

Improved Methods and Standards for Telomerase Assay: Quantitative Histopathology using Antibody Staining

Telomerase is a reverse transcriptase enzyme that catalyzes the end capping of the chromosomes (telomeres) with the repetitive DNA sequence motif (TTAGGG). It is expressed during development and in 85-90% of all human cancers, but not in normal adult, non-stem cell somatic tissues, which makes it an attractive tumor diagnostic marker. However, assays for telomerase have been cumbersome and without a universally accepted standard to compare between laboratories. To this end, we have improved the sensitivity and reproducibility of the TRAP assay system using capillary electrophoresis and a high-throughput RApidTRAP (robot-assisted TRAP) system. In addition, we have developed a candidate telomerase reference material for TRAP and RT-PCR based assays. This well-characterized candidate reference material was evaluated for potential diagnostic use across biochemical-based and direct imaging-based technologies. Quantitative measurements of telomerase will serve potentially as a traceable reference for use with faster, clinically amenable analytical methods and may promote the development of novel technologies, such as an IHC standard protein chip.

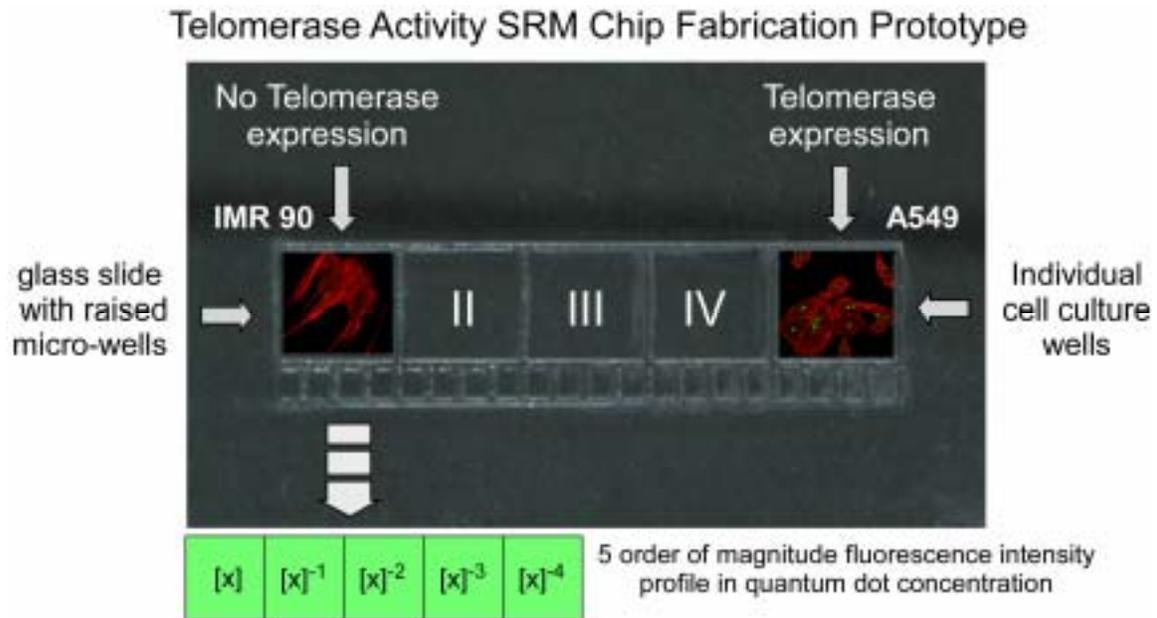
D.H. Atha and J.P. Jakupciak (Div. 831)

We have used confocal and conventional microscopy and fluorescent-labeled telomerase antibodies to quantify telomerase in cultured human lung tumor cells and in human fibroblast cells as a control. These direct measurements were compared to telomerase activity measured by RApidTRAP/PCR, hTERT mRNA RT-PCR, and flow cytometry to determine the sensitivity and the minimum number of cells required for accurate analyses. This combination of measurement of telomerase by tissue staining, TRAP/PCR activity, mRNA expression, and flow cytometry will provide a more complete quantification of telomerase levels in tissues at various stages of cancer.

In collaboration with the Polymers Division we have developed an IHC standard protein chip for telomerase expression that can be used in a wide range of histological and antibody staining applications. A prototype chip, fabricated by photolithography, is shown below. Cells with different levels of telomerase expression are seeded onto the chip and imaged. The chip uses cancer cell lines, which are known to express different levels of telomerase. At the low-end of expression IMR-90 cells are used while at the high-end of expression A549 cells are used.

The lack of quantitative imaging methods and reagents impedes the validation of image-based biomarkers. Improved methods for biomarker quantification and an established traceable value assignment would be useful to customers in a wide range of areas that include clinical diagnostics, diagnostic industries, instrument manufacturers, academic and government research agencies. Quantifiable measurements of cancer biomarkers, such as telomerase, are critical for accurate assessment of the presence of cancer and for determining the degree of cancer progression. Since telomerase has been shown to be a

primary candidate biomarker for cancer, improvement in measurement methods of telomerase could impact numerous areas related to cancer diagnostics and provide ancillary insights to understand the proteome and transcriptome.



Publications:

Jakupciak JP, Wang W, Barker PE., Srivastava S., Atha DH. **“Analytical Validation of Telomerase Activity for Cancer Early Detection,”** J. of Molecular Diagnostics, 2004, 6, 157-165.

Jakupciak JP, Barker PE., Wang W, Srivastava S, Atha DH. **“Standards for telomerase assays: TRAP/PCR, Real-time TRAP/PCR, and hTERTmRNA/RT-PCR,”** Clinical Chemistry, 2005, 51, 1443-1450.

O'Connell CD., Atha DH., Jakupciak JP. **“Standards for validation of cancer biomarkers, Cancer Biomarkers,”** 2005, 1, 233-239.

McGruder BM., Atha DH., Wang W., Huppi K., Wei WQ., Abnet CC., Qiao YL., Dawsey, SM., Taylor PR., Jakupciak JP. **“Real-time telomerase assay of less-invasively collected esophageal cell samples,”** Cancer Letters, 2006, 244, 91-100.

Jakupciak, J.P., Gallant, N.D., Becker, M.L., Tona, A. and Atha, D.H. **“Improved methods and standards for telomerase assay: Quantitative histopathology using antibody staining,”** J. of Molecular Diagnostics, 2006, submitted.

Title: Quantum Dot-Based Quantitative Cellular Imaging for Biomedicine

Authors: Peter Barker, Yan Xiao

This technology development and physical standards work is based on development of new nucleic acid probes labeled directly or via haptens such as biotin and digoxigenin, and novel IgY antibodies.

Introduction:

These probes are detected with semiconductor nanocrystals and 3D imaging in a high throughput mode to generate data with associated performance metrics. Work has focused on HER2 gene (breast cancer), TMRSS2-ETV1 fusion genes (prostate cancer) as needful metrology improvement programs for clinical cancer and quantitative histochemistry QA/QC. These studies led to a long term evaluation of cellular uptake of functionalized qdots as part of the nanotech and cellular biometrology competencies at NIST.

Accomplishments:

Our major accomplishments include: first demonstration of FISH with qdots; a novel IgY chick antibody for human telomerase (Xiao patent submission); demonstration of high resolution imaging of cellular responses to WMD protectant roxithromycin.

Impact:

A number of press interviews for breast cancer nanotech imaging work; qdot work has expanded to a number of different gene and protein analysis systems and has been the reason for several invited presentations (Barker, Xiao). Roxithromycin qdot work was selected as best paper in the November 2006 U. S. Army Science Conference.

Future Plans:

Qdot imaging of HER2 gene and protein in high throughput mode; imaging on tissue microarrays; completion of HER2 SRM.

Outputs:

Xiao, Y. IgY antibody for human telomerase (patent submitted September 2006)

Xiao, Y., Barker P. E. (2004). Semiconductor nanocrystal probes for human metaphase chromosomes *Nucleic Acids Research* 32(3):1-5.

Xiao, Y., Barker, P. E. (2004). Semiconductor nanocrystal probes for human chromosomes and DNA. *Minerva Biotechnologica* 16:1-8. (review)(0.167; C=0)

Xiao, Y., Telford W. G., Ball, J. C., Locascio L. E., Barker, P. E. (2005). pH, FISH and semiconductor nanocrystals. *Nature Methods* 2(10): 723, 2005. (IF=6.741, C=2)

Barker, P. E. (2006). Preface to standards for healthcare: needs assessment and development. *Cancer Biomarkers* 1(2005) 207-208. (IF=not available; C=0)

Mueller, F., Houben, A., Barker, P. E., Xiao, Y., Kas, J., Melzer, M. (2006). Quantum dots: a versatile tool in plant science. *J. Nanobiotechnology* 5:4.

<http://www.jnanobiotechnology.com/content/4/1/5>

Title: Quantum Dot-Based Quantitative Cellular Imaging for Biomedicine**Authors: Peter Barker, Yan Xiao**

The most critical analytes in biomedicine can be simplistically conceptualized to nucleic acids and proteins. Some methods use ensemble methods which, in effect average the values of various nucleic acid and protein biomarkers. While this appeals to chemists, the fact of biological heterogeneity among cells demands cell-by-cell quantitation. This means not only single molecule detection (SMD), but SMD and quantitation superimposed on cell type.

A significant measurement problem with SMD in cells has been the nonquantitative nature of classically used organic fluorophores. Most photobleach, rendering replication difficult if not impossible. Whereas qualitative imaging has met needs in the clinical community in the past, the increasingly sophisticated interplay among genes and proteins means that, to extract optimal value from cell measurements for medical diagnostics or evaluation of drug effects on cells, quantitation is key for the future. In addition, quantitation on single analytes (proteins or genes or RNA species) will prove to be insufficient for clinical decision making of the future. Thus, in addition of quantitation, analyte multiplexing at the cell level will assume greater importance as the discoveries of the biotechnology revolution of the past 25 years undergo translation in service of medicine and the private sector in, for example, pharmaceutical drug development and evaluation.

With this future measurement need in mind, we have focused on a novel non-photobleaching detection tool that shows great promise for SMD and multiplexing at the level of the cell: quantum dots or semiconductor nanocrystals. This technology development and physical standards work is based on development of new nucleic acid probes labeled directly or via haptens such as biotin and digoxigenin, and novel IgY antibodies. Because generic applications of quantum dots would be too broad for a small research lab, we focus on two model systems: HER2, and FDA approved analyte of significant medical and financial proportions in breast cancer detection and management; and TMPRSS2, a promising prostate cancer biomarker early in its translational development stage.

Introduction:

These probes are detected with semiconductor nanocrystals and 3D imaging in a high throughput mode to generate data with associated performance metrics. Work has focused on HER2 gene (breast cancer), TMPRSS2-ETV1 fusion genes (prostate cancer) as needful metrology improvement programs for clinical cancer and quantitative histochemistry QA/QC. These studies led to a long term evaluation of cellular uptake of functionalized qdots as part of the nanotech and cellular biometrology competencies at NIST.

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chick antibody for human telomerase (Xiao patent submission); demonstration of high resolution imaging of cellular responses to WMD protectant roxithromycin.

Impact:

A number of press interviews for breast cancer nanotech imaging work; qdot work has expanded to a number of different gene and protein analysis systems and has been the reason for several invited presentations (Barker, Xiao). Roxithromycin qdot work was selected as best paper in the November 2006 U. S. Army Science Conference. The applications work on qdots has positioned the lab, group and division among leading labs working on these novel fluorophores, and has established a track record in successful qdot metrologies in the bioscience community.

Future Plans:

Qdot imaging of HER2 gene and protein in high throughput mode; imaging on tissue microarrays; completion of HER2 SRM.

Outputs:

- Xiao, Y. IgY antibody for human telomerase (patent submitted September 2006)
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- Barker, P. E. (2006). Preface to standards for healthcare: needs assessment and development. *Cancer Biomarkers* 1(2005) 207-208. (IF=not available; C=0)
- Mueller, F., Houben, A., Barker, P. E., Xiao, Y., Kas, J., Melzer, M. (2006). Quantum dots: a versatile tool in plant science. *J. Nanobiotechnology* 5:4.
(<http://www.jnanobiotechnology.com/content/4/1/5>)

Chem-BLAST allows unambiguous chemical database searching

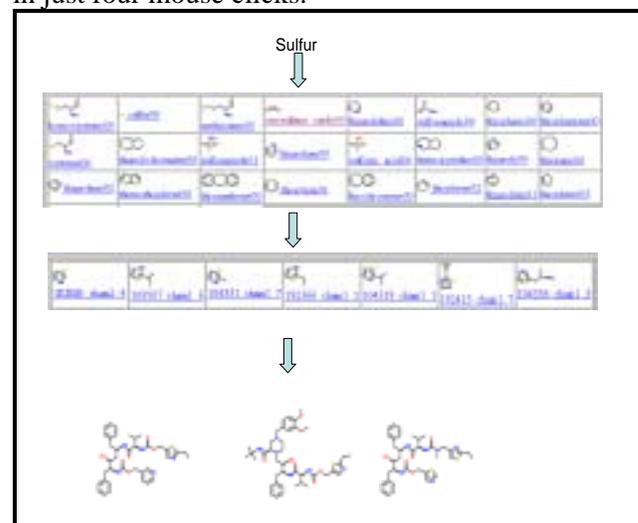
We are witnessing the emergence of a web-based data-rich era in a biochemical world. In the past decade, databases have become an integral part of research and development in the biomedical sciences. Bioinformatics plays an essential role in deriving knowledge from complex biochemical data. In order to enable reliable exchange of these data over the Web, we are developing intuitive and user friendly Web interfaces for organizing and retrieving chemical data that is based on chemical structure, not chemical name.

T. N. Bhat and A.D. Nguyen (Div. 831)

A common way of identifying or specifying chemical compounds is by name. IUPAC names are well-known examples. However, these names are difficult to generate, and machine searching for unique matches is slow. For this reason, chemical Web page developers have been relentlessly working on associating compounds with an ever growing list of so-called 'synonyms' to match against user queries. This approach is frustrating both for database providers and users of a Web page alike. A database provider can never be sure that he has included all the 'synonyms' of a compound, and a user can never be assured that a name he is familiar with has the same meaning in the context of the database he is planning to query. The result is often missed hits or an overwhelming number of hits.

NIST has developed a new technology called Chem-BLAST (Chemical Block Layered Alignment of Substructure Technique) that has proven to be highly successful to query therapeutic drugs that can be used to treat AIDS. Chem-BLAST combines the usefulness of two-dimensional pictures of chemical structures with explicitly defined chemical structural components of interest to the user. Within an environment called a *resource description framework* (RDF), the user can choose the chemical components of the structure of interest from selections of components in the database. These selections are presented in a sequential fashion until the compound of interest is built up from its structural components.

This approach is orderly, unambiguous, and efficient. In a typical setting, a database with 10,000 compounds with four layers of RDF statements may allow a user to get a precise answer to his question in just four mouse clicks.



This figure illustrates a case where a user queries the database for drugs with sulfur containing a five member ring. The Web page displays all the drugs with such an element. In each mouse click the user builds the query from the structural elements presented to him and a total of three mouse clicks are used here.

NIST implemented Chem-BLAST in one of its most popular Web resources, the HIV Structural Database (<http://xpdb.nist.gov/hivpdb/hivpdb.html>). HIVSDB is a collaborative effort between NIST, National Institute of Allergy and Infectious Diseases, National Cancer Institute, and University of Rutgers. Chem-BLAST and the Semantic Web concept have been embraced by the W3C, the International Body responsible for establishing standards for the future World Wide Web, which sees Chem-BLAST as a way to provide precise answers for complicated questions without compromising speed or convenience for either database developers or users.

Concordance Study with MiniFiler and other STR Kits

Genetic tests that target smaller sections of DNA are more successful at recovering information from highly degraded biological specimens. The value of miniature short tandem repeat (miniSTR) assays were demonstrated during efforts to identify the 9/11 World Trade Center victims. The first commercial kit based on this miniSTR technology is now being tested and promises to greatly aid forensic labs in the future to solve previously intractable cases.

C.R. Hill, M.C. Kline, J.M. Butler (Div. 831)

A number of studies have demonstrated that successful analysis of degraded DNA specimens from mass disasters or forensic evidence improves with smaller sized polymerase chain reaction (PCR) products. If DNA is exposed to the elements or to fire for any length of time, degradation can occur due to bacterial, biochemical or oxidative processes. Within the forensic community, a core set of short tandem repeat (STR) markers have been developed for utilization in forensic casework, and large DNA databases such as the Combined DNA Index System (CODIS) have been developed incorporating these markers.

During the spring and summer of 2006, the NIST Human Identity Project Team was involved in beta-testing a new DNA testing kit that will enable improved recovery of genetic information from biological samples that are highly fragmented or otherwise environmentally compromised. The principles behind miniSTR technology were pioneered at NIST in order to aid recovery of information from the highly fragmented remains of victims from the 9/11/01 terrorist attacks on the World Trade Center twin towers.

Applied Biosystems (Foster City, CA) is working with NIST scientists to validate their AmpF ℓ STR[®] MiniFiler[™] PCR Amplification kit through examining samples amplified with this new test and presently available DNA tests. These concordance tests are necessary because each test contains slightly different PCR primers that target the same regions of human DNA. Due to the fact that sequence variation can and does exist in the flanking region of STR markers, samples need to be evaluated to verify PCR amplification performance and quantify any differences where mutations are occurring in primer binding regions.

The MiniFiler kit enables size reduction on eight of the larger STR loci amplified in the other commercial STR kits such as Identifiler (Applied Biosystems) and PowerPlex 16 (Promega Corporation, Madison, WI). MiniFiler amplifies CSF1PO, FGA, D2S1338, D7S820, D13S317, D16S539, D18S51, and D21S11 as well as the sex-typing locus amelogenin. A total of 1,308 samples were evaluated with both the MiniFiler and Identifiler STR kits: 449 African American, 445 Caucasian, 207 Hispanic, and 207 Asian individuals. Full concordance between Identifiler and MiniFiler kits was observed in 99.7% (10,437 out of 10,464) STR allele calls compared. The 27 differences observed (see dotted lines in figure) encompass the loci D13S317 (n=14) and D16S539 (n=10) as

well as D18S51 (n=1), D7S820 (n=1), and CSF1PO (n=1). Genotyping discrepancies between the Identifiler and MiniFiler kits were confirmed by re-amplification of the samples and further testing using the PowerPlex 16 kit. DNA sequence analysis was also performed in order to understand the nature of the genetic variations causing the allele dropout or apparent repeat unit shift.

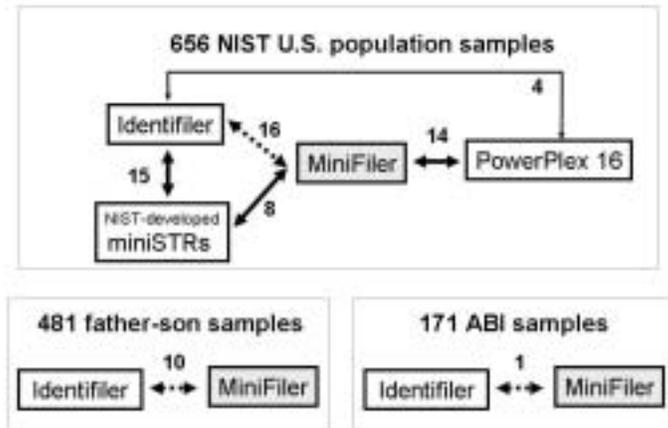


Figure: a description of differences noted between the various tested STR kits during concordance studies conducted

Publications:

Butler, J.M., Shen, Y., McCord, B.R. (2003) The development of reduced size STR amplicons as tools for analysis of degraded DNA. [J. Forensic Sci 48\(5\) 1054-1064.](#)

Biesecker, L.G., Bailey-Wilson, J.E., Ballantyne, J., Baum, H., Bieber, F.R., Brenner, C., Budowle, B., Butler, J.M., Carmody, G., Conneally, P.M., Duceman, B., Eisenberg, A., Forman, L., Kidd, K.K., LeClair, B., Niezgodna, S., Parsons, T., Pugh, E., Shaler, R., Sherry, S.T., Sozer, A., Walsh, A. (2005) DNA identifications after the 9/11 World Trade Center attack. [Science 310:1122-1123.](#)

Dixon, L.A., Dobbins, A.E., Pulker, H., Butler, J.M., Vallone, P.M., Coble, M.D., Parson, W., Berger, B., Grubweiser, P., Mogensen, H.S., Morling, N., Nielsen, K., Sanchez, J.J., Petkovski, E., Carracedo, A., Sanchez-Diz, P., Brion, M., Irwin, J.A., Just, R.S., Loreille, O., Parsons, T.J., Syndercombe-Court, D., Schmitter, H., Gill, P. (2006) Analysis of artificially degraded DNA using STRs and SNPs--results of a collaborative European (EDNAP) exercise. [Forensic Sci. Int. 164: 33-44.](#)

Butler, J.M. (2006) MiniSTRs: past, present, and future. [Forensic News](#) (Applied Biosystems), October 2006 [[.pdf](#)]

Hill, C.R., Kline, M.C., Mulero, J.J., Lagace, R.E., Chang, C.-W., Hennessy, L.K., Butler, J.M. (2006) Concordance study between the AmpFISTR MiniFiler PCR Amplification Kit and conventional STR typing kits. *submitted.*

CSTL FY2006 Technical Activity Report

Disclaimer: This project was supported by National Institute of Justice Grant Number 2003-IJ-R-029, which is an interagency agreement between [NIJ](#) and the [NIST Office of Law Enforcement Standards](#), awarded by the National Institute of Justice, Office of Justice Programs, US Department of Justice. Points of view in this document are those of the authors and do not necessarily represent the official position or policies of the US Department of Justice. Certain commercial equipment, instruments and materials are identified in order to specify experimental procedures as completely as possible. In no case does such identification imply a recommendation or endorsement by the [National Institute of Standards and Technology](#) nor does it imply that any of the materials, instruments or equipment identified are necessarily the best available for the purpose.

Examination of Software for Rapid Analysis of Single Source DNA Profiles

A significant bottleneck in getting DNA profiles into DNA databases to help solve crimes is the data review process. Expert system software has been evaluated in terms of performance on single-source DNA samples.

C.R. Hill, M.C. Kline, A.E. Decker, J.M. Butler (Div. 831) and D.L. Duewer (Div. 839)

Forensic DNA testing typically involves two different types of samples: (1) single source reference samples from convicted offenders, suspected perpetrators or biological relatives of missing persons and (2) casework evidence that is often a mixture of victim and perpetrator and may be compromised in terms of quality and quantity of material. While casework samples often are challenging to analyze, the sheer number of the relatively easily analyzed reference samples is itself a bottleneck that requires development and validation of high-throughput data review procedures. As of September 2006, the National DNA Index System maintained by the FBI Laboratory contained over 3.6 million convicted offender profiles but only 150,000 forensic casework profiles. Thus, 96% of samples on the national DNA database are single source reference samples.



integrity
interpretation
innovation

In January 2006, the NIST Human Identity Project team purchased the Forensic Science Service FSS-i3 Expert System Software from Promega Corporation (Madison, WI). Short tandem repeat (STR) typing data for over 1,000

samples have been evaluated thus far with the FSS-i3 software and compared with manually evaluated results. Several Excel-based software tools have been developed to aid conversion of data formats and comparison of manually produced and expert system derived allele calls. These tools are available at:

<http://www.cstl.nist.gov/biotech/strbase/software.htm>. Our experience with the concordance studies has been the subject of several presentations and likely future publications. Future studies will involve examining the ability of expert systems to decipher DNA mixtures similar to what would be encountered in forensic casework.

NIST scientists also play an advisory role on the National Institute of Justice (NIJ) Expert System Testbed (NEST) project and have aided review of data obtained from forensic DNA laboratories with various expert system software programs.

