemical methods analogous to those used in microplate assays yet with significantly improved efficiency. GigaMatrix assays also leverage the vast number of assays, reporters, and substrates that have been developed for general liquid phase screening. Moreover, as GigaMatrix plates are reusable, the amount of plastic waste generated is greatly reduced. Approximately 24 tons of plastic waste is generated annually in screening 100,000 wells per day in a 96-well format (assuming 84 g plastic/plate×1,000 plates/day×260 days/year). These benefits are realized through miniaturization, parallel processing, and automation with the goal of achieving FACS throughputs with microplate functionality.

GigaMatrix Plates

At the core of the GigaMatrix platform are reusable plates (Fig. 2) that are fabricated using techniques originally developed by the fiber optics industry. The plates consist of a high-density array of cylindrical through-holes mounted in a plastic carrier[20] that conforms to the SBS microplate footprint. Plates with approximately 100,000, 400,000, and one million wells having well diameters (and volumes) of 200 μm (190 nl), 100 μm (47 nl), and 65 μm (18 nl), respectively, have been fabricated and utilized. The wells are arranged in a slightly irregular close packed hexagonal array. Currently all plate formats have wells that are 6 mm in depth. Maintaining a high length to diameter aspect ratio, nearly 100:1 in the case of the million-well plate, helps minimize evaporation so that plates can be handled in a normal laboratory environment without extraordinary care. The proprietary materials from which the GigaMatrix plates are made satisfy the following requirements: (1) properties that allow for passive loading by surface tension, (2) minimal fluorescence at typical fluorescent screening wavelengths, (3) optical isolation between wells, (4) no inhibition of cell growth, (5) relatively break resistant, (6) tolerant to harsh cleaning solutions and conditions, and (7) withstand repeated sterilization cycles.

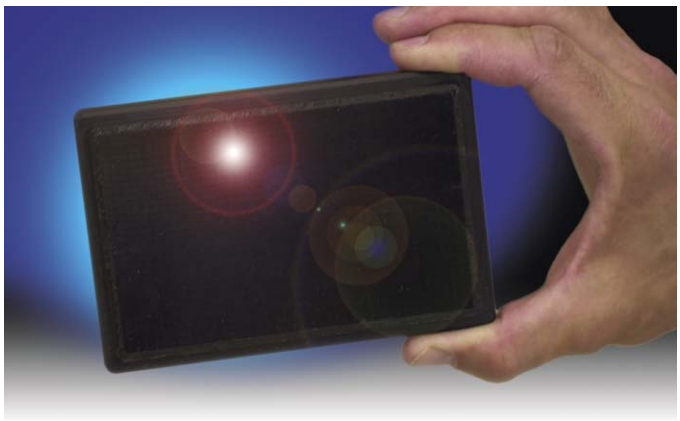

Figure 2. A GigaMatrix plate.

Enzyme Assays

In general, an assay is designed such that in the presence of the desired bioactivity a fluorescent signal is generated. For an enzyme discovery screen, DNA library clones (in the form of plasmids replicating within a bacterial host) are typically grown in the presence of a fluorescently labeled substrate. When an enzyme correctly acts upon the substrate, a fluorescent product is liberated which can then be detected. In this type of screen, enzyme/substrate interaction is dependent upon one or both components having the ability to traverse the bacterial cell wall and membrane. In cases where there is a lack of cell permeability to enzyme and substrate, alternative methodologies are employed (screening DNA libraries in the form of lytic lambda bacteriophages, for example). A fluorescent signal from a well indicates the presence within that well of a potentially useful library clone, i.e., a putative hit. From the putative hits, clones are isolated and further characterized. This basic process, originally developed for microplates, has proven to be successful at both discovering novel enzymes and identifying favorably evolved variants.[9–11] GigaMatrix screening typically consists of the following four steps: (1) Sample preparation, (2) plate filling and incubation, (3) detection and hit recovery, and (4) hit validation. The biological and biochemical methods utilized have been detailed previously.[21] The basic principles of each of these steps are briefly described in the following sections.

Sample Preparation

Typically, library clones must be grown within a host organism (e.g., *E. coli*) in order for the recombinant DNA to be expressed as proteins that can be assayed. The methods and strains used for host introduction depend on the cloning vector in which the library is produced and generally follow established protocols.[22] Once library clones are within a host, they are combined with growth medium, selective antibiotic (where relevant), and fluorescently labeled substrate to form a suspension. In doing so, clones must be diluted to a concentration such that random distribution into a GigaMatrix plate will result in a *seed density* (the average number of clones per well at the time of plate loading) appropriate for the particular screen.

Plate Filling and Incubation

All of the wells of a GigaMatrix plate are filled simultaneously without the need for complicated dispensing equipment. Plates are manually placed in contact with the assay suspension which is held in a simple container. Surface tension at the air-water interface draws the assay suspension into all of the wells. The passive nature of this "wicking"

technique necessitates that plate surfaces be thoroughly cleaned and free of hydrophobic contaminants. When necessary, pressure-driven filling devices are used to fill plates manufactured with hydrophobic surfaces and/or treatments.

The random placement of clones within the well matrix generally follows the Poisson distribution. The preferred seed density used for screens is an important parameter that must be determined empirically for each assay. Many assays can be run at a seed density of approximately one. In this scenario, ~37% of the wells contain a single clone, ~26% contain more than one clone, and the remainder contain no clones. Assays with high signal-to-noise can often tolerate much higher seed densities (up to 10) whereas lower performance assays may be run at seed densities of less than one to minimize the number of wells with multiple samples. In whole cell assays, it is not uncommon for one clone in a library to grow at a faster rate than another. Differential growth rates in a well that has been seeded with more than one clone can result in dramatically altered clone ratios in that well after several generations. This can lead to the discovery of a hit that is difficult to isolate because it represents only a small percentage of the total number of cells in that well.

After filling, the plates are manually loaded into an incubator to promote cell growth and allow reactions to occur. Typical incubation conditions are 37°C for 1–2 days. Because of the high length to diameter aspect ratio of the wells, evaporation is minimized. By sealing individual plates in enclosures and using a humidified incubator, evaporation is limited to about 1% (v/v) per day at 37°C. Temperature variations during incubation can result in condensation on the surface of the plate and should be avoided. In a typical *E. coli* strain, each clone will replicate to 10,000–50,000 clones in 24–48 hours.

Detection and Hit Recovery

After incubation, the plates are transferred to the automated detection and recovery station where fluorescence imaging is used to detect the expression of bioactive molecules. Putative hits (from fluorescing wells) that contain the desired bioactivity appear as a bright spot in an otherwise dark background (Fig. 3). Since the identity of the clone(s) in a given well is unknown at the time of detection, putative hits are recovered and transferred to a standard microplate for subsequent isolation, identification, and characterization. The details of the detection and recovery system are discussed below.

Hit Validation

Samples from the microplate containing putative hits are spread on agar plates to obtain isolated clones. These isolates are then individually tested for activity in a secondary screen under the same conditions used in the initial GigaMatrix screen. The secondary screen is typically carried out in 384-well microplates but can also be done in GigaMatrix plates. Isolated clones that are active in the secondary screen are then archived, DNA sequenced, and further characterized.

GigaMatrix Screening Equipment

The GigaMatrix platform contains the custom automated equipment described below.