Presentations:

Becky Hill presentation at the Expert Systems Workshop held in conjunction with the 2nd Annual Present and Future Technological Advances in Human Identification Conference (Roanoke, VA), March 27, 2006, "NIST Experience with FSS-i3 software" [[.pdf](#)]

Amy Decker presentation for Promega Technology Tour (Phoenix, AZ), June 22, 2006, "NIST Experience with FSS-i3 Software" [[.pdf](#)]

Becky Hill presentation at 17th International Symposium on Human Identification (Nashville, TN), October 11, 2006, "NIST Experience Using v4.1.3 of FSS-i3 Software" [[.pdf](#)]

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Supplying Training and Educational Materials to the Forensic DNA Community

Useful and accessible training materials benefit the application of technology utilized in the forensic DNA field. Training workshops are being conducted and education materials released to aid understanding of fundamental principles involved in human identity testing.

J.M. Butler, M.D. Coble, P.M. Vallone (Div. 831)

Members of the NIST Human Identity Project Team within the CSTL Biochemical Science Division are helping to train the scientific and legal communities involved in forensic DNA technology. In 2006, ten different training workshops were conducted as part of forensic conferences or as specific training to individual forensic DNA laboratories around the country. Hundreds of PowerPoint slides were also made available on the NIST STRBase website so that these valuable educational materials may be used by others.

In late February 2006 at the American Academy of Forensic Sciences (AAFS) meeting in Seattle, Washington, Dr. Butler and Dr. Bruce McCord, a colleague from Florida International University, conducted an all day workshop on advanced topics in forensic DNA analysis and provided instruction to 200 people representing federal, state, and local crime laboratories from 30 different states, industry leaders, college professors, expert witnesses, and defense lawyers. The training materials presented are available on the NIST STRBase website: <http://www.cstl.nist.gov/biotech/strbase/training.htm>. Dr. Butler's book, *Forensic DNA Typing: Biology, Technology, and Genetics of STR Markers*, is now in its second edition and used worldwide by forensic scientists, lawyers, and college students studying this dynamic field.

Also at the 2006 AAFS meeting, the Department of Justice released a new CD-ROM entitled "Principles of Forensic DNA for Officers of the Court" funded under the President's DNA Initiative. Dr. Butler developed some of the content of this training tool, which is also available on-line at <http://www.dna.gov>. In addition, Dr. Butler serves on the advisory group for the DNA Forensics Program of the American Prosecutors Research Institute (APRI) and is working to develop a standard curriculum for training prosecutors nationwide. In the advisory group's first meeting held at the end of January 2006, several crime scenarios with hypothetical biological evidence were designed. In the coming months, NIST will be generating data for these cases, which will be used in future training courses taught by APRI.



In 2006, Dr. Coble and Dr. Vallone helped teach workshops on mitochondrial DNA and quantitative PCR techniques used for DNA quantitation. Slides from these workshops are available at <http://www.cstl.nist.gov/biotech/strbase/YmtDNAworkshop.htm> and <http://www.cstl.nist.gov/biotech/strbase/qPCRworkshop.htm>.

Dr. Butler provided specific laboratory training to the New York City Office of Chief Medical Examiner Forensic Biology Section (New York City, NY), the Minnesota Bureau of Criminal Apprehension DNA Section (St. Paul, MN), and the New Jersey State Police DNA Laboratory (Hamilton, NJ).

Through funding from the National Institute of Justice to the NIST Office of Law Enforcement Standards, the NIST Human Identity Project Team develop new technologies, conduct interlaboratory studies to define measurement needs, and produce standard reference materials to aid calibration of forensic DNA laboratories.

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Development of 26 miniSTR Loci to Aid Human Identity Testing

Genetic tests that target smaller sections of DNA are more successful at recovering information from highly degraded biological specimens. The value of miniature short tandem repeat (miniSTR) assays were demonstrated during efforts to identify the 9/11 World Trade Center victims. Additional loci are being developed at NIST to help expand the capabilities of miniSTR testing.

C.R. Hill, M.C. Kline, M.D. Coble, J.M. Butler (Div. 831)

A number of studies have demonstrated that successful analysis of degraded DNA specimens from mass disasters or forensic evidence improves with smaller sized polymerase chain reaction (PCR) products. If DNA is exposed to the elements or to fire for any length of time, degradation can occur due to bacterial, biochemical or oxidative processes. Within the forensic community, a core set of short tandem repeat (STR) markers have been developed for utilization in forensic casework, and large DNA databases such as the Combined DNA Index System (CODIS) have been developed incorporating these markers.

An initial effort to reduce the STR amplicon size for CODIS loci resulted in a set of miniplexes to analyze degraded DNA. However, new autosomal STR loci are being examined because many of the CODIS core loci contain repeat flanking regions that are not amenable for redesigned primers (e.g. D7S820) or have large allele ranges (e.g., D21S11 and FGA) that make it impossible to create small PCR products. We have therefore scanned the literature for new STR loci and the 26 additional loci were chosen based on their small sizes (less than 150 bp), variability in a set of more than 600 U.S. population samples, and location on certain chromosomes. The candidate loci are all either located on chromosomes that differ from the 13 CODIS core loci or are at least ~ 50 Mb apart from an existing CODIS loci on the same chromosome, and therefore unlinked from that particular marker (see figure for their exact locations in relation to the CODIS loci). In addition, by moving PCR primers closer to the STR region, we have established that it is possible to decrease the chance of allele or locus-dropout that may occur in degraded samples. In fact, the value of these new loci have been confirmed in comparing the success of the miniSTR assays for typing degraded bone samples and aged blood and saliva stains while partial profiles were observed with the majority of the samples using a commercial STR kit.

A portion of the NIST STRBase website is being devoted to information on these new miniSTR loci: <http://www.cstl.nist.gov/biotech/strbase/newSTRs.htm>. Some of our first miniSTR loci characterized--D2S441, D10S1248, and D22S1045--have been selected by the European community as recommended STRs for adding to their core genetic systems used for human identity testing. A major commercial manufacturer, Applied Biosystems, is currently developing a new miniSTR kit based in large measure on our pioneering work here at NIST.

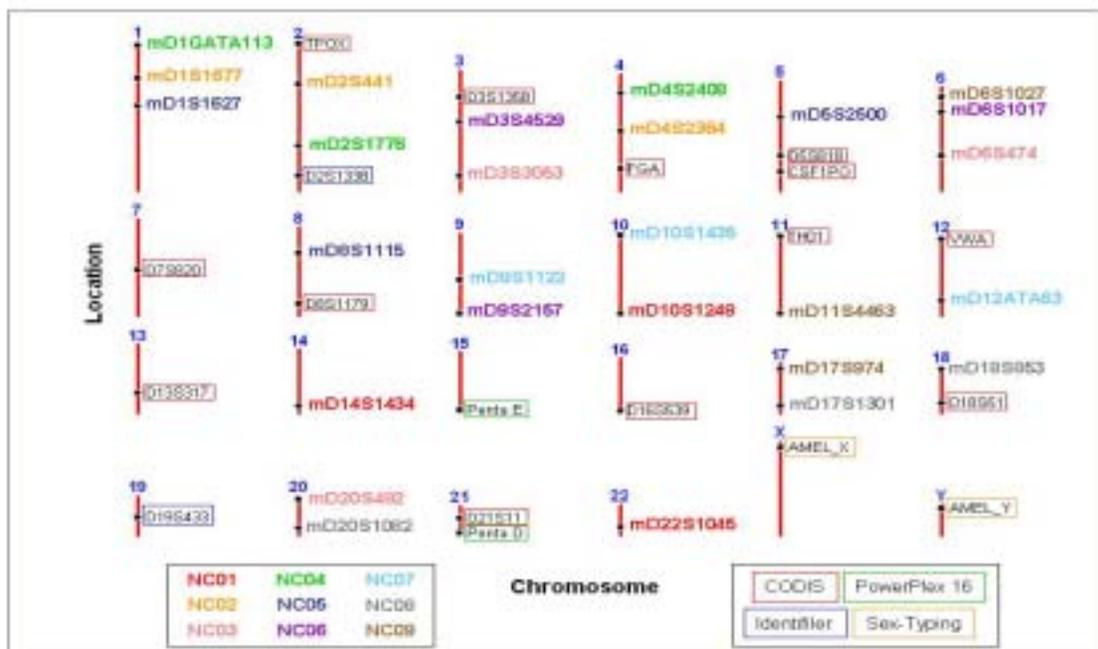


Figure: a schematic illustrating the chromosomal locations of the miniSTR markers in relation to the previously established CODIS markers

Publications:

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Coble, M.D. and Butler, J.M. (2005) Characterization of new miniSTR loci to aid analysis of degraded DNA. *J. Forensic Sci.* 50: 43-53.

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Hill, C.R., Kline, M.C., Coble, M.D., Butler, J.M. (2006) Development of additional miniSTR loci for improved analysis of degraded DNA samples. *submitted*.

Hill, C.R., Butler, J.M., Coble, M.D. (2006) Allele frequencies for 26 miniSTR loci with U.S. Caucasian, African American, and Hispanic populations. *J. Forensic Sci.* *in press*.

Butler, J.M., Coble, M.D., Vallone, P.M. (2006) STRs vs SNPs: thoughts on the future of forensic DNA testing. *submitted*.

Disclaimer: This project was supported by National Institute of Justice Grant Number 2003-IJ-R-029, which is an interagency agreement between [NIJ](#) and the [NIST Office of Law Enforcement Standards](#), awarded by the National Institute of Justice, Office of Justice Programs, US Department of Justice. Points of view in this document are those of the authors and do not necessarily represent the official position or policies of the US Department of Justice. Certain commercial equipment, instruments and materials are identified in order to specify experimental procedures as completely as possible. In no case does such identification imply a recommendation or endorsement by the [National Institute of Standards and Technology](#) nor does it imply that any of the materials, instruments or equipment identified are necessarily the best available for the purpose.

Title: Validated Raman Spectral Libraries

Authors: Steven Choquette Division 831 and Bruce Benner (839)

Program Area: Forensics and Homeland Security (HLS)

Context: ACD is working on the development of instrument corrected Raman spectral libraries for homeland defense applications. Raman spectroscopy is an attractive analytical technique for the identification of unknown chemical compounds because it requires no sample preparation, can be used through common transparent sample containers, and with current technological innovations, is very portable. However because Raman is an emission process, each instrument has a unique response function. As a result, Raman libraries of chemical compounds are currently vendor-specific and not transportable between systems. Since FY 2000 ACD has been involved with the development of instrument performance standards (SRM's) to correct for instrument response, and recently has been funded by DHS to develop an instrument corrected Raman spectral database. Because the Raman spectrum of a neat compound is an intrinsic property of the material, this primary data activity should lead to a standardized, universally useable, Raman database. This years' activities have involved the initial development of a validated spectral library of Toxic Industrial Compounds (TICs) and the writing of an ASTM practice for determining the resolution of a Raman spectrometer.

Approach: Nineteen compounds were chosen from a list of over 350 compiled by the US EPA. TICs are industrially important compounds that are routinely manufactured and transported in every major industrial country. Typically they are less toxic than military chemical warfare agents, but are highly reactive and much easier to obtain. The initial list comprised families of isocyanates, formates, and compounds with unsaturated double bonds. Each was assessed for purity using GC-FID and the identity confirmed with GC-MS. Several compounds were too thermally unstable for GC analysis and an NMR technique is under development to assess the purity of these materials. In addition, several reaction products, in excess of 5% were discovered during the course of the purity assessment, either as impurities or reaction products with air or the acidic protons of the solvents used in the GC analysis. These reaction products will also be included in the library as they would be likely present when the material is used in an industrial setting. Instrument corrected Raman spectra of each of the compounds were acquired with 785 nm and 1064 nm laser excitation for the compounds in their original containers (amber bottles) and also in quartz sample cells. The amber bottle typically used for reagent grade materials effects the shape of the spectra and should be included in the library.

To support our FY 2005 work on algorithms, a practice guide for determining the resolution of a Raman spectrometer was written and presented for ballot to the ASTM E 13.08 subcommittee for Raman spectroscopy. The practice guide will enable the end user of a field portable system to assess the resolution of their instrument using either pen lamps or the calcite spectrum. This information will be used in conjunction with NIST standards (SRM 2241) to calculate instrument corrections for spectra acquired using these field portable systems. This practice will facilitate searching end user generated spectra on a NIST database.

Future plans: We plan to develop a web-based version of the library (NIST CHEMWEB book) that will enable users to compare their spectra with NIST's. In addition FY 2006 we will work on writing a standard practice guide for the use of our intensity standards for the acquisition of instrument corrected Raman spectra.

Technical Highlight

“The Impact of *Bacillus anthracis* Spore Germination on Decontamination of Water Systems”

J.B. Morrow, J.L. Almeida, Lisa Fitzgerald, and K.D. Cole, Biospectroscopy Group, Biochemical Science Division

Objectives

The objective of this work was to elucidate the fate of *Bacillus anthracis* spores in the complex environment of a treated water system biofilms and evaluate commonly used disinfectants for decontamination in the event of a bioterror attack on a public water system. Biofilm communities predominate at water/surface interfaces common to nearly all ecosystems including drinking water systems and are known to harbor potential pathogenic bacteria.

Problem

Spores of *Bacillus anthracis* have been used as biological weapons and the continued threat of their use requires constant surveillance and research to protect human health. An act of terrorism to our public water systems can result in damage to human health and our nation's infrastructure causing severe economic cost.

Approach

We have been developing laboratory models for the growth of biofilms on plumbing materials and pipes for the past two years. PVC and copper pipe materials were used in either a continuously stirred tank reactor (CDC reactor) for controlled shear or a pipe loop system. Native water system biofilms were accumulated on pipe material surfaces with synthetic water containing humic acids as a carbon source. Once the biofilms were established, *Bacillus anthracis* Sterne or *Bacillus thuringiensis* (kurstaki and ATCC 33679, used as simulants) spores were added to the water system. Pipe surfaces were studied for biofilm accumulation and spore adhesion. Commonly used disinfectants, sodium hypochlorite and monochloramine disinfection of spores in solution and adhered to and biofilm organisms on the pipe materials were quantified using plate count methods.

Results

Bacillus spores are normally highly resistant to disinfection. We have found that spores readily adhere to the biofilm surfaces on plumbing materials at high levels, and when adhered they are more difficult to disinfect with chlorine and monochloramine than in free solution. Spore retention was proportional to the biofilm accumulation ($R^2 = 0.955$). The high concentrations and long contact times required for disinfection with chlorine and monochloramine make their use impractical and even create an additional environmental hazard through the release of high concentrations of toxic disinfectants and disinfection byproducts. An alternative approach we are developing is to trigger germination of the spores, a process known to change the permeability of the spores, with millimolar concentrations of an amino acid, L-alanine and a nucleoside, inosine. Germination of biofilm associated spores resulted in the release of attached spores from the pipe surfaces subsequently significantly increasing the sensitivity of both free and attached spores to chlorine and monochloramine.

Global Impacts

Enhancing the disinfectant efficacy through germination provides a means to use greatly reduced concentrations of disinfectant and lower contact times to achieve complete decontamination of the spores from the pipe surfaces. The knowledge gained from this research will allow us to better determine the extent of a deliberate contamination event and to safely remediate a water system in the event of a bioterror attack.

Future Research

We are continuing this research to better understand the complex interactions between introduced biological threats and water system biofilms and develop measurements methods. We are extending our measurements to *Escherichia coli* O157:H7, *Francisella tularensis* (the bacteria that causes tularemia), and the toxin, ricin. Future work will focus on elucidating the long-term fate and possible persistence of biological threats in water systems using Confocal Laser Scanning Microscopy (CLSM) in addition to the methods described here.

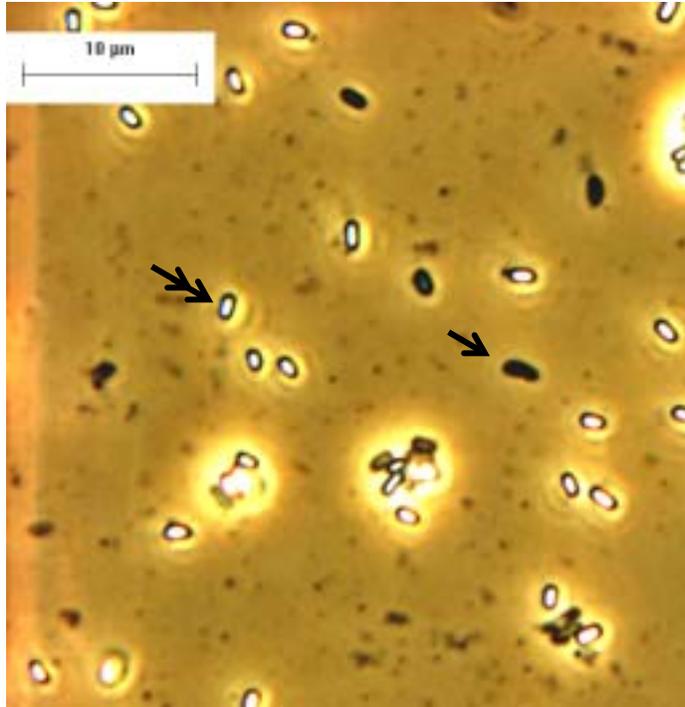


Figure 1. Phase microscopy image of *Bacillus thuringiensis* spores. Germinated (or permeable) spores appear as black rods (single arrow), ungerminated spores are phase bright (double arrow). Image captured with an Olympus BX50 Microscope, 100X magnification.

Technical Highlight

“Efficient Decontamination of *Bacillus anthracis* Spores in Water Systems”

J.B. Morrow, J.L. Almeida, L.A. Fitzgerald, and K.D. Cole, Biospectroscopy Group,
Biochemical Science Division

Objectives

The objectives of this work are to gain a better understanding of the adhesion, fate, and disinfection of *Bacillus anthracis* spores when introduced to the complex environment of a drinking water system. This knowledge will allow us to better determine the extent of a deliberate contamination event and to remediate the water system.

Problem

Spores of *Bacillus anthracis* have been used as biological weapons and the continued threat of their use requires constant surveillance and research to protect human health. An act of terrorism to our public water systems can result in damage to human health and our nation’s infrastructure causing severe economic cost.

Approach

We have been developing laboratory models for the growth of biofilms on plumbing materials and pipes for the past two years. *Bacillus anthracis* and simulant spore preparations are applied to these systems and adhesion and disinfection properties have been determined. We have initially focused on the main disinfectants used in water systems, including free chlorine (sodium hypochlorite) and monochloramine.

Results

Bacillus spores are normally highly resistant to disinfection. We have found that spores readily adhere to the biofilm surfaces on plumbing materials at high levels, and when adhered they are more difficult to disinfect with chlorine and monochloramine than in free solution. The high concentrations and long contact times required for disinfection with chlorine and monochloramine make their use impractical and even create an additional environmental hazard through high concentrations of toxic disinfectants and disinfection byproducts. An alternative approach we are developing is to trigger germination of the spores, a process known to change the permeability of the spores with millimolar concentrations of alanine and inosine. Germination of biofilm associated spores resulted in the release of attached spores from the pipe surfaces subsequently increasing the spore sensitivity to chlorine and monochloramine. Enhancing the disinfectant efficacy through germination provides a means to use greatly reduced concentrations of disinfectant and lower contact times to achieve complete decontamination of the spores from the pipe surfaces.

Future Research

We are continuing this research to better understand the complex interactions between introduced biological threats and water system biofilms and develop measurements methods. We are extending our measurements to *Escherchia coli* O157:H7, *Francisella tularensis* (the bacteria that causes tularemia), and the toxin, ricin. The long-term fate and possible persistence of

biological threats in water systems is important to determine and we are planning experiments to investigate this possibility.

A High-Accuracy Fluorescence Spectrometer for Measuring True Fluorescence Spectra

P.C. DeRose and G.W. Kramer

Vision: To qualify a research-grade fluorescence spectrometer for measuring true fluorescence spectra, enabling the certification of fluorescence Standard Reference Materials.

Purpose: Luminescence measurements have become the detection methods of choice for new clinical and biochemical assays, and related high-throughput techniques, due to their extraordinary selectivity and sensitivity. These new analytical methods are becoming increasingly more quantitative, requiring standards to calibrate the luminescence measuring instruments that they utilize and aid in the validation of the methods. Reported here is the first step in this process, which is to qualify a research-grade fluorescence spectrometer for measuring true fluorescence spectra of reference material candidates. “True” spectra are defined here as those with fluorescence intensity, relative or absolute as required, and wavelength both being reported with high accuracy and known precision, after wavelength has been calibrated and corrections for excitation intensity and detection system responsivity have been applied.

Scientific Research: Qualifying a fluorometer for measuring true spectra requires 1) excitation wavelength and bandwidth calibration, 2) emission wavelength and bandwidth calibration, 3) radiometric calibration of the excitation source as a function of wavelength (i.e., excitation correction) for excitation spectra and 4) radiometric calibration of the emission detection system as a function of wavelength (i.e., emission correction) for emission spectra. We have utilized conventional methods for calibrating intensity, using physical transfer standards (i.e., a calibrated light source, a calibrated detector and a calibrated diffuse reflector), and wavelength, using atomic lamps. Correction curves for fluorescence intensity as a function of wavelength were calculated.

Major Accomplishments: Our high-accuracy fluorescence spectrometer has been qualified to measure both relative and absolute intensity-corrected fluorescence spectra throughout the visible region and beyond (310 nm to 800 nm). Both calibrated source (CS)- and calibrated detector (CD)-based methods for spectral correction of fluorescence were developed and compared. The CS-based method gave uncertainties, typically about $\pm 5\%$ for relative spectral correction, that were about half that of the CD-based method for determining both relative and absolute spectral correction factors. We demonstrated that absolute spectral correction factors can be determined using either method without knowing the optical geometry of the instrument.

Context and Impact: Currently, fluorescence intensity is nearly always expressed on a relative scale, making the comparison of intensity between different fluorometers difficult or impossible. Fluorescence spectra are often not corrected for the detection system’s responsivity as a function of wavelength and in some cases are not even corrected for the intensity of the excitation source as a function of wavelength, resulting in a quantitatively incorrect and perhaps even qualitatively misleading spectral shape. This includes most of the fluorescence spectra found in the literature. The availability of

SRMs for relative spectral correction of emission and intensity validation will enable the shape and absolute intensity, respectively, of fluorescence spectra to be compared over time and between instruments with relative ease by users, even non-experts. SRMs 2940 and 2941 have already been certified for spectral correction and intensity validation using our high-accuracy instrument and others are soon to follow.

Future Plans: We are presently working to extend the wavelength region, where true fluorescence spectra can be measured, of our fluorescence spectrometer into the NIR region from 800 nm to 1000 nm, a region into which many new biological and clinical assays are beginning to extend. We are also adding accessories to our instrument to enable the routine measurement of absolute fluorescence quantum yields, an area where the need for standards is also increasing.

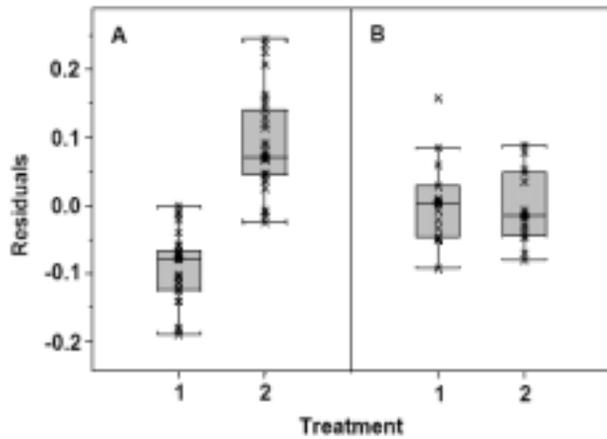
Repair of Oxidative DNA Damage is Deficient in Lymphoblasts of Women with *BRCA1* Mutations and Breast Cancer

H. Rodriguez (NCI, NIH), P. Jaruga (831), D. Leber, S. G. Nyaga (NIA, NIH), M. K. Evans (NIA, NIH), and Miral Dizdaroglu (831)

Program: Health and Medical Technologies

*Breast cancer is the second leading cause of cancer deaths among women. Inherited mutations that affect a single allele of the breast cancer 1 and 2 genes (*BRCA1* and *BRCA2*) predispose women to high risk of breast and ovarian cancers, although the magnitude of this risk is controversial. Hereditary breast cancers accounts for approximately 5-10% of all breast cancers among women, whereas other breast cancers are considered to be sporadic. On the other hand, 30–60% of familial breast cancers result from inherited mutations on *BRCA1* and *BRCA2*. *BRCA1* plays an important role in maintaining the genome integrity, at least in part, through its role in the repair of DNA damage. Thus, the *BRCA1* product, *BRCA1* is involved at multiple steps in the cellular response to DNA. Further evidence shows that *BRCA1* plays a role in the repair of other types of oxidative DNA damage as *BRCA1*-deficient cells exhibit chromosomal abnormalities and are hypersensitive to oxidative damage caused by DNA-damaging agents such as ionizing radiation and hydrogen peroxide. In this work, we investigated the cellular repair of several oxidatively induced DNA lesions in lymphoblasts of women with *BRCA1* mutations in comparison to those of women with no detectable *BRCA1* deficiency.*

Mutations in breast and ovarian cancer susceptibility genes *BRCA1* and *BRCA2* predispose women to high risk of these cancers. Here, we show that lymphoblasts of women with *BRCA1* mutations, who had been diagnosed with breast cancer, are deficient in the repair of some typical products of oxidative DNA damage, namely 8-hydroxy-2'-deoxyguanosine and 8,5'-cyclopurine-2'-deoxynucleosides. Cultured lymphoblasts from 10 individuals with *BRCA1* mutations and those from 5 control individuals were exposed to 5 Gy of ionizing radiation to induce oxidative DNA damage and then allowed to repair this damage. DNA samples isolated from these cells were analyzed by using measurement methods developed in our laboratory. We used liquid chromatography/mass spectrometry and gas chromatography/mass spectrometry to measure 8-hydroxy-2'-deoxyguanosine, (5'S)-8,5'-cyclo-2'-deoxyadenosine, (5'R)-8,5'-cyclo-2'-deoxyguanosine and (5'S)-8,5'-cyclo-2'-deoxyguanosine. After irradiation and a subsequent period of repair, no significant accumulation of these lesions was observed in the DNA from control cells. In contrast, cells with *BRCA1* mutations accumulated statistically significant levels of these lesions in their DNA, providing evidence of a deficiency in DNA repair. In addition, a commonly used breast tumor cell line exhibited the same effect when compared to a relevant control cell line. The data suggest that *BRCA1* gene product *BRCA1* plays a role in cellular repair of oxidatively induced DNA lesions. The failure of cells with *BRCA1* mutations to repair 8,5'-cyclopurine-2'-deoxynucleosides indicates the involvement of *BRCA1* in nucleotide excision repair of oxidative DNA damage. This work suggests that accumulation of these lesions may lead to a high rate of mutations and to deleterious changes in gene expression, increasing breast cancer risk and contributing to breast carcinogenesis. The measurement of oxidative DNA damage and its repair in human tissues will contribute to the understanding the role of *BRCA1* in risk assessment for breast cancer and may lead to development of therapeutics for prevention and treatment. We plan to further investigate the effect of *BRCA1* on the repair of oxidative damage to DNA in human tissues and measure other major lesions of DNA damage.



Levels of 8-hydroxy-2'-deoxyguanosine in DNA of lymphoblasts from women with BRCA1 mutations and from control women. A: Cells with BRCA1 mutations; B: Control cells, 1: Non-irradiated, 2: Irradiated at 5 Gy.

Publication:

Rodriguez, H., Jaruga, P., Nyaga, S. G., Evans, M. K. and Dizdaroglu, M., “Lymphoblasts of women with BRCA1 mutations are deficient in cellular repair of 8,5'-cyclopurine-2'-deoxynucleosides and 8-hydroxy-2'-deoxyguanosine,” *Biochemistry* (in press).

Quantitative measurements of cells adds insight into biological noise

JT Elliott, M. Halter, A.Plant

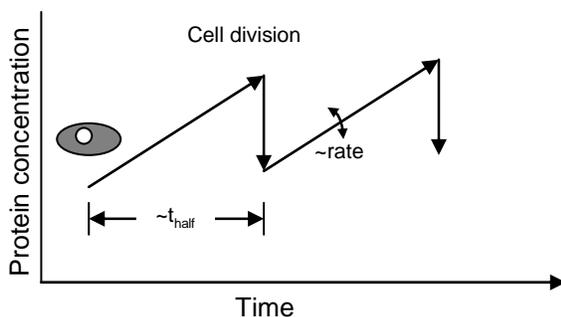
One of the major goals of systems biology is to understand and predict cellular responses to extracellular signals present in the cell's local environment. Many biochemical techniques, such as Western blots, measure an average result by combining many cells for analysis. When cell-by-cell data are taken, it can be seen that there is a range of responses within the population of cells; although genetically identical, each cell does not display the same phenotype. By careful control of experimental conditions, it can be confirmed that this distribution is highly reproducible and is not the result of experimental noise. Although it is not frequently recognized, the observed distributions in response are due to fluctuations, or noise, in the intracellular processes that are responsible for the cell's response. Without adequate models to describe the origin of this distribution, the observed data obtained in an experiment can be ambiguous.

Observing a population of genetically identical cells that are expressing constitutively produced green fluorescence protein shows that different cells exhibit different levels of fluorescence. Any one cell does not experience the entire range of fluorescence intensities within the population; furthermore the distribution is highly robust from experiment to experiment. We have developed a simple model to predict the source of this distribution of these responses, and found that it can be explained by the population response to growth and cell division.

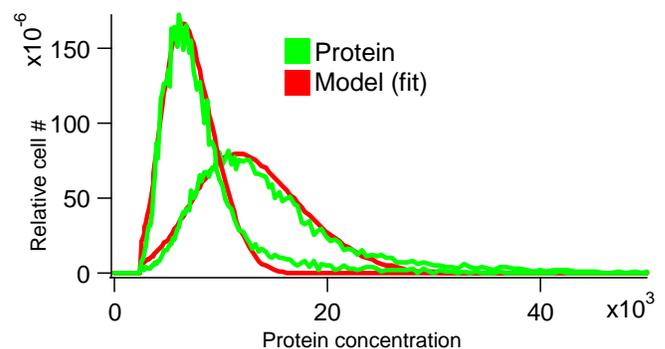
Data were collected on individual cells by flow cytometry. The model is based on a simple dynamical linear-growth and division process where the rate of growth and the division time for each cell cycle is randomly selected from a Gaussian distribution around a mean value, and allows us to predict, based on cell volumes, the average rate of cell growth, and the noise in that rate across the population, and the average time of cell division, and the noise in that time. We find that that the mean value of the observed distribution are a function of only the mean growth rate and mean division time for the cell population, and the width of the observed distribution is a function of the variations in the growth rate and division time. This variance provides insight into the biological mechanisms that are governing protein production and cell cycle time.

Our results indicate that any cell-by-cell measurement of a growing cell population will contain noise due to the cell division process (i.e. extrinsic noise) in addition to noise associated with the parameter being investigated. Our fitting procedure will facilitate extracting relevant parameters from bserved distributions and quantifying the biological noise that is inherent in cell signaling pathways.

a. Model for linear growth and division of cells



b. Observed and fitted cell-by-cell data



Nanoscale mechanical properties of collagen influence cellular response

Cells recognize their environment through specific receptor molecules, and these molecular interactions result in intracellular signaling pathways that influence cell response to other environmental entities such as pharmaceuticals. Despite this knowledge, most laboratory studies employ plastic tissue culture dishes instead of a matrix of extracellular matrix proteins because commercial and laboratory preparations of matrix proteins are notoriously irreproducible and poorly characterized. Thin films of matrix proteins such as collagen can provide robust, reproducible, and analytically tractable biomimetic cell growth matrices. These thin films can also provide new insight into the environmental parameters, such as mechanics, that direct cell response.

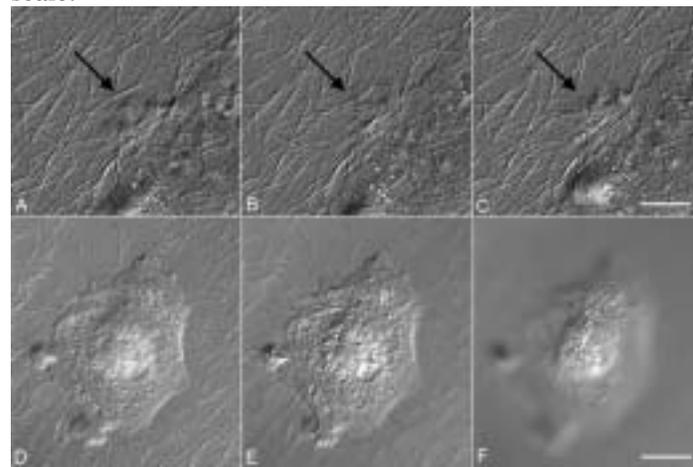
JT Elliott, K. Bhadriraju, A. Plant (Div. 831)

Cells are used in the testing of therapies and drugs, in the diagnosis of disease, and occasionally as therapies themselves. Cells are also critical research tools as surrogates for even more complex intact organisms. An important determinant of cell response is the extracellular matrix (ECM) proteins that cells adhere to. However, there is a great deal of variability in laboratory preparations of matrix proteins. As a result, comparison of data from different labs is problematic.

Using thin film technology to self assemble Type 1 collagen at alkanethiol-coated surfaces, we have demonstrated that thin films of the ECM protein collagen can be robust, analytically tractable, and mimic *in vivo* conditions. Thin films of collagen appear to be identical to thick gels of collagen with respect to a number of phenotypic parameters, and result in quantifiable phenotypic responses in cells that are highly reproducible.

Much is yet to be learned about what environmental cues elicit cellular responses. Not all of the parameters of the ECM that are important in determining cell response have identified. In addition to chemi-

cal recognition, we have found that cells are very sensitive to the mechanical nature of collagen fibrils. While it has been known that cells respond differently to bulk polymers with different compliance, we have shown for the first time that cells are very sensitive to mechanical properties of their matrix at the nanoscale.



Smooth muscle cells can be observed to move collagen fibrils in thin films of collagen (Panels A-C) as they extend and retract their cell processes. Cells manipulate the fibrils by pulling them up over the top of them, thus creating a 3-D environment for themselves; this is observed by examining cells at different focal planes (D-F).

We compared thin films of collagen that remained hydrated with thin films that were allowed to dry extensively, such as might occur during storage or shipping. Our results show that when collagen fibrils are kept fully hydrated, they remain flexible. Cells on flexible collagen fibrils are in a senescent, non-growth state, which is appropriate for most normal cells in the body. However, when collagen fibrils are allowed to dry, they become stiffer. Cells appear not to be able to move dried fibrils, and cells on dried fibrils are observed to spread more, change their gene expression and cytoskeleton arrangement, and grow and divide approximately 4 times as rapidly. This observation has potential significance in physiological processes such as wound healing and tumor growth.

D.P. McDaniel, G.A. Shaw, J.T. Elliott, K. Bhadriraju, C. Meuse, K. Chung, A. L. Plant, The stiffness of collagen fibrils influences vascular smooth muscle cell phenotype 2006 Biophys J. In Press

Cellular Biometrology

Cell-based assays are utilized extensively in the biotechnology and pharmaceutical industries during multiple phases of product and drug development. Despite the widespread use of these assays, their predictive capabilities are lacking, owing in part to the poor environmental control provided by conventional cell culture strategies, and few strategies for generating quantitative, correlated measurement data. The NIST project in Cellular Biometrology is addressing these shortcomings using integrated microfluidic systems in two ways: 1) by engineering reproducible microfluidic systems that will provide control and evaluation of the cellular microenvironment, and 2) by integrating in a single microfluidic system multiple quantitative cell-based analysis capabilities.

S. P. Forry and A. Plant (Div. 831)

Establishing robust and predictive metrics for cellular outcomes and behaviors in artificial cell culture environments has become an enormous hurdle to studying biological systems. Existing measurement tools for evaluating cellular systems are severely limited: in addition to offering poor environmental control, analyses in these systems typically produce qualitative and descriptive data, and different measurement modalities are not correlated. The appetite for quantitative and correlated measurements under well-controlled conditions is enormous, but measurement tools are lacking.

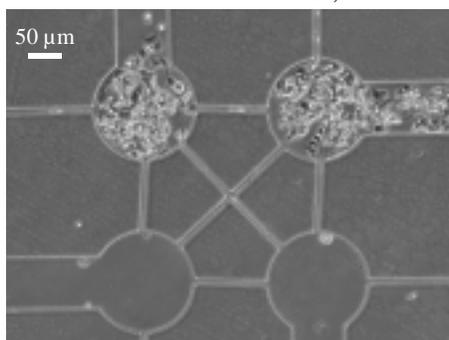
Cell behavior is powerfully modulated by local extracellular cues. However, the number of parameters that effect cell behavior is large, and include soluble signaling molecules, dissolved gases, the chemistry and mechanics of the insoluble extracellular matrix proteins, and the actions of neighboring cells. Additionally, cells respond to spatial and temporal variation in these environmental cues.

Micro- and nano-fabricated microfluidic systems can provide a level of control over the cell culture microenvironment that cannot be achieved in traditional culture condition such as plastic plates. Microfluidics systems can reproducibly produce confined and well-defined systems on the cellular length scale ($\sim 5\mu\text{m}$ - $500\mu\text{m}$) and can incorporate complex designed topographies, densities of extracellular matrix signaling molecules, nonrandom organization of cells of different type, and ability to mimic *in vivo* solution flow. Microfluidic cultures can be designed to be small and massively parallel for application in high throughput drug or toxin screening on small, defined cell populations.

Flow within the microfluidic system will be used to precisely stimulate and interrogate cells with high spatial and temporal resolution. Control of the cellular environment vis a vis extracellular matrix proteins, hormones, cytokines and other cell stimuli can in principle be achieved on a scale of a few microns. Recent technological advances in polymer microfluidic systems provide a definable, precise platform for configuring engineered biomimetic microenvironments.

Additionally, microfluidic systems will enable integration of cell culture with automated analysis on the same device. The microfluidics device will provide access to optical imaging, electrochemical inter-

rogation, and controlled lysis of desired cells and collection of cell contents for downstream analysis is envisioned. There has been significant development in the field of analytical capabilities within microfluidic devices. These enable integration in a single device of sensitive cell-based assays with a well-controlled microenvironment for continuous monitoring of the cell culture. Alternately, the microfluidic systems can provide sample prep for off-device analysis via amplification or preconcentration.



Prototype microfluidic device enables controlled introduction of cells and delivery of soluble bioactive molecules.

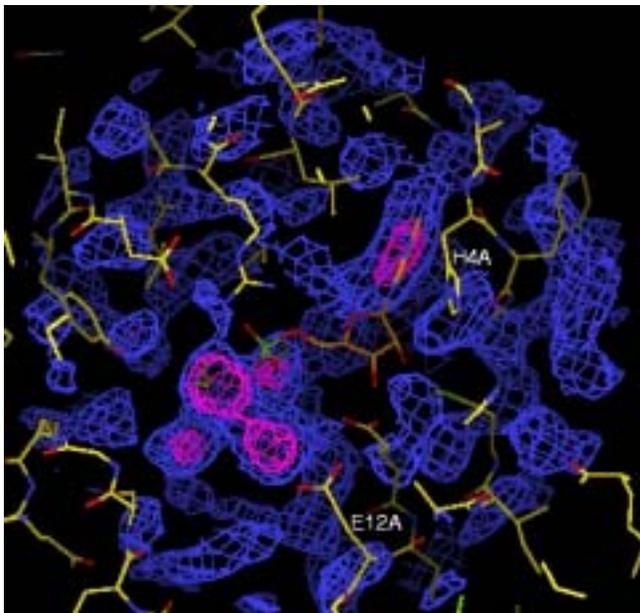
Adenylyl Cyclase Structure and Mechanism Measurements

T. Gallagher, N. Smith, S.-K. Kim, P. Reddy (831)

Pharmaceuticals and Biomanufacturing

Introduction: The enzyme adenylyl cyclase (AC) performs key signaling functions in humans and also in pathogenic bacteria such as those that cause anthrax and tuberculosis. The plague-causing pathogen *Yersinia pestis* contains an AC belonging to a newly characterized class, class IV. Of the six distinct classes of AC enzymes that have been found (distinct protein families that perform the same enzymatic reaction), three have been structurally characterized. The form present in both anthrax and whooping cough bacteria (*Bacillus anthracis* and *Bordetella pertussis*), which belongs to class II, the human form, belonging to class III, and the newly characterized form in *Yersinia pestis*, belonging to class IV, all show distinct protein folds. All are active scientific research foci; the class IV enzymes are of special interest because of the pathogenicity of *Y. pestis* and other organisms that contain them, and because of their small size (smallest known AC subunits; in *Y. pestis* the chain length is 179 amino acids) and thermostability (this AC class is also found in hyperstable archaeal organisms).

Major Accomplishments: We have expressed, crystallized and structurally characterized the class IV AC from *Y. pestis* at 1.9 Å resolution (PDB:2FJT and J. Mol. Biol. 362, 114-122, 2006). The general region of the active site was identified based on the structure and on conserved residues in multiple alignments. Recently we have obtained additional diffraction data at high resolution from crystals containing the substrate analog dideoxy-ATP, enabling modeling of the substrate complex and detailed analysis of the catalytic mechanism. Figure 1 shows a preliminary view of the electron density map for this substrate analog in the active site.



The figure shows the active site region, with initial electron density for the substrate

analog ddATP. The cluster of pink balls reports the locations of the three phosphate moieties in ddATP and a manganese ion that stimulates enzyme activity, while the additional pink blob on the upper right represents the adenine ring.

Impact: Even before complete refinement of this active-site complex structure, it reveals several features of the mechanism. For example, the residue E12A is observed as the key anchor for the manganese ion, and the adenine moiety binds near the N-terminus (near histidine H4A in Figure 1). This work will lead directly to further publications of widespread interest, especially regarding protein structure analysis, pathogen biology, enzyme mechanism, and crystallogensis.

Future Plans: After completing the refinement at 1.8 Å (the resolution of our data), the structural analysis will be combined with our earlier measurements of activity under various conditions and in several key mutants, enabling a thorough treatment of the catalytic mechanism in this novel and important enzyme.

A Multistate Model for the Fluorescence Response of R-Phycoerythrin

A. Gaigalas, T. Gallagher, K.D. Cole, T. Singh, Lili Wang, and Yu-Zhong Zhang

Although strong fluorescence makes the R-phycoerythrin (R-PE) proteins increasingly useful in biological and clinical assays, they are subject to nonlinear effects including transitions to collective dark states and photodegradation that complicate quantitative applications. This work reports measurements of R-PE fluorescence intensity as a function of incident power, duration of illumination, and temperature. Emission intensity in the band at 570 nm is proportional to incident power for low power levels. At higher incident power, the emission at 570 nm is smaller than expected from a linear dependence on power. It is proposed that R-PE undergoes both reversible emission cessation on a millisecond time scale, attributed to transitions to a collective dark state, and irreversible photodegradation on a timescale of minutes. Singlet oxygen scavengers such as dithiothreitol and n-propylgallate have protective effects against the latter effect but not the former. Electrophoretic analysis of irradiated solutions of R-PE indicates that significant noncovalent aggregation is correlated with photodegradation.

A multistate model, based on fluorescence measurements and geometric analysis, is proposed for the fluorophores in R-PE. The phycobilin fluorophores are partitioned into three groups: the phycourobilins (PUB) absorbing at 490 nm, one group of phycoerythrobilins (PEB) absorbing at 530 nm (PEB-530), and another group of PEB absorbing at 560 nm (PEB-560). The partitioning is based on geometric analysis of the fluorophores, calculated coupling interactions, and fluorescence measurements under various conditions. Two PUB fluorophores transfer energy to the PEB-530 group which transfers energy to the PEB-560 group. The electronically excited states of PEB-530 and PEB-560 can be described as exciton states since coupling exists between all of the fluorophores within each group.

The fluorescence emission from R-PE is due to radiative decay of the lowest excited state of the PEB-560 manifold. In addition to the radiative decay, these states undergo intersystem crossing to the triplet state, and transitions to a dark state. A relaxation pathway of the triplet state results in the production of singlet oxygen and consequent damage to the protein. The most likely place where damage occurs is where the PEB 560 groups are located. The reduction of fluorescence intensity due to prolonged irradiation was attributed to photodegradation. Frequency domain measurements show fluorescence intensity decrease with a time scale of about 6 ms that is attributed to the population of a collective dark state and subsequent non radiative relaxation to the ground state.

Both sources of fluorescence intensity reduction introduce problems in the quantitation of fluorescence intensity in biological assays. The fast variation is of importance in flow cytometers and scanning instruments. The slow variation (photodegradation) is more relevant to imaging with long exposures under constant illumination, such as microscope imaging applications.

Purity Analysis of SRM Cholesterol, Estradiol, Progesterone, and Testosterone Steroids by Proton NMR.

D.K. Hancock and G.W. Kramer

Final installation of our 600 MHz NMR instrument was completed in the fall of 2005. Almost immediately the usefulness of our new NMR capabilities was tested by requests to aid in the purity analyses of several SRM steroids.

Cholesterol. NMR analyses were performed to compare the SRM 911c and 911b cholesterols and to verify the presence of, and identify, a 911c impurity with a mass of 384 (=cholesterol mass-2) that had been observed by LC MS analysis of the 911c material. Based on the LC-MS results, the major impurity in the 911c material was thought to be 5,7-cholestadiene-3 β -ol, but NMR easily ruled this out. The NMR spectra however suggested the presence of 5,24-cholestadiene-3 β -ol, and its presence was confirmed by spectral comparison with a commercially available sample. Identification of this impurity allowed accurate quantification of the impurity by LC techniques. In addition to the major impurity three minor impurities were also present in 911c. Two of these minor impurities were tentatively identified as 5,25-cholestadiene-3 β -ol and cholest-7-en-3 β -ol, but standards were not available for confirmation. Although the NMR experiments in these analyses were performed to provide screening results, rather than optimal quantification, the quantitative results: 0.57 mole % 5,24-cholestadiene-3 β -ol and 0.08 mole % 5,25-cholestadiene-3 β -ol were in agreement with LC results. The other two minor impurities were present at 0.06 mole % and 0.02 mole %.