Detection System

GigaMatrix plates are optically interrogated using an epi-fluorescent imaging system (Fig. 4). Light from a high-intensity metal halide lamp (EXFO, Vanier, Québec, Canada) is relayed via a fluid filled lightguide and focused

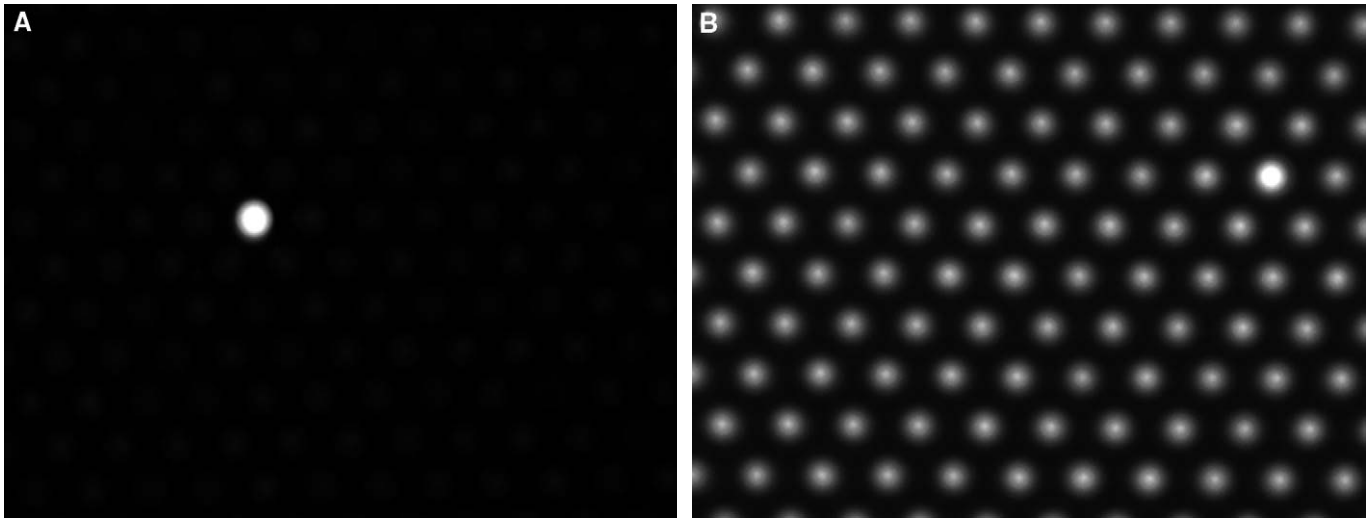

Figure 3. Examples of positive hits in a 100,000-well GigaMatrix plate. (A) A clone active on a resorufin-labeled galactopyranoside substrate (E_m=571 nm; E_x=585 nm) and (B) a clone active on an aminomethylcoumerin-labeled aminoadipic acid substrate (E_x=351 nm; E_m=430 nm).

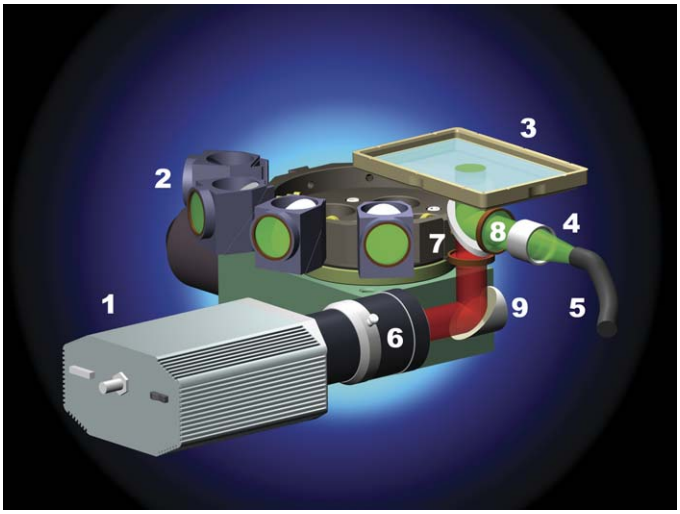

Figure 4. A 3D model of the detection system. (1) Cooled CCD camera, (2) filter wheel, (3) GigaMatrix plate, (4) excitation focusing lens, (5) fluid filled light guide, (6) telecentric lens, (7) fluorescence emission filter, and (8) fluorescence excitation filter.