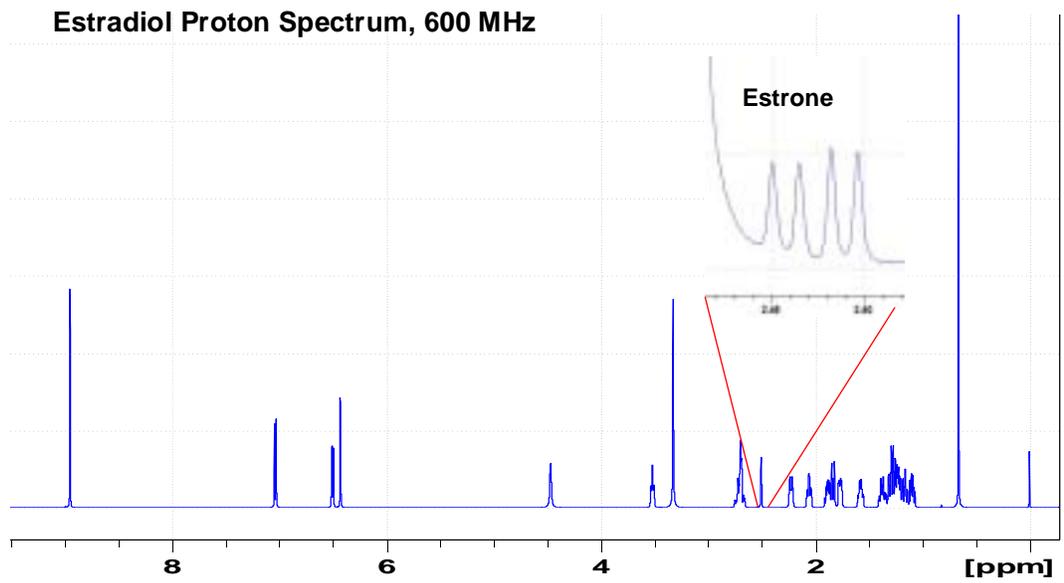
Estradiol, progesterone, and testosterone samples, selected as primary standards for the certification of SRM 971 - Steroids in Human Serum, were each quantitatively analyzed by NMR.

Estradiol. NMR spectra showed one major impurity in the estradiol sample. The impurity, present at 0.38 mole %, was initially tentatively identified as estrone. NMR analysis of an estrone sample confirmed the estrone identification. The NMR spectrum of estradiol is shown below. The inset is an expansion of the spectrum showing the doublet of doublets from the H-18 estrone impurity. In addition to the major impurity two minor impurities were present at 0.025 mole % and 0.0067 mole %. These minor impurities were not identified. Due to the severe spectral overlap of closely related structures very limited spectral data for the minor components are often present, making identification difficult, if not impossible.

Progesterone. One major impurity was present in the NMR spectra of the progesterone sample. This impurity, present at 0.86 mole %, is tentatively identified as 4-pregnen-3-ol-20-one. A commercial sample is backordered so the identity has yet to be confirmed. The NMR spectra suggest the presence of several other minor impurities, though the resolved data is very limited so the data is not definitive. Quantification of these impurities range from 0.3 mole % to 0.05 mole %.

Testosterone. The major impurity in testosterone was identified and confirmed by NMR spectroscopy as epitestosterone (17 α -hydroxyandrost-4-en-3-one). The epitestosterone is present at approximately 0.29 mole %. NMR spectra show the presence of two additional unidentified minor impurities at 0.05 mole % and 0.04 mole %.

Estradiol Proton Spectrum, 600 MHz



Title: Quantitation of Genomic DNA from Plants**Authors: M.J. Holden and R. Haynes (831), S. Rabb and M.R. Winchester (839)**

Commodity grains, developed using techniques of modern biotechnology, are prominent in the current U.S. agricultural sector. Export of raw and processed foods, especially those containing corn and soy, often need to be tested for compliance with regulations of importing countries. Accurate testing for identification significantly impacts U.S. trade in agricultural products. While international harmonization of sampling and testing procedures is as yet unrealized, improvements in the analysis of commodity grains is a goal that can be moved forward.

DNA measurements are the methods of choice for identification and analysis of the biotech crop components. These methods, particularly quantitative real-time PCR (Q-PCR), are highly sensitive, and it is now possible to achieve the regulated requirements for limits of quantitation which are often quite low (<1% w/w). While Q-PCR is a powerful technique, it is imperative that an appropriate amount of template DNA is added to the assay so that trace detection is possible. It is also important not to overload the template in the reaction as impurities co-isolated with the DNA often interfere with the amplification reaction.

Therefore, quantitation of total genomic plant DNA is important, prior to conducting quantitative PCR studies to determine that presence and the amount of transgenic material. However, quantitation of genomic DNA isolated from plants is also problematic as there is often a discrepancy between data obtained using common spectroscopic methods for detection. Therefore, we have been exploring other methods for quantitation of total DNA in order to certify genomic DNA reference materials and investigate spectroscopic inconsistencies. This year, we have investigated the use of High Performance Inductively Coupled Plasma – Optical Emission Spectroscopy (HP-ICP-OES) for the measurement of phosphorus content of acid-digested DNA. It was determined that measurement of DNA can be achieved with excellent accuracy and very high reproducibility using this method. (Traceable Phosphorus Measurements by ICP-OES and HPLC for the Quantitation of DNA. MJ Holden, SA Rabb, YB Tewari, MR Winchester; *Anal. Chem.* In press 2006). This protocol is now being applied to the analysis of maize and soy genomic DNA isolated from seeds. The methodology requires that impurities be removed from the DNA prior to analysis as biological systems contain many phosphorus compounds, some of which might co-purify with DNA. Prior to HP-ICP-OES analysis, DNA is cleaned using a series of enzymatic treatments and extraction steps to remove residual proteins and carbohydrates, and then washed to remove small molecules. It is important to note that cleaning the DNA can damage the integrity of the molecule leading to small fragments or even single stranded DNA which can have an effect on the spectroscopic measurements. Therefore, it is necessary to analyze the integrity of the DNA following these steps.

With the development of these procedures, we have produced a validated independent measure of the total DNA content making it possible to certify pure genomic DNA

reference materials. We are currently investigating the accuracy of commonly used spectroscopic methodologies for quantitating plant genomic and other DNAs.

Mitochondrial DNA Mutations as Biomarkers of Early Cancer Detection

John P. Jakupciak, 831

Samantha Maragh, 831

Mutations in the mitochondrial DNA (mtDNA) have been detected in colorectal, breast, cervical, ovarian, prostate, liver, pancreatic, and lung cancers. Mitochondrial dysfunction is causally related to neoplastic transformation via mutations that retard electron flow resulting in increased ROS production. In addition to cancer, the biological effects of mtDNA instability have been reported in degenerative diseases, neurodegenerative diseases, macular degeneration, aging and longevity and cardiovascular disease.

The utility of mtDNA mutations as biomarkers for cancer detection in tumors and non-invasively collected bodily fluids has remained poorly validated. Further, recent publications estimate that more than half of mtDNA sequence publications contain errors. A few studies using bodily fluids have been conducted, but have subsequently been shown to contain errors based on the comparison of reported sequences with databases that make up global mtDNA phylogeny. This study is therefore timely, and demonstrates that mtDNA can serve as a sensitive biomarker for cancer.

This work was supported in part by the Early Detection Research Network (EDRN) of the National Cancer Institute and in close collaboration with NCI's Biomarkers Research Group. To identify mtDNA mutations in a systematic manner, the mitochondrial genome was sequenced using the MitoChip microarray, which is faster, less expensive and more sensitive than the CE DNA sequencing reference method.

Future

These findings indicate comprehensive mtDNA sequencing can be a high-throughput tool for detecting mutations in clinical samples and has direct application for cancer detection. This entire mitochondrial DNA analysis provides a genome wide view of the cancer associated mutations in bodily fluids. Detection and monitoring of the tumor and its field via mtDNA mutation analysis could be a practical way to assess remote sites. Our results reinforce the ideas that mtDNA mutations occur frequently in the coding region and pending the development of a mitochondrial expression array alter (genetically) important OXPHOS genes. We envision conducting epidemiological studies of the mitochondrial genome to compare genotype/phenotype associations in order to understand the pathogenic basis of neoplastic and non-neoplastic diseases linked to mitochondrial dysfunction.

Publications

Jakupciak, J.P., Dakubo, G.D., **Maragh, S.**, Parr, R.L. Mitochondrial DNA as a Cancer Biomarker. *Current Opinions on Molecular Therapeutics*, in press (2007).

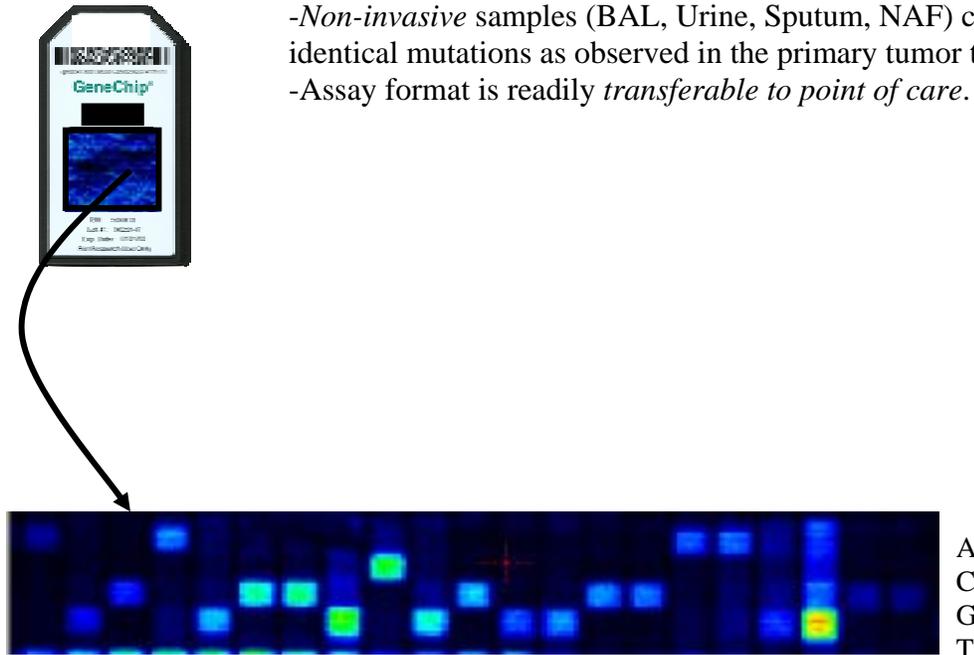
O'Connell, C.D., Atha, D.H., **Jakupciak, J.P.** Standards for validation of cancer biomarkers. *Cancer Biomarkers*, 1 (4-5), 233-239(2006)

Parr, R.L., Maki, J., Reguly, B., Dakubo, G.D., Aguirre, A., Wittcock, R., Robinson, K., **Jakupciak, J.P.**, Robert E.Thayer, The pseudo-mitochondrial genome influences mistakes in heteroplasmy interpretation. BMC Genomics. 21;7:185 (2006)

Jakupciak, J.P., Markowitz, M., Ally, D., Srivastava, S., Wang, W., Maitra, A., Sidransky, D., O'Connell, C.D. Mitochondrial DNA as a Cancer Biomarker. J. Mol. Diagnostics, 7, 258-267 (2005)

Jakupciak, J.P., O'Connell, C.D.. Standards and Standardization of Molecular Diagnostics. as Part V: Quality Assurance in Molecular Diagnostic Laboratories, in Molecular Diagnostics for the Clinical Laboratorian, Second Edition, Eds. G. Tsongalis, Humana Press, Inc. Totowan, NJ, 243-246, (2005)

- Optimized Resequencing Microarray to detect DNA mutations in early stage cancers (provided by Dr. Sidransky).
- Early Cancer* associated mutations detected in 88% of patients.
- Non-invasive* samples (BAL, Urine, Sputum, NAF) contained the identical mutations as observed in the primary tumor tissue.
- Assay format is readily *transferable to point of care*.



Scans entire mitochondrial genome

Genetic Testing Reference Materials

John P. Jakupciak, Kristy Richie

The expansion of molecular testing using nucleic acid technologies in clinical and public health practice has increased the need for appropriate, reference materials and verified quality control (QC) materials for quality assurance, test validation, proficiency testing, and development of new tests. Good laboratory practice requires the use of reference materials to establish an assay and assess the variability of the results. Once the assay is established, QC materials are required to assess the assay on a daily basis. However, despite the growing test volume, and the rapidly increasing number of tests being offered, the necessary daily use QC materials and certified reference materials are not available for most tests.

International cooperation between NIST, the Centers of Disease Control and Prevention (CDC) and the Eurogentest network is facilitating a strategy to initiate the acceptance and recognition of validated materials to serve as international standards for genetic testing. In a concerted effort we intend to develop standards for clinical genetics laboratories, including cytogenetics, biochemical, and molecular genetics specialties. In response to the need of standards for HD genetic testing, NIST is validating a panel of HD genomic material for use as an SRM for HD testing. The panel of standards includes: a pair of homozygous alleles, closely-spaced normal alleles, normal/expanded alleles, borderline/expanded alleles, closely-spaced expanded alleles, and normal/large expanded alleles.

Impact

The completion of the human genome sequence and subsequent genomic research has led to an amazing increase in the number of laboratory tests for genetic diseases. The molecular testing market is forecasted to increase to \$900 million in 2006 and sales growth consistently exceeds 20% per annum. In 2005, the molecular testing market generated revenue of \$778 million. CLIA regulations require that QC materials be used during patient testing if available. In addition, proficiency surveys routinely reveal mistakes made during the testing process. The reduction of errors that could be ascribed to improper calibration or controls.

Publications

Jakupciak, J.P., Richie, K.L. QC Materials for Huntington's Disease Genetic Testing. Accreditation and Quality Assurance, in press (2007).

Levin, B.C., Richie, K.L., Jakupciak, J.P. Advances in Huntington's Disease diagnostics: development of a standard reference material. Expert Rev Mol Diagn. Jul;6(4):587-96 (2006)

Program: Forensics and Homeland Security

Title: Characterization of SRM 2372 Components for Human DNA Quantitation

Authors: M.C. Kline, A.E. Decker, P.M. Vallone, J.C. Travis, M.V. Smith, J.M. Butler (831) and D.L. Duewer (839)

Abstract: Candidate Standard Reference Material 2372 Human DNA Quantitation standard (SRM 2372) is being developed by NIST's Human Identity Project team in response to numerous requests from the forensic human DNA identity community. SRM 2372 is expected to provide a well-characterized, homogenous, and stable DNA quantitation standard to this community and others where DNA concentration is a major factor in the quality of downstream applications results. While the certified values for each component are the absorbances at five ultraviolet wavelengths, these values define a "conventional" DNA concentration and several DNA quality metrics. An interlaboratory involving 32 laboratories using 12 different DNA quantification methods was conducted to evaluate the practical utility of the conventional concentration assigned to each component.

Purpose: Characterize SRM 2372 components to assure they are fit-for-purpose for calibrating secondary standards for human DNA quantification assays, including the various quantitative Polymerase Chain Reaction (qPCR) methods. In addition to establishing traceability for secondary and working standards, this SRM will allow individual laboratories to validate the DNA calibration of materials included in commercially available DNA quantification kits. The DNA concentration in some widely used commercial materials has varied by up to a factor of two.

Major Accomplishment: The three components of SRM 2372 have been prepared and packaged: component "A" is a single-source male material prepared at NIST, "B" is a multiple-source female material prepared at NIST, and "C" is a commercially prepared mixed male and female material. The three materials were prepared to have very similar DNA concentration of approximately 50 ng/ μ L. Absorbance measurements from the Biochemical Sciences Division's second generation National Reference Spectrophotometer were used to define a "conventional" DNA concentration for each component. An interlaboratory study was conducted to establish the commutability of the conventional DNA concentration assigned to the results of 12 different field methods comparing the components to one another. Homogeneity testing within each component has been completed.

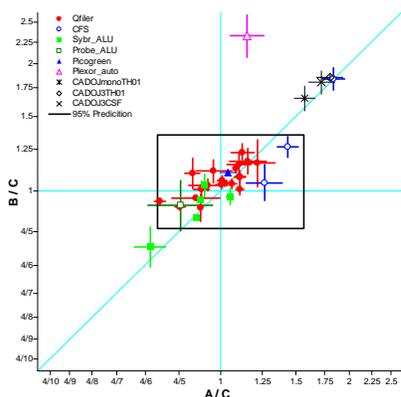


Figure 1 compares interlaboratory results for SRM 2372 components A and B, each standardized to component C. The resulting relative values, labeled A/C and B/C, indicate that the materials are commutable among most of the quantification methods but that a few assays are sensitive to the elevated salt concentration of component C and one assay is sensitive to the relative male/female composition of the materials. Error crosses represent approximate 95% confidence intervals. The boxed area defines an approximate 95% confidence interval on the mean ratios.

Impact: The SRM 2372 materials are fit for purpose.

Future Plans: Complete the certification process, get it released for sale and educate the target community on how to most efficiently use the SRM 2372 materials. Publish a paper on the preparation and use of the SRM materials.

Disclaimer: This project was supported by National Institute of Justice Grant Number 2003-IJ-R-029, which is an interagency agreement between [NIJ](#) and the [NIST Office of Law Enforcement Standards](#), awarded by the National Institute of Justice, Office of Justice Programs, US Department of Justice. Points of view in this document are those of the authors and do not necessarily represent the official position or policies of the US Department of Justice. Certain commercial equipment, instruments and materials are identified in order to specify experimental procedures as completely as possible. In no case does such identification imply a recommendation or endorsement by the [National Institute of Standards and Technology](#) nor does it imply that any of the materials, instruments or equipment identified are necessarily the best available for the purpose.

A UV-transmitting, High-Durability, Phosphate Glass for Fluorescence Standard Artifacts

G.W. Kramer and P.C. DeRose

We have recently completed the certification of SRMs 2940 and 2041, Relative Intensity Correction Standards for Fluorescence Spectroscopy, with orange emission and green emission respectively. These fluorescent glass artifacts are the first new fluorescence spectral correction CRMs issued by an NMI in 25 years (NIST certified SRM 936, Quinine Sulfate Dihydrate in 1979 – R. Velapoldi and K. Mielenz). We plan to produce a series of such standards for use from the near ultraviolet to the near infrared. The orange and green emitting standards were produced in a borate glass; however, this matrix is not suitable for ultraviolet/violet and blue SRMs because it does not transmit well in the ultraviolet region. Accordingly, we began a search for a suitable glass matrix. The ideal glass should be transparent far into the ultraviolet, should be rugged and resistant to corrosion from handling and atmospheric humidity, should be a good solvent for the inorganic metal oxide fluorophor dopants, and should melt at a reasonable temperature with a low viscosity so that bubble entrapment would not be a problem. For easy production, the ideal glass should be resistant to devitrification, anneal easily, have a sufficiently low coefficient of expansion to avoid cracking during production, and be hard enough to polish easily.

To develop suitable glasses, we set up a small melting and fabrication facility where we can produce test melts on a small (50 g) scale as well as cutting and polishing the glass into prototypes for testing. In doing this, we were both mentored and encouraged by folks who really know glass. Without the assistance of Doug Blackburn and Wolfgang Haller (both retired from the old glass group in MSEL), from Jack Fuller and Jeff Anderson in the NIST Optical and Glass Shops, and from Joe Hayden at Schott Glass, this project could not have been accomplished.

From the literature, we assembled a list of glass types with acceptable properties that we discussed with our mentors, who immediately eliminated many of the possibilities. Other candidates were ruled out because our melt oven was then limited to 1200 °C. We eventually discovered a calcium aluminophosphate glass that seemed to have what we wanted. However, there was a problem, most known fluorescing metal oxide dopants failed to produce fluorescence in our glass. This turned out to be caused by impurities from the ingredients used to make the glass and from the inexpensive silica/alumina crucibles we were then using. Switching to high purity alumina crucibles solved most of the problem, and a study of ingredients from several vendors led us to sources of suitable ingredients.

The basic formulation for our glass is 47 mole % CaO, 3 mole % Al₂O₃, and 50 mole % P₂O₅ (the dopant concentrations is <0.01 mole %). The precise ingredients used for a given batch depend upon the redox chemistry we are trying to carry out. Many of the metal oxide dopants we are studying exist in multiple oxidation states, not all of which fluoresce. The desired oxidation state in the glass is achieved by controlling the local atmosphere in and around the glass as it is melted, through the choice of ingredients and/or by carrying out the melt in a suitable gaseous atmosphere. For example, copper I ions in glass are fluorescent, while copper II ions are not. Doping with copper I oxide is not sufficient to ensure that the copper will remain in that state in the glass. To produce copper I in glass, reducing melt conditions are employed. Our best results with copper have come by supplying some of the phosphate from ammonium dihydrogen phosphate (at 1200 °C the liberated ammonia burns and consumes the local oxygen) in a forming gas (5 % H₂ in N₂) atmosphere. The resulting glass is colorless and fluorescent (copper II glass is the familiar blue color and does not fluoresce in the uv/vis region).

Most phosphate glasses have bad reputations for stability. Many (especially those with Group I cations) are rather soft and corrode easily in humid air. Phosphate glasses with Group II cations are more resistant to water damage. The addition of a small amount (<8 mole %) of alumina) has been shown to improve both the hardness and corrosion resistance. A DI water corrosion test showed our glass to be only an order of magnitude less stable than ordinary window glass. A similar study on the borate glasses used to make SRM 2940 and SRM 2041 showed them to be almost an order of magnitude more easily corroded than our phosphate glass.

The uv/visible spectrum of our calcium alumino phosphate glass exhibits transmittance down to 220 nm, which is better than some quartz or fused-silica materials. However, to achieve this performance, the ingredients and the crucibles must be essentially free of metallic impurities, especially iron. Just a few $\mu\text{g/g}$ can dramatically raise the uv cut-off wavelength. The glass shows good solubility for most metal oxides, and we have used it to make over 30 different metal-oxide-doped fluorescent glasses and now have several SRM candidates that we are testing for photostability. Once we select the most promising glasses, we will make production batches in the MEL glass-making facility and send them to Optiglass, Ltd. for cutting and polishing prior to certification. The discovery of this calcium alumino phosphate glass will enable us to produce SRM 2942 (blue emission) and SRM 2943 (uv/violet emission). It also looks very promising as a matrix for SRM 2944 (red emission) and SRM 2945 (nir emission). We thank John Seiber and Tony Marlow for helpful X-ray fluorescence analyses.

Interchanging and Archiving Analytical Data and Metadata Using the Analytical Information Markup Language (AnIML)

Gary W. Kramer, Patrick H. Gleichmann, and Kordian S. Placzek (Div. 831), Reinhold Schaefer, Alexander Roth, and Ronny Jopp (Fachhochschule Wiesbaden) and Peter J. Linstrom (Div. 838)

Interchanging analytical data and their associated "scientific metadata" across space and time, from application to application, and to/from applications and databases has often been hampered by multiple, proprietary, incompatible data formats. Over the past 15 years, chemical spectroscopy and chromatography data have been transferred using pre-web-based technologies in conjunction with either the ASTM AnalYTical Data Interchange (ANDI) standards or the IUPAC JCAMP-DX (Joint Committee on Atomic and Molecular Physics Data Exchange) standards. While these procedures allow the interchange data from instrument to instrument, data interchange from instrument to application (e.g., importing data from an instrument into an Excel spreadsheet), from application to application, or from application to and from databases is not as well supported. Modern laboratory management concepts such as electronic laboratory notebooks require simple, common mechanisms for interchanging data between instruments, applications, and databases. The rapid pace of information technology and computing hardware innovation has exacerbated the problem of archiving of scientific data. Analytical information stored on early digital media (for example, 8-inch floppy disks) 20 years ago may be less accessible today than such information stored in a paper format 20 years prior. The difficulty is not solely being able to still read the data on the storage media, but it is also being able to decipher the proprietary formats used by obsolete applications and operating systems.

In an effort aimed to solve the interchange and archiving dilemma for analytical chemistry data, ASTM SubCommittee E13.15 on Analytical Data is creating the Analytical Information Markup Language (AnIML) for describing chromatography and spectroscopy data and their metadata. Based on XML (eXtensible Markup Language) and its associated technologies, AnIML facilitates access to analytical data by building in descriptions of the data and metadata with delimited tags in the same way that HTML (HyperText Markup Language) describes the display of items on a webpage. AnIML is built around a core schema that defines ways for describing almost any data. A separate Technique schema describes Technique Definition files that are used to constrain the data description mechanisms in the core for a given analytical technique to those commonly accepted, to delineate the metadata items commonly associated with such domain data, and to permit content extension by vendors and users without changing the core schema.