onto a GigaMatrix plate through an excitation filter (Omega Optical, Brattleboro, VT) mounted in a standard microscope filter cube. Fluorescent emission is then imaged through an optical filter in the same cube onto a high-resolution cooled CCD camera (Roper Scientific, Tucson, AZ) by a telecentric lens (Edmund Scientific, Barrington, NJ). Software adjustments to the camera's image exposure time provide over 7 logs of dynamic range in signal intensity [here dynamic range is defined as: (maximum practical integration time of 10 sec)/(min integration time of 1 ms)×(12 bits of camera pixel depth) = $10{,}000 \times 4096 = 4.1 \times 10^7$]. Ten filter cubes are mounted on a computer controlled rotary stage to provide rapid changes in excitation and emission spectra for different assays and to perform multi-spectral analyses.

GigaMatrix plates are mechanically scanned across the imaging system to create an array of partially overlapping images. The optical magnification of the imaging system, and thus the number of images captured per plate, is set to maximize system throughput while maintaining enough pixels per well for effective image processing. Digitized images are processed on-the-fly (i.e., processing of the current image occurs while moving to the next image) to both identify and locate putative hits. The image processing is performed using functions provided by a commercially available image processing library (Matrox, Dorval, Québec, Canada). Initially, an intensity histogram of the image is used to automatically determine the threshold for image binarization. Connected pixels within the binary image are then grouped into objects or "blobs." The results of measurements of the size, shape, and intensity profile of blobs are then used to discriminate putative hits from undesired background image features such as certain airborne particulates common in a non-cleanroom environment. The positions of wells containing putative hits are then located by computing the centroid of the pixels contained within the blob. Finally, these measurements of well position in pixel coordinates are algorithmically converted into actual mechanical or physical coordinates and used as inputs to the automated recovery system. Approximately 45 sec are required to collect and process a 7×8 array of images from a 100,000-well plate.

As is true with microplate readers, the assay detection sensitivity of the system (i.e., its ability to detect putative hits) is often not limited by its optical sensitivity. As currently configured, the GigaMatrix detection system can detect fluorescein in water at approximately 10 nM with a signal to background ratio of greater than two. However, in assays that involve whole cell cultures, the background fluorescence of the cell culture itself is often much brighter than the limit of optical detection. For instance, when imaged at fluorescein wavelengths, an *E. coli* cell suspension in LB growth medium has a natural fluorescence equivalent to approximately 100 nM fluorescein in water. Under UV excitation conditions, the background fluorescence is even more significant (Fig. 3). Therefore, users of the system are guided to use fluorescent substrates that excite/emit with as long a wavelength as possible.

Hit Recovery System

Because samples are distributed randomly across the plates, it is necessary to recover putative hits for identification and subsequent characterization. Automated recovery is required due to the nature of the small and irregularly spaced

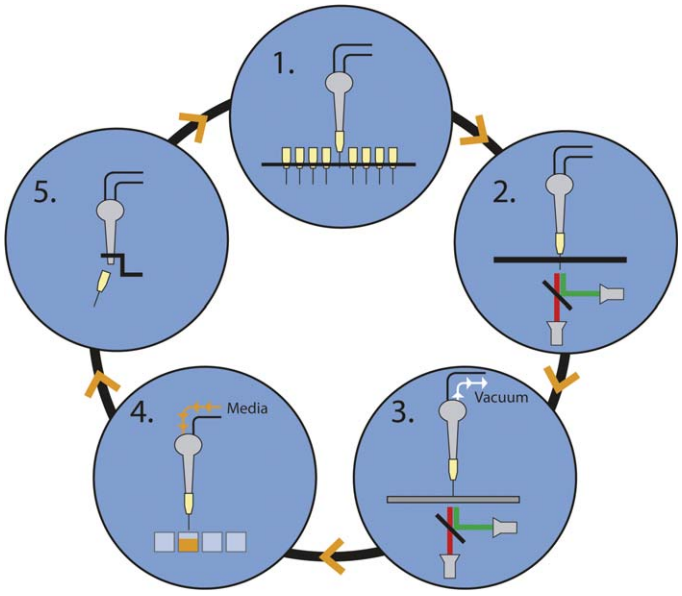

Figure 5. Basic steps of the hit recovery process: (1) Mount and locate the tip of a sterile recovery needle; (2) align the recovery needle to the well containing the putative hit; (3) aspirate the contents of the well into needle; (4) dispense the needle contents into microplate well; and (5) remove and dispose of the used needle.