Once in AnIML format, analytical data can be interchanged over the web, converted to other formats, validated, or visualized in multiple formats using existing XML-based tools. The "self-describing" XML-based format used in AnIML allows applications to utilize the data easily based on the tags that describe each data entry. This affords the possibility for creating generic programs that can visualize, display, and print AnIML-based spectroscopic and chromatography data from any source. Using AnIML formats, casual users would no longer have to have access to or have to learn proprietary vendor software just to look at analytical results. Since data formats are open and self-documenting in XML and since most XML is text-based, data stored in AnIML format should be accessible for as long as the media it is stored on can be read. Furthermore, AnIML ensures the integrity of the data through the use of digital signatures and provides for the data tracking, verification, and validation necessary for use in regulated industries.

The AnIML effort is international with participants from Germany, France, the United Kingdom, and Jamaica. Most of the participants are from instrument manufacturers or the pharmaceutical sector, e.g., Agilent, GlaxoSmithKline, Waters, Shimadzu, the TopCombi Group, Jasco, ADC Labs, Thermo

Electron Corp., Bristol-Myers Squibb. Further information about AnIML can be found at <http://animl.sourceforge.net>.

Publication:

“Automated Generation of AnIML Documents by Analytical Instruments, “ A. Roth, R. Jopp, R. Schäfer, and G.W. Kramer, *Journal of the Association for Laboratory Automation*, **2006**, **11**, 247.

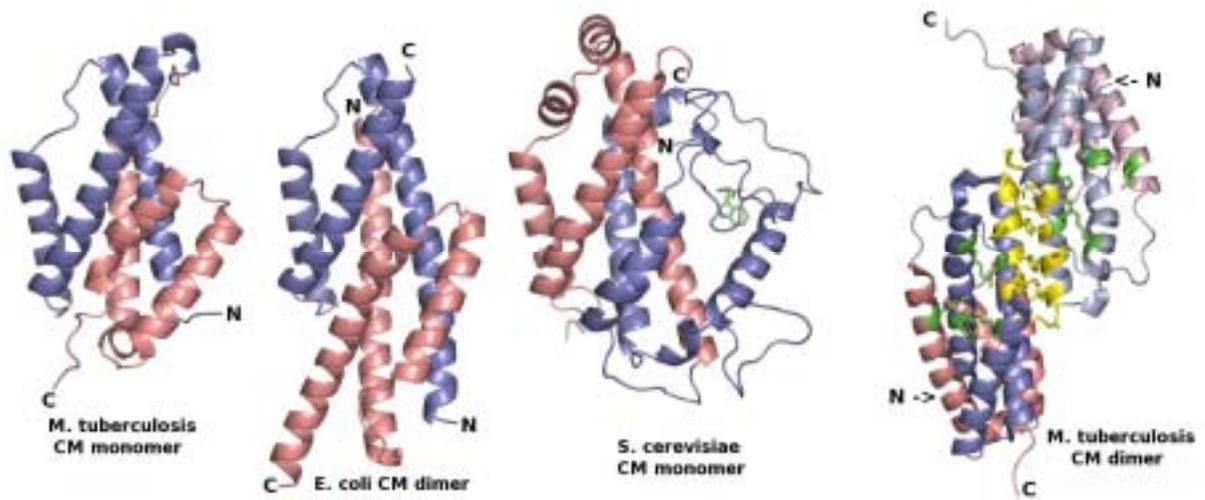
Structural Characterization of the Secreted Chorismate Mutase (Rv1885c) from *Mycobacterium tuberculosis* H₃₇R_v

J.E. Ladner, S.-K. Kim, S.K. Reddy, B.C. Nelson, G.B. Vasquez, A. Davis (Div. 831), A.J. Howard (Illinois Institute of Technology, Chicago, IL), S. Patterson, G.L. Gilliland, P.T. Reddy (Div. 831)

Pharmaceuticals and Biomanufacturing

Introduction: Chorismate mutase (CM) occupies a central role in the biochemical pathway leading to the synthesis of the aromatic amino acids and a number of other essential aromatic compounds in bacteria, fungi, algae, and plants, but this pathway is absent in mammals. CMs exist in multiple forms both functionally and structurally. Some examples of the monofunctional CMs from *Bacillus subtilis* and *Serratia rubidaea* lack allosteric control and exhibit simple Michaelis-Menten kinetics. Other examples of the monofunctional CMs from *Saccharomyces cerevisiae* and *Aspergillus nidulans* exhibit complex multi end product effector control. On three occasions, CMs have evolved to become one catalytic component of a bifunctional protein. The CMs from *E. coli* (EcCM) and *S. cerevisiae* (ScCM) are all α -helical structures whereas the CMs from *B. subtilis* and *Thermus thermophilus* have α/β barrel structures.

Major Accomplishments: The *Mycobacterium tuberculosis* Rv1885c CM is synthesized with an amino terminal signal sequence which is cleaved off upon expression in *E. coli* with the mature protein beginning with Asp₃₄. We have determined the crystal structure of this CM using 1.7 Å resolution data. The protein folds into an all α -helical bundle similar to the structures of EcCM and ScCM. The catalytic site is formed within a single chain of the dimer with no contribution from the second chain similar to the catalytic site of ScCM but different from the catalytic site of EcCM which includes residues from both the chains. The monomer of this CM resembles the monomer of ScCM and the dimer of EcCM but functions as a dimer itself. Further studies have been initiated to determine whether the function is retained if the dimer formation is disrupted.



The first three panels show the comparison of the structure of *M. tuberculosis* CM monomer on the left (first half of the chain blue and second half pink) with the *E. coli* CM dimer

(one monomer blue and one pink), and the *S. cerevisiae* CM monomer (first half is again blue and second half is pink). The dimer of *M. tuberculosis* CM is shown on the far right colored in the same way as the monomer but with the addition of the active site residues in green and the dimer interface residues in yellow.

Impact: Tuberculosis still claims 8 million humans per year. One can take advantage of the nonoccurrence of CMs in humans and try to develop antimicrobial drugs or vaccines using this pathway to combat human pathogens such as *Mycobacterium tuberculosis*. Since this particular CM is secreted it may not be a good drug target but it might prove to be a good vaccine candidate.

Future Plans: *M. tuberculosis* has a second CM which is probably trimeric, we have initiated crystallization trials with it.

Publication: S.-H. Kim, S.K. Reddy, B.C. Nelson, G.B. Vasquez, A. Davis, A.J. Howard, S. Patterson, G.L. Gilliland, and J.E. Ladner, "Biochemical and Structural Characterization of the Secreted Chorismate Mutase (Rv1885c) from *Mycobacterium tuberculosis* H₃₇R_v: an *AroQ Enzyme not Regulated by the Aromatic Amino Acids", *J. Bacteriology* (2006), in press.

Title: Thermodynamics of DNA/DNA and DNA/RNA hybridization reactions

Authors: Brian Lang and Fred Schwarz

Program: Pharmaceuticals and Biomanufacturing

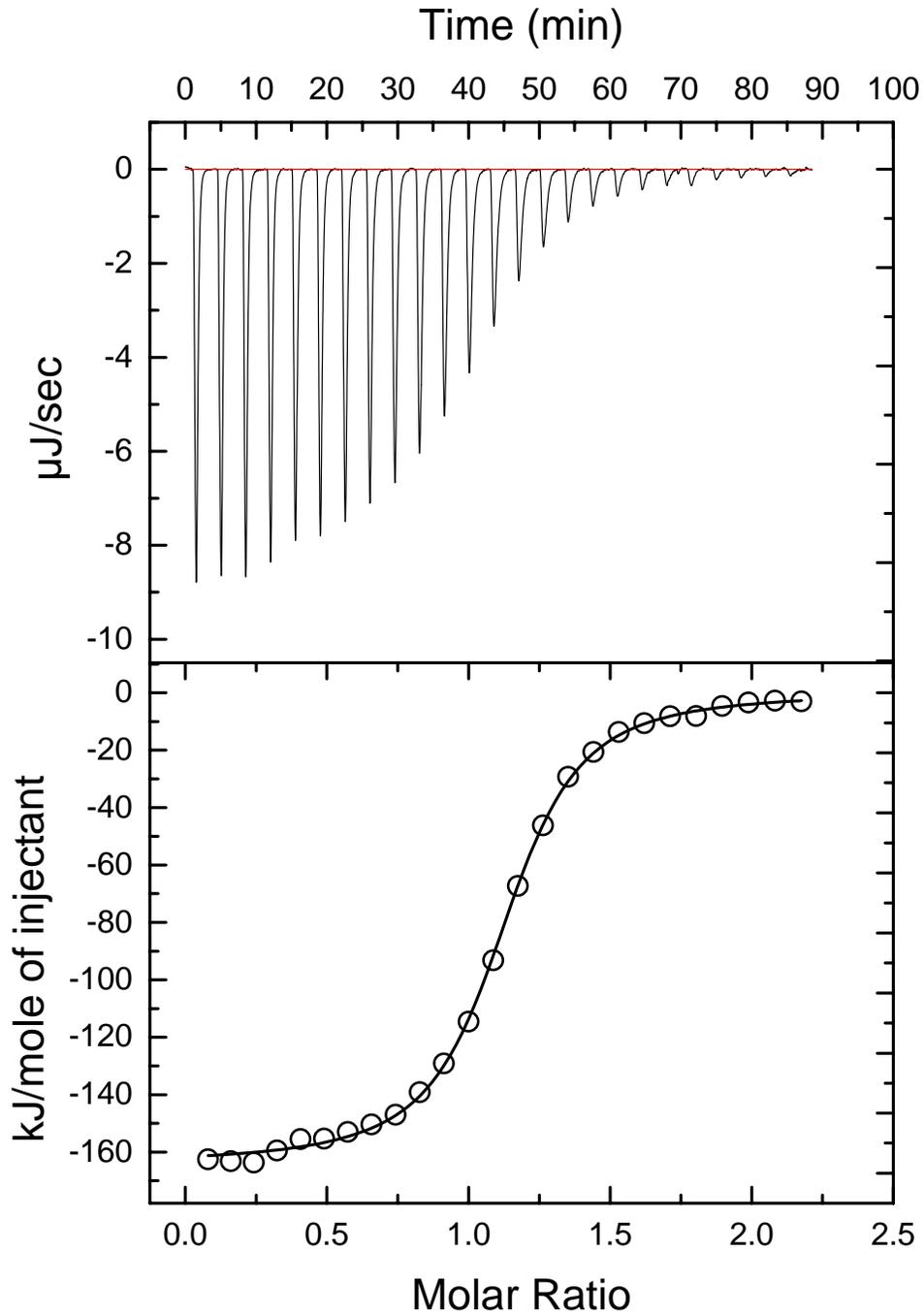
The conditions which stabilize DNA-DNA and DNA-RNA double-strands in solution are important considerations for applications such as the design of PCR and gene chip array measurements, as well as gaining a fundamental understanding of these important reactions. The stabilization of these DNA-DNA and DNA-RNA duplexes is measured in terms of thermodynamic quantities, the standard molar enthalpy changes $\Delta_r H^\circ$, standard molar Gibbs free changes $\Delta_r G^\circ$, and dissociation temperatures T_m . Although there are models which can be used to predict thermodynamic quantities for DNA-DNA and DNA-RNA duplexes, the models have not been validated over a wide range of conditions. In fact, most predictive models rely on UV melting data extrapolated to 37 °C. Consequently, we have performed a series of detailed measurements on a model system, namely an oligomer (10 bases on each strand) of single-stranded DNA binding to its complementary DNA and RNA strands.

We used isothermal titration calorimetry (ITC) to measure equilibrium constants and molar enthalpy changes for the DNA hybridization reactions over a wide range of temperature, pH, and salt (sodium chloride) concentration in order to obtain an understanding of how the thermodynamic quantities depend on changes in these parameters. Differential scanning calorimetry (DSC) was also used to determine the thermodynamic quantities ($\Delta_r H^\circ$, $\Delta_r G^\circ$, T_m) for the reverse reaction (melting of double-stranded DNA). DSC results on the melting of DNA duplexes gave results consistent with existing predictive models (based on UV melting). However, we did find an inconsistency with the results obtained from the ITC measurements. This inconsistency was explained by the presence of a transition between two different conformational states in the single-stranded oligonucleotides, which occurs between the temperatures of the ITC and DSC measurements.

This study has shown that the standard molar Gibbs free energy change, $\Delta_r G^\circ$, for the binding reaction is temperature dependent and contrary to the assumptions used in existing predictive models. Also, it was found that the dependence of $\Delta_r G^\circ$ on salt concentration between 0.1 M and 1.0 M is both non-linear and less than what the current models would predict. Additionally, both $\Delta_r G^\circ$ and $\Delta_r H^\circ$ for the binding reaction exhibit only a slight dependence on pH from pH = 6.0 to pH = 8.0. Thus, these results allow for the prediction of the thermodynamic behavior of this prototypical model system over a wide range of temperature, pH, and salt concentration and also provide insight into the behavior of many similar reactions.

Publication: Brian E. Lang and Frederick P. Schwarz, "Effect of temperature, pH, and ionic strength on the thermodynamics of DNA/DNA and DNA/RNA hybridization reactions." Submitted.

Figure: (top) the ITC scan of 10 μl aliquots of 177 μM DNA(TG) into 15.8 μM DNA'(CA) in pH = 7.0 PBS buffer at 298.15 K. (bottom) The binding isotherm for this titration.



Processing, Analysis and Alignment of Carbon Nanotubes– Where Smaller is Better.

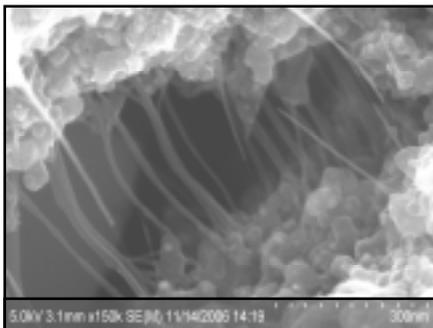
G.G. Giulian (841/CARB), S.N. Jones (821/MEL) N.G. Abdulaev (CARB/UMBI), A.E. Vladar (821/MEL), M.T. Postek (821/MEL) and J.P. Marino (831/CARB)

Program: Nanotechnology

Abstract: Researchers at the National Institute of Standards and Technology (NIST) and the Center for Advanced Research in Biotechnology (CARB) Rockville, MD have developed a simplified method to process carbon nanotubes (CNTs) into size fractions that appear to have broad application basic research, biotechnical and industrial applications. The research team applied grit shearing followed by electrophoresis-based methods to generate sub-1000 nm length CNTs which were then characterized using Scanning Electron Microscopy (SEM) and Nuclear Magnetic Resonance (NMR). These CNT building blocks “nanoblocks” were dried-down for individual nanotube examination (SEM) or prepared for bulk aqueous studies (NMR).

Introduction: Previous efforts at manipulating CNTs have typically focus on longer nanotubes (>1 micron), so that materials studied were “nano” scaled in diameter only. While longer tubes are easier to manipulate with existing methods, these CNTs do not exhibit mechanical, electrical and biological properties of a true nanoscale material (diameter and length) - *smaller is better*. For example, longer nanotubes tend to aggregate and fall out of solution. A 2 to 5 micron long CNT is on the same scale as a typical human cell, which makes these tubes too long for many applications. True nanoscale tubes “nanoblocks” are as much as 100x shorter making them much easier to mix with composite materials, interact with biological samples and even align in solution at relatively low magnetic field strengths. Previous methods to shorten and debulk CNTs have tended to result in up to 90% of the starting material turned into carbon “dust” – amorphous carbon particles. Nanoscale length fractionation has also been difficult as the CNTs tend to rapidly aggregate and clog many separation systems.

Major Accomplishment(s): The new method used by the NIST team to debulk and shorten CNTs is similar to home rock-polishing. The approach may replace or enhance processing methods that use strong acids or oxidizers and high-power ultrasonication. The older methods make it more difficult treat more than very small amounts of carbon nanotubes, whereas “grit shearing of CNTs is easily scalable” the



The Nanometer-Scale Metrology Division used high resolution Scanning Electron Microscopy (SEM) to image small de novo industrial grade CNT bundles from 1-20 nm in diameter spanning gaps up to 1000 nm (1 micron) that have been generated using the new CNT processing methods.

researchers reported. After the grit shearing step the processed CNT material is separated by hydrodynamic size (length) using methods modified from existing low-cost protein separation technology. The *nanoblocks* are resolved into size fractions comparable to many proteins. Prior efforts have used DNA-wrapped CNTs to facilitate size separation by nanotube diameter. However, DNA-wrapping is too expensive for practical use in large-scale size separation of CNTs. In contrast, the work done at NIST used Sodium Dodecyl Sulfate (SDS) to disperse the nanotubes and facilitate electro-elution separation. It was also determined that the mechanical shearing step is equally effective with a number of dispersants, so more exotic dispersants are not needed for the process

Impact and Future Plans: Currently, the high cost of processing ‘high grade’ CNT’s significantly limits the broad use of CNTs in nanomanufacturing applications. Future plans will therefore focus on scaling

up CNT *nanoblock* generation and standardizing characterization of these materials to provide the tools for generating low-cost, high-quality CNT materials to facilitate “next generation” nanotechnology applications.

Outputs: Initial findings from this work were presented in an invited talk (G.G. Giulian) at the 4th International Workshop on Nanoscale Spectroscopy and Nanotechnology (NSS4) held in Rathen, Germany, September 17-21, 2006.

Optical Spectroscopy of Active Membrane Proteins

Richard W. Hendler (NIH and Div. 831) and Curtis W. Meuse (Div. 831)

There is currently an increased awareness of the importance of active membrane proteins and the need to greatly expand studies in this area. For example, the NIH roadmap for future research includes significant funding for the isolation, crystallization, and study of membrane proteins. The best crystals are made in procedures which employ detergent. In published studies of one of us (RWH), performed with the active membrane proton pump bacteriorhodopsin (BR), it was demonstrated that the briefest exposure of a membrane protein system to the most dilute detergent dramatically alters specific membrane lipid-amino acid associations and the normal kinetics of the system. Before committing large expenditures of money and effort, there should be a quality control procedure to establish that any crystallized preparation shows the same behavior as in the membrane. Furthermore, based on published studies (RWH), there are steps that can be taken either to prevent a deterioration of normal function or to reverse those which have occurred. With a crystal known to function in its proper mode, we can apply mathematical approaches based on linear algebra that can deconvolute the overlapping optical spectra, in a time-resolved study, into the individual pure states that make up the kinetic sequence. Even when a crystal of a protein is found to follow a perturbed kinetic sequence we can isolate kinetic states which are similar to those of the native protein for comparison. As a first step in developing standardization procedures that can be applied to membrane protein crystals, we are planning parallel time-resolved FTIR and optical studies on the same sample. In addition to proper lipid protein interactions, native behavior requires a certain minimum amount of hydration. In a crystal, this is in the form of the water of crystallization. Using our side-by-side FTIR and optical spectrometers, we can determine the minimum number of water molecules (using the strong IR vibrational mode for H₂O) required for normal kinetics (determined optically). The ultimate goal is to relate conformational changes of a protein to its function, which for BR is the electrogenic transfer of protons across the membrane.

In FY2006, we were able to localize the precise steps in the kinetic sequence of the laser pulse-initiated photocycle of BR, where most of the membrane potential is generated by the proton current. We also, in collaboration with Paul Smith (NIH), designed and built special optics and supports for measuring the kinetics of the BR photocycle on the standard 1 inch barium fluoride allowing us to use a single sample for both the optical and IR spectrometers.

Future plans (near and long term)

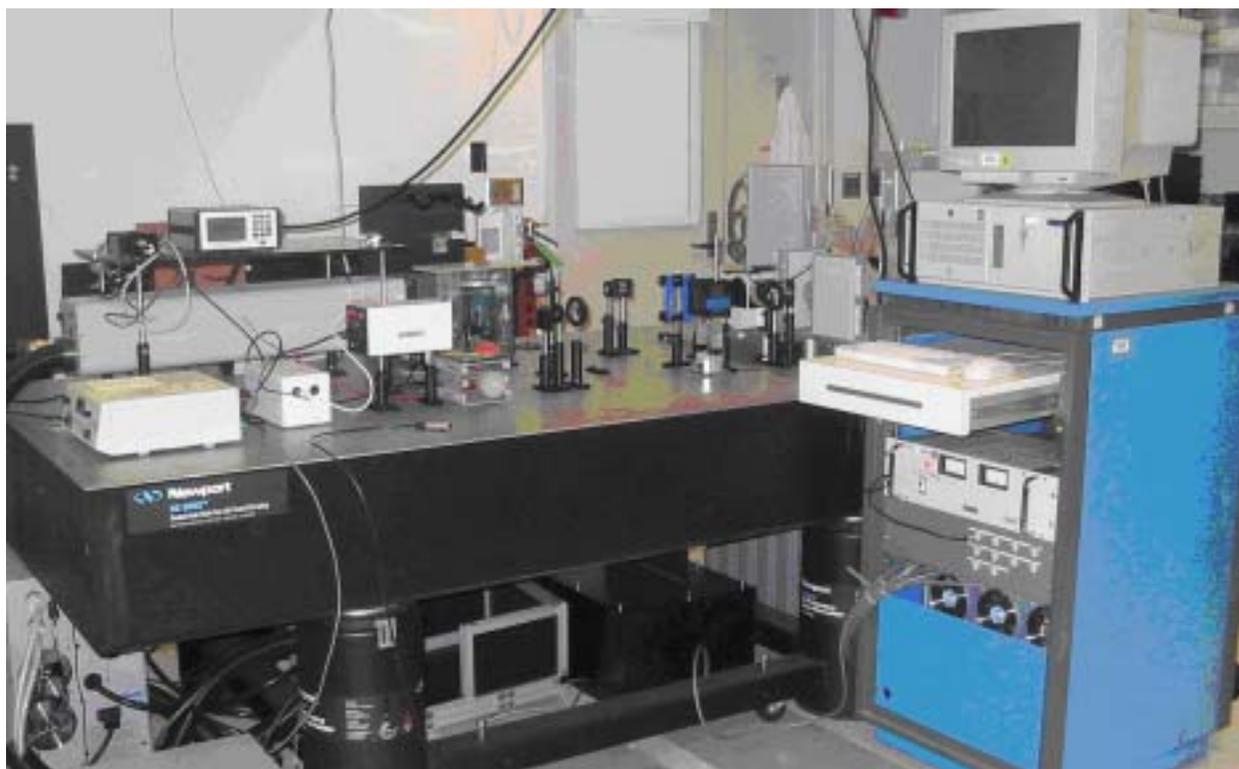
1. To perfect the step scan capability of our FTIR spectrometer to allow the obtaining of clean time-resolved spectra at both 100 and 200 KHz.
2. To try to determine the minimum ratio of H₂O molecules to BR that allows normal photocycle behavior.
3. To determine if profound changes in photocycle behavior under different conditions of pH and temperature are due to changes in the mosaic or raft organization of membrane lipids resulting in different membrane-protein interactions.
4. To build or participate in the building of a new instrument that uses microscope optics combined with fiber optics and an intensified charge coupled device detector that enables the obtaining of optical and IR spectra on single crystals of membrane protein.

5. To obtain time-resolved X-ray diffraction data on crystals previously standardized by our combined optical and FTIR spectroscopic analyses.

Publications

Richard W. Hendler, John W. Kakareka, Paul D. Smith, Thomas J. Pohida, and Curtis W. Meuse. "Proton-pumping Capabilities of the M-fast and M-slow Photocycles of Bacteriorhodopsin" Submitted to *Journal of Physical Chemistry B*.

Richard W. Hendler, Richard I. Shrager, and Curtis W. Meuse. "The Ability of Actinic Light to modify the Bacteriorhodopsin Photocycle Revisited: Heterogeneity vs. Photocooperativity" Ready for submission to *Journal of Physical Chemistry B*.



Time-resolved multichannel optical data are collected by a unique spectrophotometric system. This second-generation 96-channel spectrophotometer has: higher resolution (16-bits), higher maximum sampling rate (200 kHz), independent programmable gain and offset for each analog channel, logarithmic as well as linear scheduling of samples, and higher system reliability. This state-of-the-art optical system allows a complete photocycle of the protein to be obtained with each laser pulse.

Quantitative Optical Spectroscopy of Biologicals

C. Meuse (Div. 831) and J. Hubbard (Div. 855)

Biopharmaceuticals, protein arrays, proteins for research purposes, and protein standards must have well characterized molecular compositions and conformations to allow comparisons of treatment protocols, disease diagnosis, etc. In collaboration with the FDA and the New York Academy of Sciences, NIST hosted a meeting, in December of 2005, entitled “Follow-on Biologicals: Scientific Issues in Assessing the Similarity of Follow-on Protein Products” to discuss the measurements that can be used to characterize proteins. A particular theme of this meeting was to identify techniques that can distinguish between measuring molecular averages and the distribution of properties such as glycoform and conformations.

NIST is developing a method to obtain more information about molecular conformational distributions using spectroscopy. This will allow us to distinguish between an ensemble of molecules with only a limited number of molecular conformations and an ensemble of molecules with an extended distribution of conformations that happen to have the same average values. We form a P2 orientation order parameter that depends not only on an average orientation angle, θ , but also on the deviation from that average, i.e., the width of the orientation distribution, $\delta\theta$. Comparisons of $\delta\theta$, obtained from the analysis of internal reflection optical measurements on self-assembled alkanethiol monolayers tethered to gold surfaces with different amounts of order and with parameters derived from a molecular dynamics simulation of the same systems show good agreement. In FY2006, we studied the hydrogen deuterium exchange properties of several protein monolayer-on surface-systems to allow comparisons of our parameter between protein monolayer samples with different degrees of order. The results of our hydrogen deuterium exchange experiments further suggest that our, $\delta\theta$, parameter is measuring conformational heterogeneity. This seems reasonable based on the idea that there can be no single molecular orientation when dealing with nonspecifically adsorbed flexible protein molecules, i.e., each protein has its own orientation.

Future Plans

In FY07, we will extend our studies of the quantitation of protein structural heterogeneity to include different proteins and protein systems such as fibronectin on polymer surfaces, crystallizing RNase and bacteriorhodopsin undergoing its proton pumping photocycle.

Publications

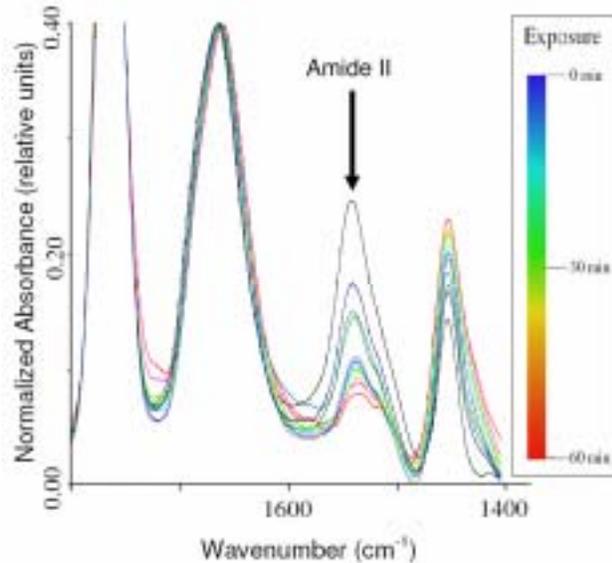
Dennis P. McDaniel, Gordon A. Shaw, John T. Elliott, Kiran Bhadriraju, Curtis W. Meuse, Koo-Hyun Chung, and Anne L. Plant “The stiffness of collagen fibrils influences vascular smooth muscle cell phenotype” Accepted by *Biophysical Journal*.

Jack R. Smith, Marcus T. Cicerone, Curtis W. Meuse “Measuring Hydrogen Deuterium Exchange in Protein Monolayers” Submitted to *Applied Spectroscopy*.

Jack R. Smith, Marcus T. Cicerone, Curtis W. Meuse “Tertiary Structure Changes in Albumin Upon Surface Adsorption Observed Via Fourier Transform Infrared Spectroscopy” Submitted to *Biomaterials*.

Figure description

NIST researchers have made measurements of deuterium exchange for hydrogen several anisotropic films of proteins. Our results indicate that exchange measurements can be generalized to describe protein conformational changes in protein monolayers.



The spectral changes in the infrared region of the amide group in a monolayer of bovine serum albumin (BSA) adsorbed to the surface of poly(D,L-lactic acid) and subsequently exposed to deuterated 0.01 M sodium phosphate buffer over the course of 1 h. The spectra are color-coded by exposure time according to the key (inset). The dark blue spectrum corresponds to the non-deuterated case, while the red spectrum corresponds to 1 h exposure. The thickness of the adsorbed BSA layer the films that produced these spectra, as measured via ellipsometry, ranged from (3.0 to 6.9) nm. Spectra are the averaged results of 3 experimental trials.

Quantum Dot-Based Quantitative Cellular Imaging for Biomedicine

Lab testing for the HER2 in breast cancer patients (about 186,000 new cases annually in the U. S.) currently suffers from a 20% error rate. Because this test stratifies patients into the ~ 30% of patients who will benefit from trastuzumab, an expensive humanized mouse monoclonal therapeutic, this error rate represents a significant healthcare and economic problem for the U. S. economy. NIST biomarker technology evaluation expertise can have significant impact through development of a universal, precision fixed-cell material standard for HER2 testing, with associated data on certified analyte values and defined uncertainties of measurement. Such a standard, by benchmarking all labs testing for HER2 nationwide should lead to a more accurate test and consequently, more effective medical evaluation of therapeutic options for breast cancer patients.

However, metrology of cellular analytes, especially those characterized by heterogeneity of gene copy number or protein expression, represents a challenging task. The wider healthcare community involved in HER2 testing and clinical management of breast cancer patients has called upon NIST to address this significant measurement need in the NIST Needs Assessment Workshop on HER2 Testing (2002).

Such a standard material has been initiated at NIST. Our first task was to improve cellular biomarker measurement reagents and quantitative microscopic imaging under conditions that parallel current clinical pathology lab testing. In the course of a series of cancer biomarker validation studies for early detection and patient stratification, we have addressed photobleaching and weak signal/noise ratios of currently used cellular cancer biomarker probes (nucleic acids and antibodies) by introducing newly discovered non-fading fluorophores (CdSe/ZnS quantum dots) during detection. We have quantified the bioimaging properties of CdSe/ZnS in biological systems including the breast cancer analytes HER2 gene and receptor, human telomerase protein and the TMPRSS2-ETV1 gene fusion recently discovered to be common in prostate cancer.

In addition, we are configuring such assays on a high throughput slide processing platform that will perform both FISH (fluorescent in situ hybridization for DNA

probes) and fIHC (fluorescence immunohistochemistry for antibody-based quantitation of selected cellular proteins) to provide a new national reference material for HER2 testing for breast cancer clinical management. We have designed non-mammalian IgY antibodies (avian) for several of these protein analytes as model systems for the comparative metrology of affinity reagents in human proteomics of small cell populations and fixed pathology specimens.

Finally, we have explored deconvolution z-plane imaging of stacks of fluorescent images in the above systems to more quantitatively capture in-focus fluorescent signals in an effort to improve measurement of cellular biomarkers for cancer diagnostics and patient stratification.

Because recent work indicates that, for most cancers, a single biomarker (DNA or antibody probe) may not suffice to significantly improve sensitivity, specificity and positive predictive values (PPV) of early cancer detection tests, we are exploring multispectral imaging for simultaneous quantitation of up to 25 different cancer biomarker analytes detected with quantum dot fluorophores in fixed tissue biopsy sections. This imaging approach from clinical cytogenetics (SKY technology) with interferometry systems in-house at NIST should be amenable to biomarkers we evaluate at NIST, and which we plan to implement in robotically actuated microfluidics systems to increase throughput and decrease unit costs in massively parallel processing of such assays.

Our systems (HER2 BAC-DNA and IgY antibody probes, and associated high and low HER2 expression cell lines; telomerase IgY antibody with high and low telomerase expressing cell lines; fusion region BAC-DNA probes and prostate cancer cell lines with or without gene fusions) have also contributed to a new 5-year Cellular Biometrology Competency (2006-2011) in which the DNA and protein probes, and cell systems we have characterized with quantum dot measurement technologies, will be utilized in collaboration with a team of NIST scientists and engineers as clinically relevant biological metrology systems. Combining our expertise in these biological systems with NIST expertise in microfluidics, we anticipate miniaturization, multiplexing and high-throughput implementation that will benefit from economies of scale in such cancer biomarker lab testing.

P.E. Barker, Y. Xiao (Div. 831)

Accomplishments:

Major accomplishments include: publications listed; NIST-NCI workshops organized on nanotechnology, proteomics and cancer biomarkers; first demonstration of FISH with quantum dots in human metaphase chromosomes; design of novel IgY chick antibodies for HER2 and for human telomerase that exhibit superior performance characteristics compared with mammalian antibodies (Xiao patent submission); high resolution imaging of cellular responses and iNOS protein expression to WMD protectant roxithromycin in human airway cells exposed to sulfur mustard.

Impact:

The impact of the work can be judged by dissemination of NIST work in the press (NPR and CNN stories) on breast cancer nanotech imaging work; quantum dot work has expanded to a number of different gene and protein analysis systems and has been the topic of invited scientific and technical presentations (Barker, Xiao). Roxithromycin quantum dot collaboration with Walter Reed was selected as best paper at the November 2006 U. S. Army Science Conference; NIST-NCI Cancer Biomarkers Lab project was initially funded (2000) and renewed (2005)(Barker, PI). Quantum dot work at NIST inspired a collaboration in which German physics graduate student Mueller traveled to NIST to work on quantum dot imaging in plant systems (see Mueller et al. 2006 publication).

Future Plans:

Completion research and production goals for HER2 fixed cell material; field testing, implementation and final certification of the NIST HER2 Reference Material. Quantum dot imaging of HER2 gene and protein in high throughput mode in a series of automated slide processor and automated imaging systems; quantum dot imaging on tissue

microarrays and evaluation of histopathology tissue preparation and fixation and antigen retrieval methods. Support of NIST Cellular Biometrology Competency (2006-2011); additional collaborations with Walter Reed

Patent and Publications:

Xiao, Y. An IgY antibody for human telomerase (patent submitted June 2006)

Barker, P. E. (2006). Preface to standards for healthcare: needs assessment and development. *Cancer Biomarkers* 1(2005) 207-208.

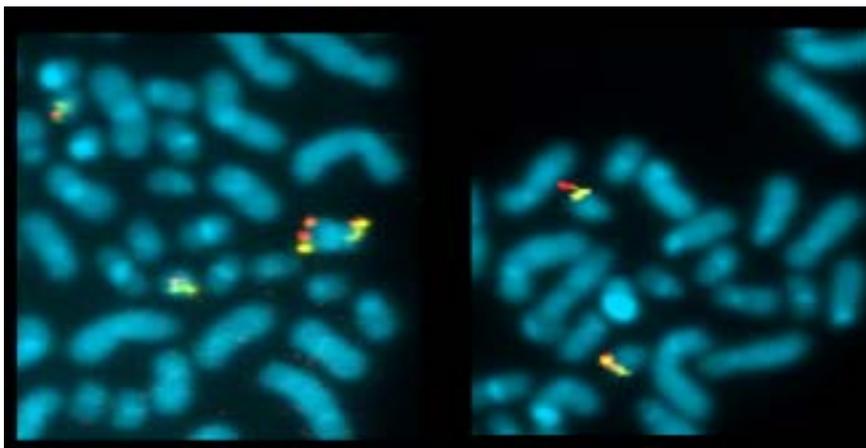
Mueller, F., Houben, A., Barker, P. E., Xiao, Y., Kas, J., Melzer, M. (2006). Quantum dots: a versatile tool in plant science. *J.Nanobiotechnology*5:4.
<http://www.jnanobiotechnology.com/content/4/1/5>

Xiao, Y., Telford W. G., Ball, J. C., Locascio L. E., Barker, P. E. (2005). pH, FISH and semiconductor nanocrystals. *Nature Methods* 2(10): 723, 2005.

Xiao, Y., Barker P. E. (2004). Semiconductor nanocrystal probes for human metaphase chromosomes *Nucleic Acids Research* 32(3):1-5.

Xiao, Y., Barker, P. E. (2004). Semiconductor nanocrystal probes for human chromosomes and DNA. *Minerva Biotechnologica* 16:1-8.

Hammond, E., Barker P.E., Taube, S., and Gutman, S. (2003). Standard reference material for Her2 testing: report of a National Institute of Standards and Technology-sponsored Consensus Workshop. *Appl Immunohistochem Mol Morphol.* 2003 Jun;11(2):103-6.



Left Panel: (Red, Green) rearranged prostate cancer TMPRSS2-ETV1 fusion gene on human chromosome 21.

Right Panel: (Red, Green), normal cell with same FISH probes from chromosome 21.

Surface Plasmon Resonance as a Proteomics Tool

Surface plasmon resonance (SPR) spectroscopy is essentially a refractive index measurement that is sensitive to changes in mass at an interface with a metal substrate. This makes the technique suitable for measuring label-free protein interactions where a protein is immobilized to the sensor surface and ligand binding is measured from solution flow over the sensor. Typically, binding constants are determined for a known protein-ligand interactions based on the kinetics of association and dissociation. (1) SPR can be made more quantitative to give additional unambiguous physical parameters. (2) SPR can be used in conjunction with mass spectrometry (MS) to determine physical and chemical properties of protein-ligand interactions when the identity of the ligand is unknown.

A. Peterson (Div. 831)

SPR resonance curves can be fit with a Fresnel reflectivity model that interprets the sensor interface as optical layers with quantitative optical properties. A measurement at a single wavelength results in ambiguous determination of refractive index and optical thickness for the immobilized protein layer. Using multiple SPR scans at multiple wavelengths will allow for an unambiguous determination of the optical properties for the biofilm. A unique refractive index and optical thickness will allow for determination of protein coverage and allow for quantitative saturation measurements. Surpassing this experimental bottleneck allows for a multitude of reliable measurements including determining the effects of protein orientation on saturation binding, and determining equilibrium binding constants directly instead of from association/dissociation kinetics.

The ability of SPR to measure label-free protein interaction kinetics has great advantages when combined with MS to determine the identity of unknown ligand binding partners. Typically, immunoprecipitation and gel electrophoresis techniques are used to pull-down protein

interactions in a long and multi-step process for which the results are unknown until after visualized with a stain after the electrophoresis process. The contributions of nonspecific binding are hard to evaluate. In contrast, SPR can make measurements real time to evaluate ligand binding to an immobilized protein. The basic steps involve (1) immobilization of the protein to an appropriate attachment chemistry, (2) incubation of cell extract (or serum, etc.) and real-time monitoring, and (3) elution and/or digest of the specific complex followed by identification by MS. SPR can be used for real-time immobilized ligand capture and as a separation technique whereby elution of the ligand off the surface is performed under a controlled method to separate specific ligand binding from nonspecific interactions.

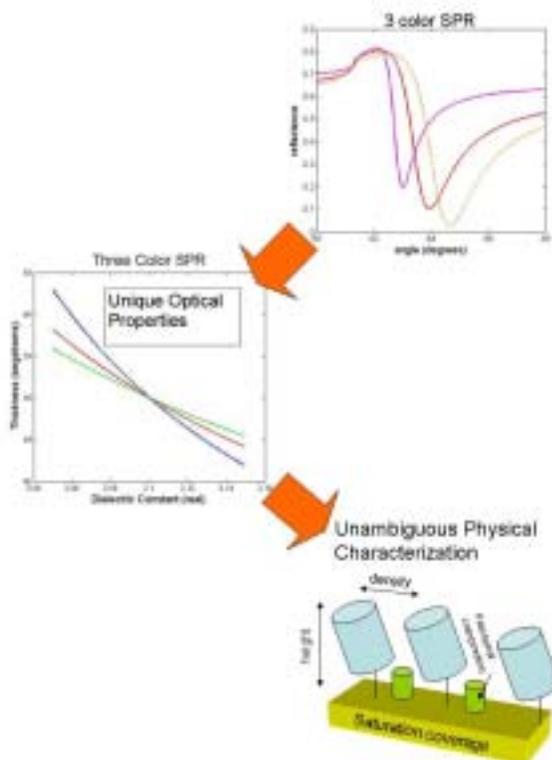


Illustration of data collection, analysis of optical properties, and conversion to physical parameters for a multi-component biofilm.

Title: Characterization of the Secreted Chorismate Mutase from the human pathogen *Mycobacterium tuberculosis*

Authors: Sook-Kyung Kim, Jane Ladner and Prasad Reddy

Objective: Tuberculosis is a dreadful human disease and accounts for more casualties than any other single infection. Multidrug resistant *Mycobacterium tuberculosis* is classified as a bioterrorism agent by the Center for Disease Control and Prevention and the National Institute of Allergy and Infectious Diseases. This work is designed to target and knockout the enzyme chorismate mutase that is present in *Mycobacterium tuberculosis* but not in humans with the idea of developing antitubercular drugs.

Problem: Chorismate mutase is present only in bacterial and lower eukaryotic organisms but is absent in higher eukaryotes such as humans. Hence, this enzyme is a popular target in metabolic engineering and drug development. Chorismate mutase is a central enzyme in the shikimate pathway that is responsible for the synthesis of aromatic amino acids and a number of other key intermediates of commercial interest. It is intriguing to determine the biochemical properties and three dimensional structure of chorismate mutase in *M. tuberculosis*.

Approach: *Mycobacterium tuberculosis* genome sequence revealed two genes for chorismate mutase, Rv1885c and Rv0948c. Rv1885c and Rv0948c chorismate mutase genes were amplified by polymerase chain reaction. Amplified genes were cloned into a protein expression vector. Both the recombinant plasmids were introduced into *E. coli* strain MZI for protein expression. Expressed chorismate mutases were purified to homogeneity and the biochemical properties of both chorismate mutases were studied. In addition, we determined the three dimensional crystal structure of Rv1885c chorismate mutase.

Results: The gene Rv1885c from the genome of *Mycobacterium tuberculosis* encodes a 199 amino acid monofunctional and secreted chorismate mutase (*MtCM) with a 33 amino acid cleavable signal sequence, hence belongs to the *AroQ class of chorismate mutases. Consistent with the heterologously expressed *MtCM having periplasmic destination in *E. coli* and the absence of a discrete periplasmic compartment in *M. tuberculosis*, we observed that *MtCM secretes into the culture filtrate of *M. tuberculosis*. *MtCM functions as a homodimer and exhibits a dimeric state of the protein at as low as 5 nanomolar protein concentration. *MtCM exhibits simple Michaelis-Menten kinetics with a K_m of 0.5 ± 0.05 mM and a k_{cat} of 60 s^{-1} per active site (at 37 °C and pH 7.5). The crystal structure of *MtCM has been determined at 1.7 Å resolution (Protein Data Bank ID: 2F6L). The protein is all alpha helical and the active site is formed within a single chain without any contribution from the second chain in the dimer. Analysis of the structure shows a novel fold topology for the protein with a topologically rearranged helix containing Arg₁₃₄. We provide evidence by site directed mutagenesis that the residues Arg₄₉, Lys₆₀, Arg₇₂, Thr₁₀₅, Glu₁₀₉, and Arg₁₃₄ constitute the catalytic site; the numbering of the residues includes the signal sequence. Our

investigation on the effect of phenylalanine, tyrosine, and tryptophan on *MtCM shows that *MtCM is not regulated by the aromatic amino acids. Consistent with this observation, the x-ray structure of *MtCM does not have an allosteric regulatory site.

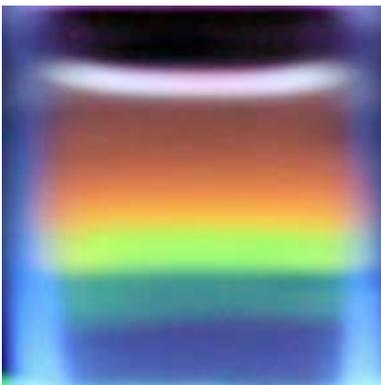
Future Plans: We are in the process of synthesizing small molecule inhibitor. We will test for inhibition of chorismate mutase activity as well as the effect of the inhibitor on the growth of *Mycobacterium tuberculosis*. We hope this research would lead to new drug discovery for *Mycobacterium tuberculosis*.

Photo-Assisted Tuning of Si Nano-Crystal Photoluminescence

Jonghoon Choi, Nam Sun Wang, and Vytas Reipa

Silicon is rather inefficient light emitter due to the indirect-band gap electronic structure, requiring a phonon to balance electron momentum during interband transition. Fortunately, momentum requirements are relaxed in 1 to 5 nm diameter Si crystals as a result of quantum confinement effects and bright photoluminescence in the UV/VIS range is achieved. Photoluminescent Si nanocrystals along with C and SiC based nanoparticles are considered bio-inert and may lead to the development of biocompatible and smaller probes than metal chalcogenide-based quantum dots. Published Si nanocrystal production procedures typically do not allow for the fine control of the particle size. An accepted way to make H-terminated Si nanocrystals consists of anodic Si wafer etching with subsequent breakup of the porous film in an ultrasound bath. However, a rather poly-disperse mixture is produced after the ultrasonic treatment leading to distributed band gap energies and degree of surface passivation. From the technological point of view, a homogeneous size nanoparticle mixture is highly desirable.

In this study, an efficient way to reduce H-terminated Si nanocrystal diameter and narrow size distribution through photo-catalyzed dissolution in the HF/HNO₃ acid mixture was demonstrated. Si particles were produced using lateral etching of Si wafer in HF/EtOH/H₂O bath followed by sonication in a deaerated methanol. Initial suspensions exhibited broad photoluminescence in the red spectral region. Adding an HF/HNO₃ acid mixture to the suspension and exposing it to 340nm light carried out the photo-assisted etching. Photoluminescence and absorbance spectra, measured during the dissolution show gradual particle size decrease as confirmed by the photoluminescence blue shift. Simultaneous narrowing of photoluminescence spectral bandwidth suggests that dissolution rate varies with particle size. Photoluminescence quantum yields up to 60% were observed following acid treatment indicating efficient passivation of the nanocrystal surface states.



Multicolor photoluminescence pattern in the stationary Si nanoparticle suspension during the UV catalyzed acid dissolution reflecting median particle diameter variation from 3.2 nm (red) to 1.5 nm (blue).

Scanning Temperature Gradient Focusing

Authors: David Ross (831), Karin Balss (836), Stacey J. Hoebel (SURF), Barbara Jones (831), Matt Munson (831), Constantin Malliaris (SURF), and Wyatt Vreeland (831).

CSTL Program: Technologies for Future Measurements and Standards

Context and Approach: This project is part of an ongoing effort to develop new microfluidics-based technologies for chemical and biochemical analysis. In order to realize the promise of microfluidic technologies for fast, portable, and inexpensive analyses, robust methods for sample concentration in small volume microchannels are required. Temperature Gradient Focusing (TGF) is a technique, recently developed at NIST, that can be used for the simultaneous concentration and separation of a wide range of biomolecular analytes. TGF works by balancing the electrophoretic motion of an analyte against the bulk flow of buffer through a microchannel or capillary. A temperature gradient is applied along the length of the channel and a buffer with a temperature-dependent ionic strength is used to create a corresponding gradient in the electrophoretic velocity of the analyte. Consequently, the bulk flow velocity and the electrophoretic velocity will sum to zero only at a single point along the gradient and all of the analyte will move toward that zero velocity point where it will accumulate or focus. Different analytes with different electrophoretic mobilities will focus at different points and are thereby separated.