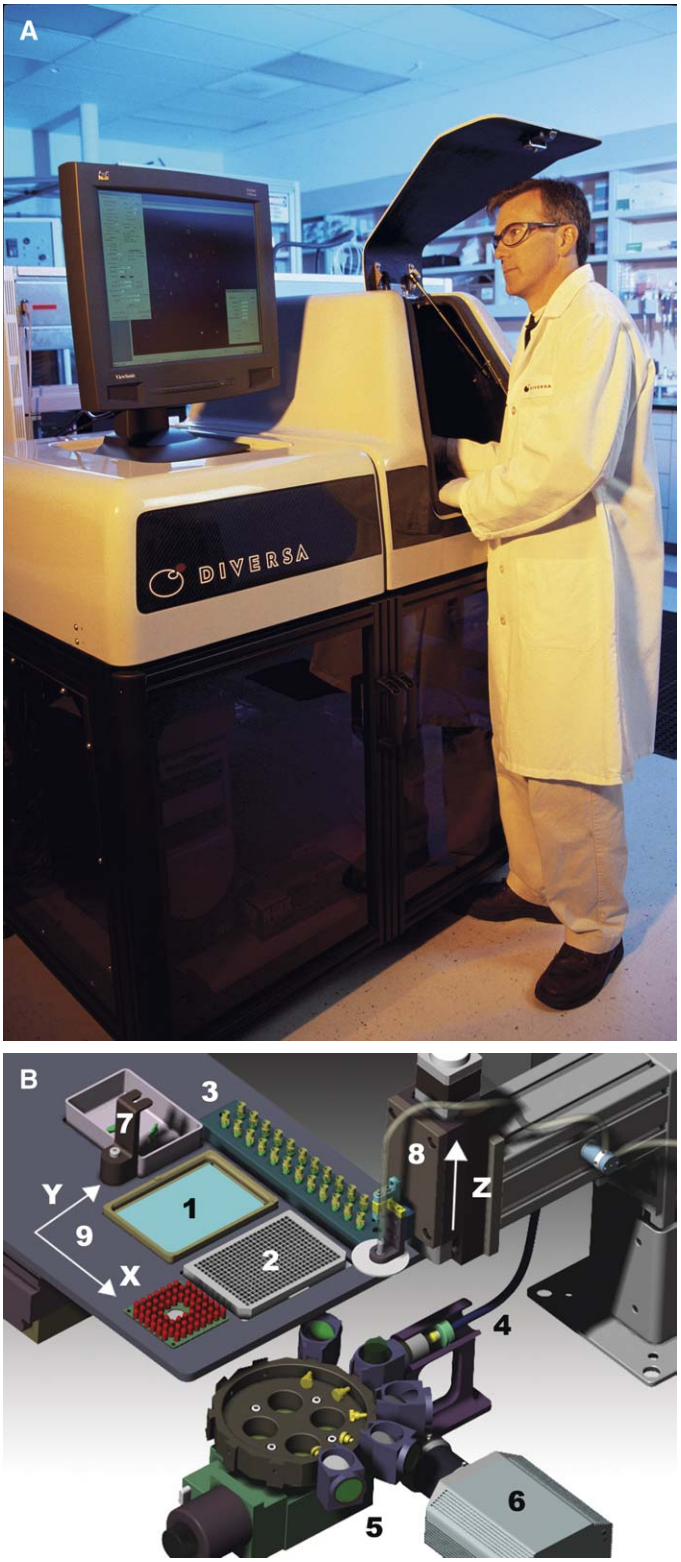

Figure 6. (A) Automated detection and recovery system and (B) exploded 3D model view of the major detection and recovery station components. (1) GigaMatrix plate, (2) recovery microplate, (3) recovery needles, (4) illumination optics, (5) filter wheel, (6) cooled CCD camera with telecentric lens, (7) needle removal device, (8) needle axis translation stage, and (9) XY translation deck.