One potential drawback to TGF is the limited peak capacity; only a small number of analyte peaks (~ 2-3) can be simultaneously focused and separated. For many applications, it is necessary to separate and quantitate a large number of components from a complex sample mixture. In this work we developed a new method that can be used with TGF to provide separations with both high resolution and high peak capacity in short microfluidic channels.

Major Accomplishments: The new method, scanning TGF, was demonstrated to enable high resolution separations of the full range of amino acids. The use of scanning TGF effectively injects and focuses each analyte for a controlled amount of time; separations are more repeatable and quantitative. Peak areas and heights (which are proportional to the sample concentration) have a typical reproducibility of 3% or better. In addition, the counter-flow can be used to exclude matrix interferents from the separation channel. For example, Samples containing high concentrations of serum proteins can be analyzed without treating the channel walls to prevent protein adsorption, because the proteins never enter the channel (see Figure 1). Consequently, the technique can be used to measure the binding of a small molecule (such as a drug) to serum proteins.

Impact: This work has improved our capabilities for separation and detection of complex samples in portable and inexpensive microfluidic systems.

Future Plans: Further work on TGF and related technologies is ongoing. Scanning TGF has become the standard operating procedure in our lab.

Publications: "Scanning Temperature Gradient Focusing " Stacey J. Hoebel, Karin M. Balss, Barbara J. Jones, Constantin D. Malliaris, Matthew S. Munson, Wyatt N. Vreeland, and David Ross, *Analytical Chemistry* 2006, **78**, 7186-7190.

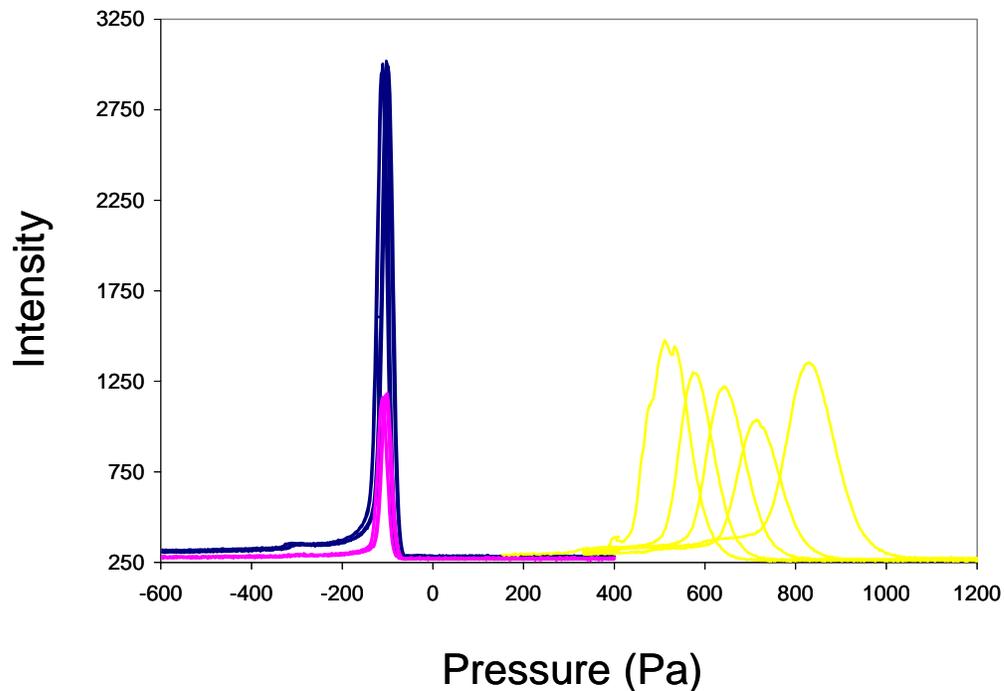


Figure 2. Scanning TGF measurement of a small molecule (fluorescein) in serum protein sample. The blue curves show five repeated measurements of the small molecule concentration in samples without serum proteins. The magenta curves show five repeated measurements of the small molecule concentration in samples containing serum protein. The reproducibility of the peak position with the addition of serum proteins to the sample indicates that the serum proteins are not entering the separation channel. The yellow curves show five repeated measurements of sample similar to those used for the magenta curves, but after the separation channel was intentionally exposed to the serum protein solution. The difference in peak height between the blue and magenta curves is due to the binding of the small molecule to the serum proteins and can be used to measure the binding constant.

Development of Standards and Methods for Measuring Gene Expression Assay Performance

Marc Salit, Jenny McDaniel, Mary Satterfield (Div. 831)

DNA Microarrays are being used to measure gene activity – Gene Expression – across whole genomes, inexpensively and rapidly. The genes being expressed in a cell contribute strongly to the nature of the cell, and their measurement can be used to indicate cell status. Microarray results have been shown to be significantly variable across different platforms, across different laboratories, and even within laboratories in single studies. While this reported variability has presented a major barrier to more broad adoption of the technology, the inability to routinely quantitatively assess performance impedes refinement of the technology, its method of application, and limits confidence in results.

Biochemical Science Division researchers are developing methods and standards that can be used to assess microarray performance quantitatively, with a focus on “spike-in,” or externally added controls. Along with developing standard controls, NIST is developing methods to use them to assess performance.

External RNA Controls Consortium Status

NIST has been hosting an industry-led consortium, the External RNA Control Consortium, in an effort to develop standard approaches to assess gene expression assay performance. This group, now with more than 90 self-declared member organizations, with participation by more than 150 individuals from those organizations, is working together to develop “spike-in,” external controls that can be added to samples to assess assay performance.

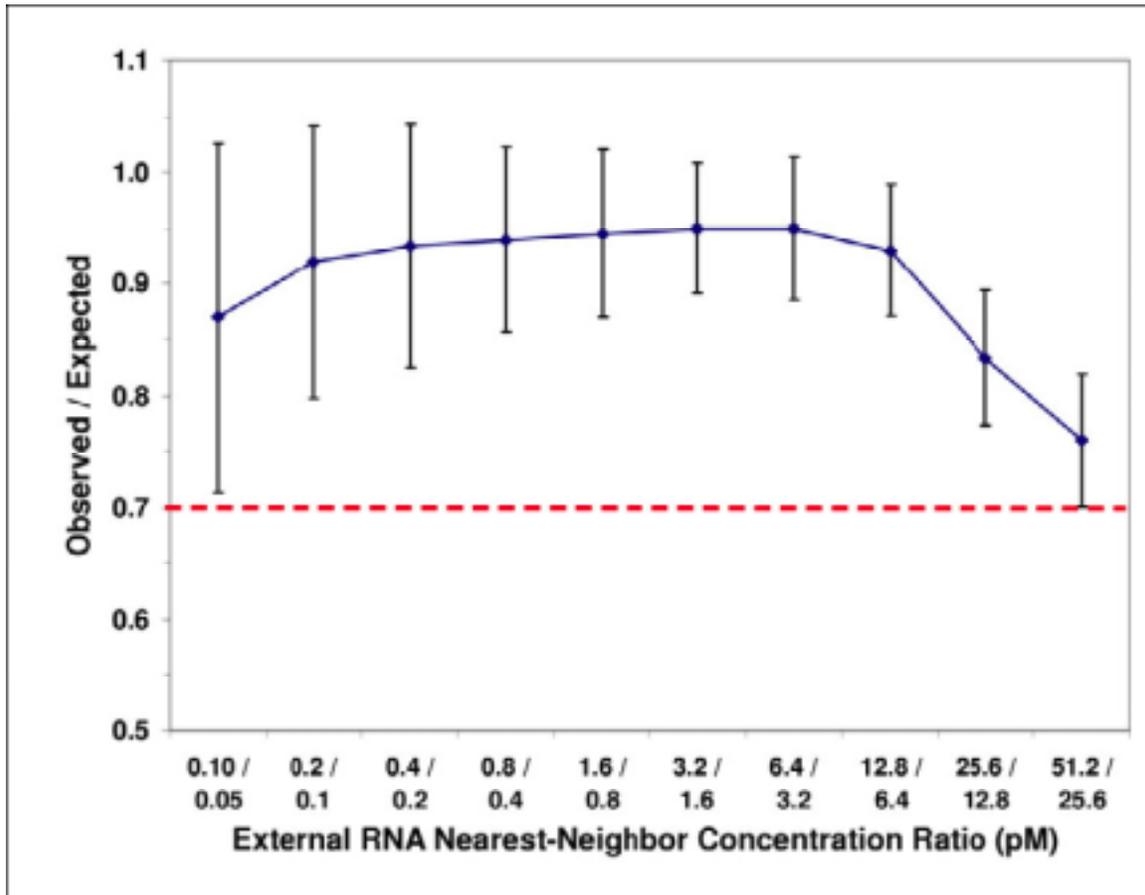
Along with these spike-in controls, working under the auspices of the Clinical Laboratory Standards Institute (CLSI), the ERCC has developed and published a guidance document, “*Use of External RNA Controls in Gene Expression Assays; Approved Guideline.*”¹ This document provides protocols for preparation and use of the spike-ins, along with discussion of the metrics that should be determined to assess performance.

A DNA sequence library has been developed to make these controls from, with 176 different sequences. These sequences are unique with respect to known genomes, and to each other. Most of these sequences were deposited in the library by ERCC members, and NIST arranged for 48 to be synthesized.

RNA is being made from these sequences, and will be distributed to 12 microarray and quantitative PCR laboratories for evaluation in a collaborative study. The design of the

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collaborative study is described in a paper, “*Proposed methods for testing and selecting the ERCC external RNA controls*”²



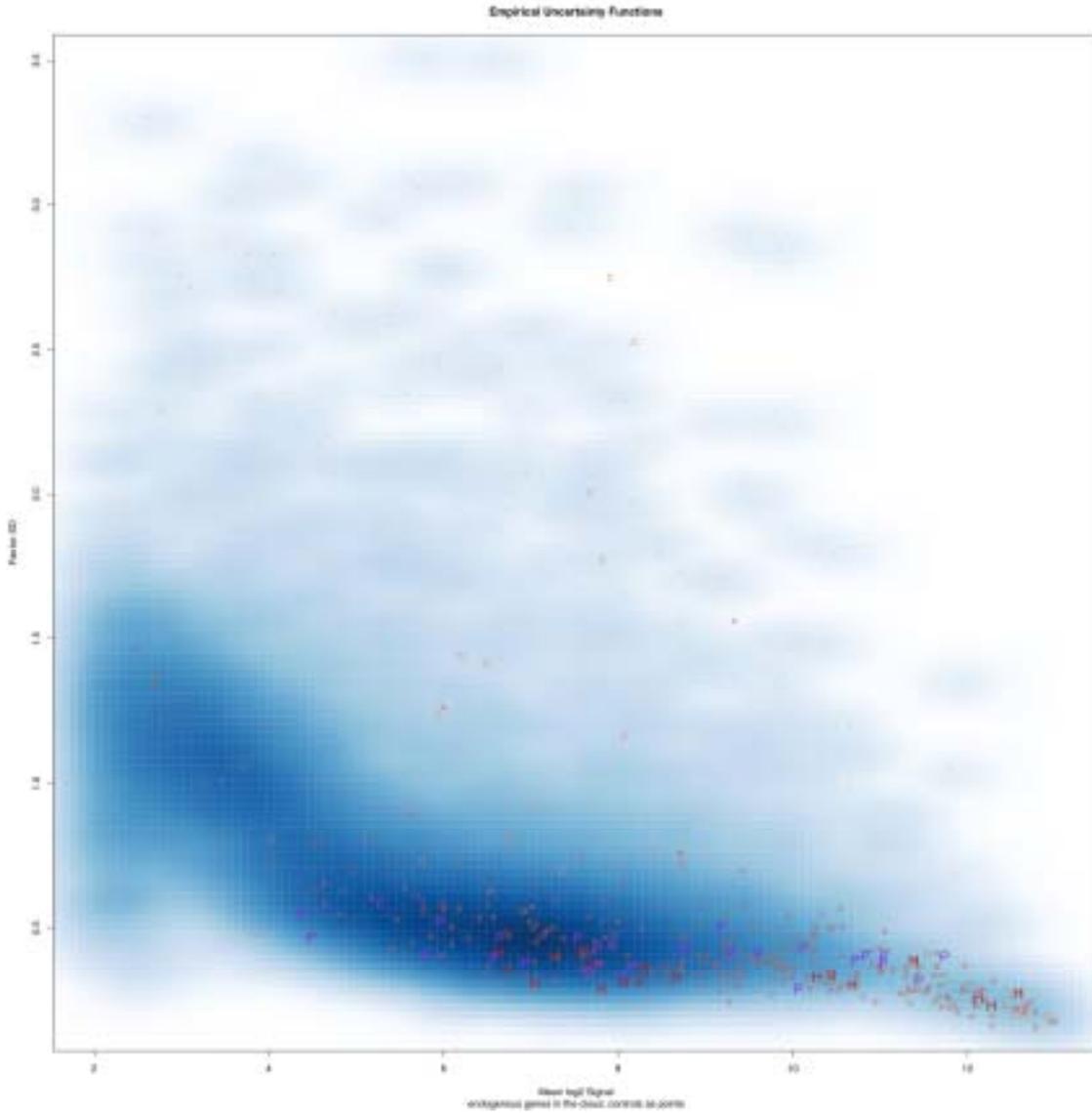
(possible figure for the external RNA testing program)

How well do spike-in controls predict performance of genes under study?

To understand further the degree to which observed spike-in performance can be used to establish performance of the genes under study, the NIST team, including researchers from both the Biochemical Science and Analytical Chemistry Divisions, are collaborating with researchers from Imperial College (IC) in London. Together, we are evaluating a large database of experiments from IC that used a common set of spike-in controls, and establishing that the response from these controls varied in a manner similar to that observed of the non-control genes. Along with the spike-in controls, the variability of so-called endogenous controls is being evaluated, laying the groundwork for further standards and method development.

² *BMC Genomics* 2005, **6**:150

The figure below shows the variability as a function of signal for the bulk of the genes in an array experiment as a density cloud, the endogenous controls as 'o' or 'c', and the spike-ins as 'P' or 'H.'



Title: Microarray Scanner Validation

Author: Mary Satterfield

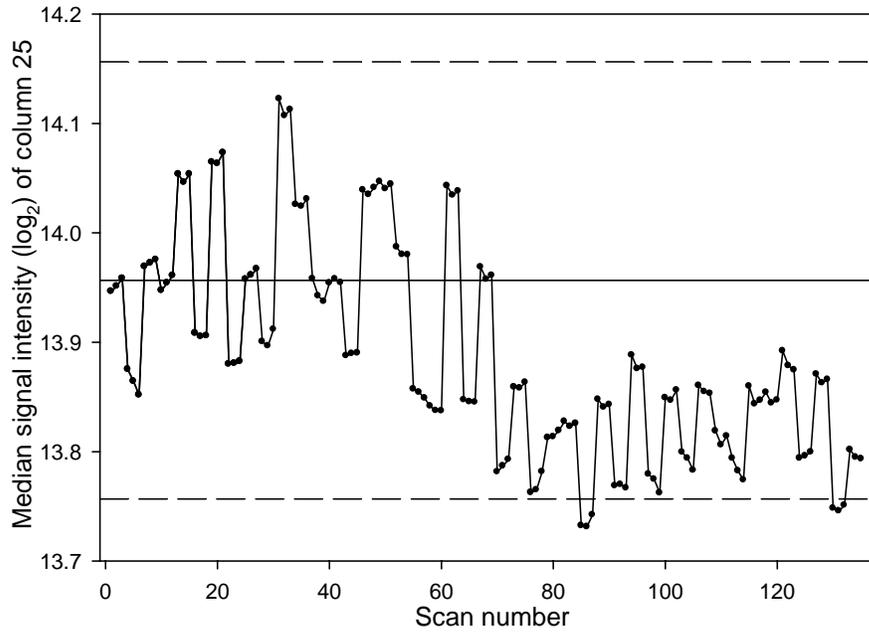
DNA microarrays have the potential to enable specific care based on an individual genome, bringing personalized medicine closer to reality. However, the measurement needs of the technology, including validation, uncertainty, and traceability, must be better understood and met before microarrays are accepted for use in clinical practice. One frequently overlooked source of variability in a microarray experiment is the microarray scanner. Our Gene Expression Metrology Team is developing a method and materials to establish scanner performance, validating signal measurement in microarray experiments by providing quantitative, objective evidence that a scanner is performing consistently from day-to-day. The quantitative performance measures will help to establish the magnitude of variability in the microarray experiment arising from scanner performance.

Under ideal conditions the microarray scanner is expected to produce a stable response to a given material day after day, a response that matches that given on the previous day and with the previous scan, as well as those scans made in the future. However, without validation materials and a quantitative history of stable performance it is difficult to tell that a microarray scanner is not working correctly. Scanner validation would provide an on-going record of scanner performance allowing the user to determine whether the scanner is contributing an undue amount of uncertainty to a microarray experiment. At this time there are no well-characterized methods to track scanner performance or to establish comparability of different scanners.

To validate microarray scanner performance a material that produces the same response under given conditions is required in order to judge the instrument performance independently from the performance of the material in use. Using dye slides with multiple identical rows of successive dilutions of Cy3 and Cy5 dyes, figures of merit including signal intensity, slope of the linear region, limit of detection, background, and signal-to-noise are under investigation. As an investigation of the suitability of this proposed tool for scanner validation, dye slides from three different lots (two “in-date,” and one expired, some slides stored desiccated per manufacturers specifications, and some stored in room air) have been scanned in triplicate daily, weekly, or at the beginning and end of the experimental period. The effects of a variety of factors on scanner performance over a five-week period as established with the dye slide were investigated. These factors (both slide- and scanner-related) include the effects of the number of scans, age of slide, slide lot, scanner unit, and scanner calibration on performance.

Tracking the figures of merit over time reveals whether they may be appropriate as measures of scanner performance. Significant differences in the Cy5 versus Cy3 dye performance are evident, with the Cy5 dye showing signs of degradation that make separation of the instrument performance from the dye performance difficult. The stability of the Cy3 slope and signal intensity of a column within the linear region observed with control charts indicates the potential of these figures of merit for scanner

validation. An additional figure of merit, background, holds promise for use with both dyes, as a way of measuring scanner performance over time and determining if the associated instrument variability is acceptable. Through the use of these proposed figures of merit, a better understanding of the variability contributed by the microarray scanner to the overall experiment is possible.



Control chart of signal intensity of one concentration from the linear range of the Cy3 serial dilution series. Triplicate scans were made twice daily morning and afternoon over a five week period. The broken reference lines represent 3 standard deviations from the mean (solid line) of the first week's data.

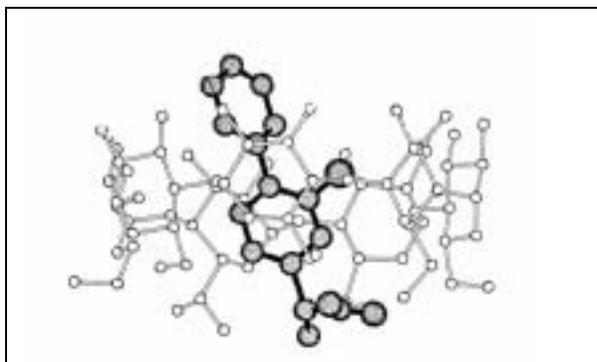
A Drug-Binding Database for Developing and Evaluating Drug Docking Algorithms

N. Todorova (UMD) and F.P. Schwarz (831)

Pharmaceuticals and Biomanufacturing or Measurement Standards

Introduction: Drugs are designed to target and inhibit the function of proteins, which constitute the pathways that advance the level of disease in cells. Drug development in the pharmaceutical industry involves high throughput screening of large libraries of diverse molecular structures to determine if they inhibit the protein target. A typical success rate for the initial screening of possible drug candidates is 3-6 hit series out of 750,000 compounds. If the structure of the protein target is known, either through crystallography or homology modeling, then the screening process and success rate can be significantly enhanced through computationally high affinity “docking” of the molecular structure into the active site on the target. Accordingly, pharmaceutical companies have made a significant investment in computational screening, with the docking-and-scoring software alone costing \$200k to \$1M per year per company. Although docking algorithms have been developed, it was found that there is very little correlation between the “fit” of the drug in the binding site and its binding affinity or potency. It is not clear as to whether newly developed docking algorithms are sufficient or as to whether the experimental data employed to validate the docking algorithm are accurate. To address the accuracy of the binding data, the binding of drugs to cyclodextrins was identified as a binding system sufficient for use in the validation of newly developed docking algorithms.

Major accomplishments: Thermodynamic binding parameters, of the drugs flurbiprofen (FLP), nabumetone (NAB), and naproxen (NPX) binding in the cavity of β -cyclodextrin (β CD) and in the larger cavity of γ -cyclodextrin (γ CD) in sodium phosphate buffer were determined from isothermal titration calorimetry (ITC) measurements over the temperature range from 293.15 K to 313.15 K. A fluorescence binding assay was developed for FLP and NAB, based on the enhancement of the drug fluorescence from an aqueous environment to the hydrophobic



Crystal structure of FLP in the β CD cavity

environment of the cyclodextrin cavity. The drug binding affinities range from 367 M^{-1} for NPX binding to γ CD at 313.15 K to 9520 M^{-1} for FLP binding to β CD at 293.15 K, over a factor of almost 30 and, thus, a viable range of values for validating binding affinities calculated from the docking algorithms. Since docking algorithms can also exhibit a dependence of binding affinity on pH and salt concentration, the binding affinities were also determined at 298.15 K as a function of pH from 6 to 8 and sodium chloride concentration up to 0.3 M. From comparisons of the results of the fluorescence assays to the

fluorescence enhancement of the drug in a hydrophobic solvent such as isopropanol, it was found that water reorganization plays an important role in the thermodynamics of high affinity drug binding in the CD cavity. The role of water reorganization in the binding reaction was also confirmed by the heat capacity changes of the binding reactions.

Impact: The results are now in press and to quote the comment of one of the reviewers on the paper, “their careful control for pH, temperature, and salt concentration is very thorough and the data are of high quality. The basis for the experiments is well established in the paper, with the role of water release/retention a key concept in the interpretation of binding thermodynamics and drug design.” This research emphasizes that water has to be taken into account when developing drug docking algorithms even for the simple model of drug binding in the cyclodextrin cavity.

Future Plans: A data base on drug binding to two isoforms of the $\pi 38 \alpha$ Map kinases, a popular protein drug target in the treatment of inflammation, is being developed for use in the development and validation of drug docking algorithms.

Publication: Todorova, N.A. and Schwarz, F.P., “*The role of water in the thermodynamics of drug binding to cyclodextrin*”, Journal of Chemical Thermodynamics in press.

This research was supported by the 2006 CSTL Exploratory Fund.

Gradient Elution Moving Boundary Electrophoresis for Microfluidic Separations

J. Shackman, M. Munson, D. Ross (Div. 831)

This project is part of an ongoing effort to develop new microfluidics-based technologies for chemical and biochemical analyses. In order to realize the full potential of miniature microfluidic devices for totally integrated and portable measurements, separation methods that can be performed on very short length scales are required. Gradient elution moving boundary electrophoresis (GEMBE) is a new technique recently developed at NIST that can perform quantitative analyses in microchannels less than one centimeter in length. GEMBE has allowed for the development of higher throughput, parallel analysis devices without requiring a significant increase in device size as seen with other techniques.