wells. Recovery of samples from GigaMatrix plates is accomplished by using vision-guided positioning to align a small needle with the opening at the end of a well containing a putative hit and aspirating the contents into the needle under vacuum. The basic steps of the recovery or "cherry-picking" process are described in Fig. 5. An automated recovery system with integrated detection system (Fig. 6) was developed that can complete these steps at a rate of approximately 20 sec per hit with the anticipation of primary hit rates on the order of a few per plate.

The requirement for sterile operation led to a design that uses disposable sterile recovery needles to minimize sources of contamination and maximize ease of use. These 30 and 32 gauge needles (EFD, East Providence, RI), with inside diameters of approximately 150 and 90μm respectively, are commonly used in the electronics industry to glue components onto printed circuit boards prior to soldering. A 0.22 μm filter (Millipore, Billerica, MA) is mounted on each recovery needle to avoid contamination to and from the fluid and vacuum lines. During the recovery process, the needles are loaded onto a counterbalanced or "floating mount" so that only very light contact with the plate is made to avoid damaging the surface of the plate or bending the needle.

Multiple aspects of the design of the recovery system contribute to the mechanical precision required to reliably interact with individual 200 μm or smaller wells. First, by locating the detection optics on the opposite side of the plate from the recovery mechanics, both the complexity of the positioning system and the opto-mechanical constraints of the detection system are minimized. Further reduction in the complexity of the recovery system, and thus sources for potential positioning errors, is realized through the use of a novel (patent pending) configuration of components. Once mounted, the position of the tip of a needle does not change within the imaging plane (x,y) of the detection system, but rather only along the optical axis (z). In contrast, the GigaMatrix plate itself, and all other components mounted to the movable deck, move only within the imaging plane (x,y). In this way, the desired well is moved into alignment with the known and fixed position of the needle tip.

Precise, single-well recovery from a million-well GigaMatrix plate requires alignment of the center of the recovery needle to the center of the well to within approximately ±15 μm. The precision 2-axis translation stage and microstepping electronics (Danaher Precision Systems, Salem, NH) that performs this alignment has positional accuracy sufficient to satisfy this requirement. However, relatively large (> 500 μm) variations in needle tip position are common with these small diameter disposable and often slightly bent needles. For this reason, image based position feedback is used to compensate for needle-to-needle variability. Basically, after the needle is mounted, the (x,y) position of the center of the tip of the needle is determined by the detection system and converted from pixel coordinates into physical coordinates. Next, the translation stage holding the GigaMatrix plate is moved

by the difference between the measured physical coordinates of the needle and the hit to bring the two into alignment. Finally, the needle translates along z (i.e., the direction of the optical axis) until it gently touches the top of the well opening. The accuracy of this method is demonstrated in Fig. 7.

All of the software for the detection and recovery system is written in Visual Basic. The user interface provides the flexibility for users to: selectively navigate around plates or automatically scan entire plates; manually or automatically select hits; adjust numerous image capture, processing, display, and archiving parameters; select sample and output plate formats; and perform numerous other functions and controls.

Plate Cleaning System

To satisfy the need for a continuous supply of consistently clean and sterile plates, a custom automated GigaMatrix plate cleaning system was constructed (Fig. 8). Our design strategy was to leverage the proven hardware and software architecture used in our microplate based HTS workcells. The system uses an articulated robot arm (Thermo CRS, Burlington, Ontario, Canada) to transfer plates through a series of custom cleaning stations. Special features in the sides of the GigaMatrix plates facilitate accurate robotic handling of wet plates. Each station is a self-contained microprocessor controlled device that performs a single step in the cleaning process. There are stations for ultrasonic cleaning, fluid removal, and flushing/rinsing. In the fluid removal stations, plates are passed over a slot connected to a high flow rate vacuum blower to suck out any fluid in the plates. Similarly, within the rinse stations, water and cleaning solutions are forced through the plate by a high-pressure nozzle. A de-ionized water rinse ensures no cleaning solutions are left behind that could affect cell growth or the surface tension properties of the plates. The output of the system after the final fluid removal station is a rack of clean and dry plates that are ready for reuse after autoclaving. Cleanliness is verified by fluorescence imaging of the material at multiple excitation wavelengths and looking for visible signal that could be misinterpreted as a positive well. Sterilization is validated by overnight incubation of plates filled with sterile growth media, followed by culturing the contents on solid medium to verify the lack of colony formation. Under normal use, GigaMatrix plates can be used indefinitely.

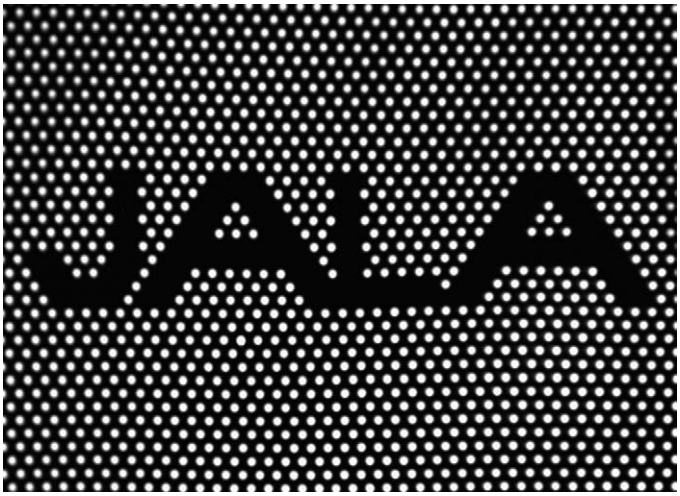

Figure 7. Selected 200-µm diameter wells are aspirated from a portion of a 100,000-well GigaMatrix plate filled with 1 µM fluorescein to demonstrate precision recovery.

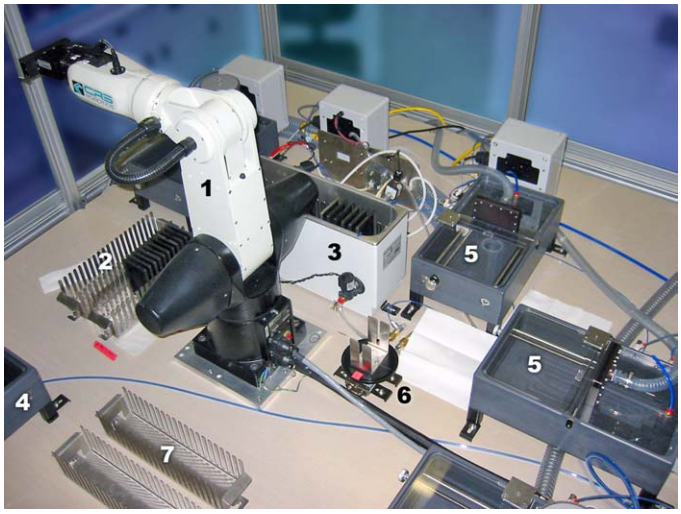

Figure 8. Automated GigaMatrix plate cleaning system. (1) Articulated robot arm, (2) input plate rack, (3) ultra sonic cleaning bath, (4) fluid removal stations, (5) fluid flushing stations, (6) regrip station, and (7) output plate rack.

Applications and Results

The GigaMatrix platform has proven to be an effective and efficient tool for harvesting potentially useful biomolecules from a diverse microbial world. Hundreds of unique enzymes covering a range of protein types and functions have been identified using this technology, including ß-xylosidases, ß-galactosidases, α-amylases, cellulases, α-L-arabinofuranosidases, proteases, and other non-protease amidases. A significant majority of these discovery clones represent gene sequences not found in the public gene databases.

Depending on the well densities and assay conditions used, throughputs of 10^6 to 10^8 assays per day have been achieved. Background fluorescence is a leading contributor to false positives and can affect the overall productivity of the platform. On a typical screening day with a moderate performance assay (with signal to background ratio of approximately 2:1 such as shown in Fig. 3A), 20 to 30 GigaMatrix plates per day with a seed density of one clone per well are screened by one person working alone. This yields a typical daily throughput of over two million assays per day per person using 100,000-well plates (Table 1).

Table 1. Typical productivity of 100,000-well GigaMatrix screens for moderate performance assays

Enzyme	No. clones screened	No. confirmed hits[a]	No. screening days[b]
Protease	3.9×10^7	156	15
Cellulase	3.6×10^7	282	28
ß-xylosidase	2.0×10^7	70	9
α-L-arabinofuranosidase	1.2×10^7	73	4

[a]Number of confirmed hits includes redundant clones.
[b]Number of screening days spent loading and analyzing screening plates and recovering primary hits.

For comparison, it would require approximately 29 days of screening in 384-well plates using one of Diversa's automated microplate workcells that can process about 180 microplates per day to match the single day throughput of a typical GigaMatrix screen. Further, the microplate-based assay estimate does not include automated hit-picking and would require preparing and disposing of over 500 times more solutions/reagents and disposal of thousands of microplates. Peak throughputs of over 100 million assays per day have been achieved using million-well plates for assays with low background fluorescence (Fig. 3B) and where biological biases can be tolerated to allow processing multiple clones per well. Hit rates (defined as the number of confirmed hits per number of clones screened) are highly dependent upon many factors, independent of the screening methodology, including the nature of the library screened, the target bioactivity, and the selectivity of the assay. The hit rates achieved using GigaMatrix technology are comparable to those achieved using microplate methodologies with similar assays in both head-to-head comparisons and through analysis of historical data.

The GigaMatrix platform has also been used successfully for protein optimization. The excitation/emission characteristics of a fluorescent protein were modified in a recent molecular evolution project. For this project, the plates were imaged with three filter sets, each having different spectral characteristics to detect subtle changes in protein fluorescence. A false-color composite image was then produced using the grey-scale images from each filter set and displayed on the monitor. With this multi-spectral system, wild-type proteins would appear green. Variants with emission spectra shifted toward shorter wavelengths would appear blue or cyan and those shifted toward longer wavelengths would appear red. Several variants, mostly shifted towards blue, were identified from the evolved library and are currently being characterized (unpublished data).

Since its deployment as a screening system for discovery programs in 2001, the GigaMatrix platform has become a mature and established screening methodology at Diversa. Our experience has shown that only three days are required to train laboratory personnel to effectively utilize the methods and equipment of the GigaMatrix platform. In over three years of daily operation, the oldest and most used detection and recovery workstation has required minimal maintenance (semiannual changing of the light source lamp and lubrication of the precision motion stages). Such ease of operation and high reliability has contributed to the rapid acceptance of this technology at Diversa. Further, the number of novel enzymes and the rapidity at which they were discovered have demonstrated the effectiveness of the GigaMatrix platform as a screening tool.

Acknowledgments

The development of the GigaMatrix platform was a multi-disciplinary effort requiring the skills of many engineers and scientists. The authors gratefully acknowledge Betsy George who first demonstrated the concept and the members of the research and development team: Patti Kretz, Dan Kline, Chuck Tweedy, Scott Beaver, Ian Storer, and Tom Todaro. The authors thank the following scientists for their first-hand experience with, and successful implementation of, the GigaMatrix platform in their screening projects: Connie Hansen, Joslin Gemsch, Ajit Botejue, Yoko Phillips, and Lilian Parra-Gessert. They also thank Dan Robertson and Jay Short for their vision and support. Work on the million-well GigaMatrix plate technology was supported in part by NIH Grant No. 1R43 GM066474-01.

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