Gradient Elution Moving Boundary Electrophoresis (GEMBE) is a novel method for performing electrophoretic separations. GEMBE utilizes the electrophoretic migration of chemical species in combination with variable hydrodynamic bulk counter-flow of the solution through a separation capillary or microfluidic channel. Continuous sample introduction is used, eliminating the need for a sample injection mechanism. Only analytes with an electrophoretic velocity greater than the counter-flow velocity enter the separation channel. The counter-flow velocity is varied over time so that each analyte is brought into the separation column at different times, allowing for high-resolution separations in very short channels. The new variable of bulk flow acceleration affords a new selectivity parameter to electrophoresis analogous to gradient elution compositions in chromatography. Because it does not require extra channels or access ports to form an injection zone and because separations can be performed in very short channels, GEMBE separations can be implemented in much smaller areas on a microfluidic chip as compared to conventional capillary electrophoresis.

GEMBE separations of small dye molecules, amino acids, DNA, and immunoassay products have been demonstrated. A low-cost polymeric eight-channel multiplexed microfluidic device was also fabricated to demonstrate the reduced area requirements of GEMBE; the device was less than one square inch in area and required only $n+1$ fluidic access ports per n analyses (in this instance nine ports for eight analyses). Parallel separations of fluorescein and carboxyfluorescein yielded less than 3 % relative standard deviation (RSD) in inter-channel migration times and less than 5 % RSD in both peak and height measurements. The device was also used to generate a calibration curve for a homogeneous insulin immunoassay using each of the eight channels as a calibration point with less than 5 % RSD at each point with replicate analyses.

Impact:

This new separation method allows for reduced size requirements for portable microfluidic devices. Higher analysis element density will allow for more efficient measurements on smaller devices.

Future Plans:

Research using GEMBE is on going as the technique's fundamental figures of merit are explored, and the method is optimized. The potential to measure complex samples, such as found in biology, is being investigated, as the counter flow allows for the exclusion of separation degrading matrix interferants.

Output:

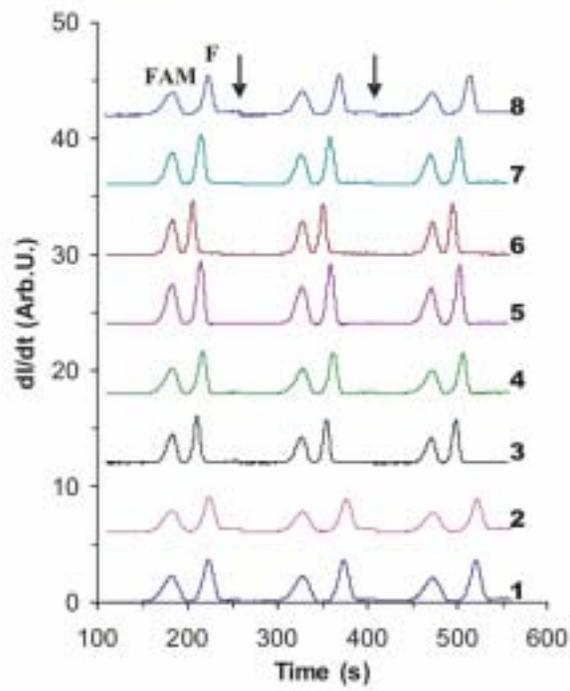
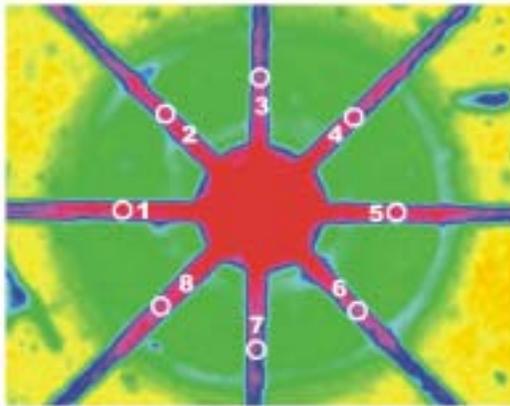
Shackman, J.G.; Ross, D. “**Electrophoretic Separations in small spaces: Gradient Elution Moving-Boundary Electrophoresis (GEMBE)**”, In *Proceedings of μ TAS 2006 Conference*, Kitamori, T.; Fujita, H.; Hasebe S. Eds.; Society for Chemistry and Micro-Nano Systems, Tokyo, 2006; pp. 912-914.

Shackman, J.G.; Munson, M.S.; Ross, D. “**Gradient Elution Moving Boundary Electrophoresis (GEMBE) for High-Throughput Multiplexed Microfluidic Devices**”, *Analytical Chemistry* In Press 2006.

Shackman J.G.; Ross, D. “**Gradient Elution Moving Boundary Electrophoresis (GEMBE)**”, Recommended for U.S. Patent by NIST Patent Review Committee, Nov. 2006 (Disclosure 06-011).

Figure:

Eight channel multiplexed microfluidic GEMBE separation. (Top) False color fluorescence image of detection region of microchip; the 7.1 mm long channels contain fluorescein and carboxyfluorescein (FAM). The common control port and the eight radially arranged channels are seen as red (blue at the outer edges of the image). The eight detection points are shown as the small white circles. (Bottom) Derivative intensity (dI/dt) plots for parallel separation of fluorescein (F) and FAM (500 nmol/L of each). Sample was loaded into each of the eight sample reservoirs; traces relate to each channel with three sequential runs (delineated by arrows).



Gene family workflow system for genome analysis

Arlin Stoltzfus (CARB and Biochemical Science Division, NIST), Tom Hladish (CARB), Vivek Gopalan (CARB)

Programs: Health, Pharma

Context and approach: Genome sequences are an enormous resource, but the practical ability to tap this resource depends on comparative methods that use data from different species. Genome annotation, drug target discovery, biomolecule engineering, and medical genetics rely on such comparisons, but most such efforts are still a long way from the ideal of applying a probabilistic model of evolutionary change to all relevant data. The success of a robust approach to comparative analysis depends on software tools that facilitate the storage, exchange, processing and visualization of genomic data together with phylogenetic trees.

To facilitate phylogeny-based analysis of comparative data, we have developed a software pipeline to assemble sequence family data sets that contain cleaned gene and protein sequence data, taxonomic identifiers, alignments, phylogenies, intron positions, and other data.

Major accomplishment: We have implemented the EGFAM (Eukaryotic gene families) pipeline, which uses data drawn ultimately from GenBank, SwissProt, Pfam, and NCBI's organismal taxonomy. In this analysis pipeline, sequence data and intron annotations are checked; families are assembled and redefined to remove duplicates and outliers; sequences are aligned; phylogenies are inferred; and ancestral states are inferred for some characters. The EGFAM pipeline has been implemented using workflow management software that provides generalized methods for specifying dependencies, running programs, and logging effects.

The use of workflow software provides a layer of abstraction that allows flexibility and makes it easier for us to update the steps in the pipeline, or to carry out quality-control analyses. So far we have tested data integrity through the pipeline.

Impact: This pipeline has made it possible for us to distribute data on thousands of sequence families. So far we have not monitored the use of these data sets by other scientists.

Future Plans: A major challenge in genome analysis is to generate sequence family data sets of high quality by automated methods. This seems to be largely a matter of cleaning annotations, removing outliers and optimizing the multiple sequence alignments. In the future we plan to use the workflow system to develop methods for evaluating and improving multiple alignment quality using the BAliBase alignments as an external reference. We hope to develop methods of alignment evaluation and alignment quality control that are of general use in bioinformatics.

Outputs: Sequence family data sets produced by this method are available via our Nexplorer web site. A publication on the workflow system is in preparation (Hladish, Gopalan and Stoltzfus, in preparation).

Title: Thermodynamic studies of ketoreductase-catalyzed reduction reactions in organic solvents

Authors: Yadu B. Tewari (831), Michele M. Schantz (839), David J. Vanderah (831)

Program: Pharmaceuticals and Biomanufacturing (Pharma)

In recent years biocatalysis in organic media has been used for synthesis of rare chemicals that are useful intermediates for the pharmaceutical and agrochemical industries. Lipase-catalyzed esterification and transesterification reactions in organic solvents have been used for the stereoselective resolution of racemic mixtures. Recently, ketoreductase-catalyzed reactions have been used to obtain the desired chiral alcohols by reduction of secondary ketones. These chiral alcohols are useful intermediates in multi-step chiral synthesis for pharmaceuticals, agrochemicals, and liquid crystals. For these reasons, we have carried out thermodynamic studies (equilibrium constants and molar enthalpies of reaction) of representative ketoreductase-catalyzed reactions which have included 2-alkanones, cycloalkanones, 1-phenyl-1-alkanones, 2-substituted cyclohexanones and other substituted secondary ketones. These reactions have been studied both in organic solvents and in supercritical carbon dioxide (SCCO₂). The thermodynamic results obtained in these studies are essential both for the basic understanding of the energetics of these reactions and for the practical utilization (process optimization and heat-balance calculations) of these reactions.

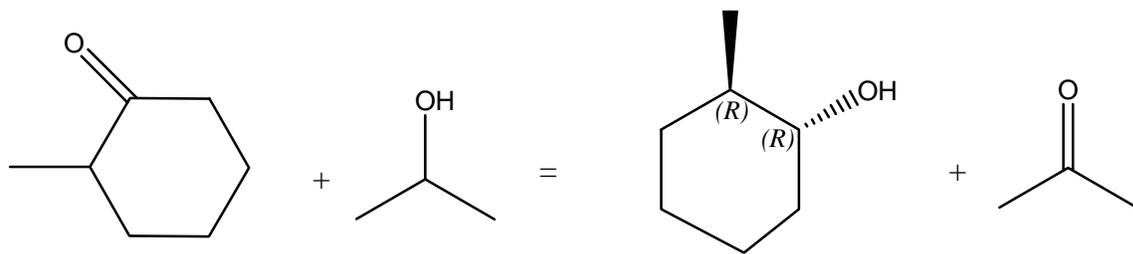
Publications:

Y.B Tewari, M.M. Schantz, K.W. Phinney and J.D. Rozzell, "A thermodynamic study of the ketoreductase-catalyzed reduction of 2-alkanones in non-aqueous solvents." J. Chem. Thermodyn. 37 (2005) 89-96.

Y.B Tewari, K.W. Phinney, and J.F. Liebman, "A thermodynamic study of the ketoreductase catalyzed reactions. 2.Reduction of cycloalkanones in non-aqueous solvents." J. Chem. Thermodyn. 38 (2006) 387-394.

Y.B Tewari, N. Kishore, J. D. Rozzell, D.J. Vanderah, and M.M. Schantz, "A thermodynamic study of ketoreductase-catalyzed reactions.3.Reduction of 1-phenyl-1-alkanones in non-aqueous solvents." J. Chem. Thermodyn. 38 (2006) 1165-1171.

Y.B Tewari, J.F. Liebman, J. D. Eozzell, D.J. Vanderah and M.M. Schantz, "A thermodynamic study of ketoreductase-catalyzed reactions.4.Reduction of 2-substituted cyclohexanones in *n*-hexane." J. Chem. Thermodyn. (in review).



2-methylcyclohexanone

2-propanol

trans-2-methylcyclohexanol

acetone

Thermodynamics of reactions catalyzed by D-hydantoinase and by N-carbamoyl-D-amino acid hydrolase

Yadu B. Tewari, Brian E. Lang, and Robert N. Goldberg (831)

The enzymes D-hydantoinase and N-carbamoyl-D-amino acid hydrolase are used for the commercial production of optically pure amino acids. These optically pure amino acids are, in turn, widely used to produce a wide variety of biotechnology products that include semi-synthetic antibiotics, active peptides, pharmaceuticals, hormones, pesticides, and sweeteners. Interest in these enzymes is also increasing as evidenced by numerous molecular biology studies involving the enzymes and the reactions that the enzymes catalyze. Following the suggestion of an industrial contact who pointed out to us the absence of any thermodynamic investigations, we initiated a series of equilibrium and calorimetric measurements on these reactions with the aim of providing reliable thermodynamic data that could enhance the efficient bioprocess engineering of the reactions catalyzed by these enzymes.

We used HPLC, calorimetry, and equilibrium modeling calculations to study several representative reactions catalyzed by these enzymes. For the reactions catalyzed by D-hydantoinase, we were able to measure values of the apparent equilibrium constants K' . However, the reactions catalyzed by N-carbamoyl-D-amino acid hydrolase proceeded to completion and equilibrium measurements were not possible. Calorimetric measurements were performed on several individual and combined reactions. In those cases where measurements were not possible, we used property values for structurally similar reactions to provide estimates for the needed property values. The thermodynamic results obtained in this study provide quantitative data that can be used for the efficient bioprocess engineering of these enzyme-catalyzed reactions. The results obtained in this investigation are the first to be reported in the literature on the thermodynamics of these reactions.

Publication: "Thermodynamics of Reactions Catalyzed by D-Hydantoinase and N-carbamoyl-D-amino acid hydrolase," *The Journal of Chemical Thermodynamics*, in press.

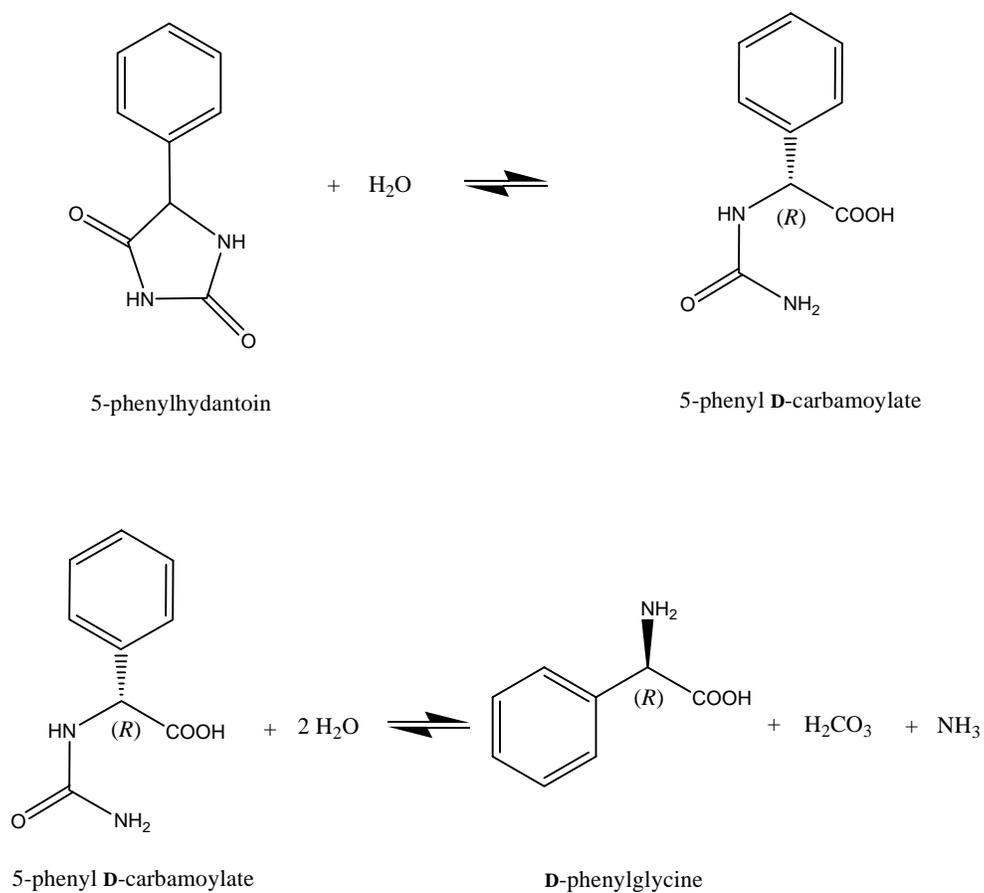
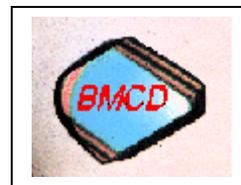


Figure caption: Representative reactions catalyzed, respectively, by D-hydantoinase and by N-carbamoyl-D-amino acid hydrolase.

The New Biological Macromolecular Database (Version 4.0)

M. Tung and T. Gallagher (831)



Pharmaceuticals and Biomanufacturing

Introduction: Since biological function is based on the structure of proteins, major efforts have been dedicated to the determination by x-ray crystallography of the structures of thousands of proteins from key organisms. These methods require high-quality crystals (diffracting to at least 3 Å), so efficient methods of crystallizing proteins are actively being sought, and have resulted in the accumulation of large amounts of crystallogenesis data. The Biological Macromolecular Crystallization Database (BMCD) first developed at NIST in 1985 has archived crystallogenesis data principally from the Protein Data Bank (PDB), now the Research Collaboratory for Structural Bioinformatics PDB. Previous versions of the BMCD drew data from the PDB by accessing the reports on a one to one basis. Recently, improved methods of accessing and importing crystallization data from the PDB have been developed, enabling the BMCD to be updated on a more automated and frequent basis. Recent research has also focused on determining correlations between the physical and chemical properties of a protein and its tendency to form crystals yielding high resolution x-ray diffraction data for structural determination.

Major Accomplishments: The BMCD has now been updated to a β 4.0 test version (BMCD4) and now includes over 8000 entries with crystallization data on proteins and other macromolecules. New entries in the BMCD4 have been expanded to include macromolecule sequence and sequence-derived information, enabling the analysis of relationships between protein properties, crystal growth conditions, and the geometric and diffraction properties of the crystals. The BMCD4 (<http://xpdb.nist.gov:8060/BMCD4>) also offers enhanced search capabilities that can retrieve specified subsets of entries for analysis. For example, utilizing the expanded content and other database features, a strong correlation was found between the distributions of crystal density and diffraction resolution.

Impact: It is expected that with the expansion of the entries in the PDB (eg. recently about 4,000 entries per year) that the BMCD4 will expand accordingly. It is anticipated that the number of hits of this new version of the BMCD would be greater than the number of hits of 1 M per yr of the former version (2.0) of the BMCD.

Future Plans: It is planned to enhance new features on the text search engine in order to perform numeric range searches such as protein crystallization at a specific pH level, and to perform searches with different citation formats. It is planned to add a multidimensional backend to display statistical and summarized features of a particular protein entry taken from the files on the protein crystallization conditions, rather than just a list of files on the protein entry.

A novel metrology system for quantitative profiling of serum proteins.

W.-L. Liao, I. V. Turko (Div. 831)

Program: Pharmaceuticals and Biomanufacturing

Introduction: A novel strategy for quantitative profiling of serum proteome is described. This strategy includes scaling up and ammonium sulfate depletion of original sample, affordable chemistry of stable isotope labeling for big protein sample, separation of labeled unfolded proteins, and quantification by MALDI MS. A number of pre-analytical variables, such as collection, processing, storage and transportation can alter serum proteome. To define conditions of serum handling, analytical methods to evaluate a potential impact should be in place. Stable isotope labeling allows relative quantification of proteins by mass spectrometry and can be used in assessing pre-analytical variables. Stable isotope labeling includes unfolding of proteins with urea, modification with chemicals which introduce stable isotopes and mixing of two samples to be compared at 1:1 ratio. Chromatographic separations before labeling/mixing will cause quantitative variations in samples and make the following quantification irrelevant. It is safe to perform separations after labeling/mixing since the protein ratios are established. Albeit proteins are unfolded they possess charge and size and can be separated by various electrophoretic and chromatographic approaches.

Major accomplishments: Serum proteins were first unfolded in 8 M urea and labeled with a pair of acrylamides, none-deuterated or D₀-acrylamide and deuterated or D₃-acrylamide. Three workflows for separating labeled unfolded proteins were then compared that is (1) liquid-based IEF/2D-PAGE; (2) FPLC/2D-PAGE; and (3) whole gel elution/FPLC. Whole gel elution/FPLC (Fig. 1) which combines electrophoretic separation based on protein molecular weight with the following chromatographic separation in the presence of 8 M urea based on protein charge resulted in quantification of a highest number of serum protein including those which abundance falls in a category of at least 10⁻⁵ to albumin. It makes this robust workflow suitable for quantitative profiling of protein changes in serum associated with pre-analytical variables.

Future plans: Develop criteria for the above metrology system for measuring proteomic changes due to pre-analytical processing.

Publications: Manuscript is pending WERB approval.

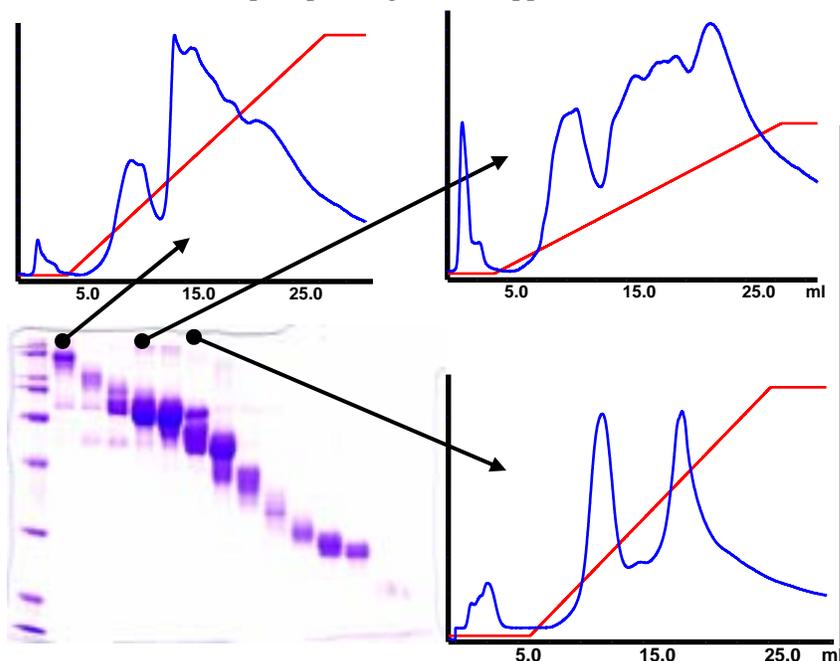


Figure 1. Whole gel elution/FPLC workflow. Pellet of 30-40% ammonium sulfate saturation was first separated into 14 fractions using a Whole Gel Eluter. The proteins in these fractions were further separated on HiTrap Q XL column (1 ml) in 20 mM TrisHCl (pH 8.0)/8 M urea using 0.0 – 0.3 M NaCl linear gradient. Three representative chromatographic profiles are shown.

CSTL Program: Forensics and Homeland Security

Title: Forensic Application of the Affymetrix Human Mitochondrial Resequencing Array

Authors: Peter M. Vallone, John P. Jakupciak and Michael D. Coble

Abstract: In the field of forensic DNA testing, sequencing regions of the mitochondrial genome is performed when insufficient genomic DNA is present for traditional autosomal short tandem repeat (STR) testing. Typically only subsets of the mitochondrial control region (HV1 and HV2) are sequenced for forensic analysis. Sequencing coding region polymorphisms in the mitochondrial genome can be useful for resolving individuals who have the identical HV1 and HV2 control region sequence. Various methods and strategies have been established to interrogate coding region polymorphisms. These range from SNP assays probing sites most likely to differentiate individuals based on their HV1/HV2 sequence to the use of mass spectrometry to pyrosequencing. Our goal is to evaluate the potential of the Affymetrix GeneChip Mitochondrial Resequencing Array (ver 2.0) for forensic applications.

Purpose: To obtain experience with an array based platform and assess its potential for forensic usage. To determine the accuracy of a commercial resequencing array technology. Is the platform robust enough (provide accurate and reproducible base calls) for routine usage within the forensic community?

Major Accomplishments: We are investigating the reproducibility, base call accuracy, sensitivity, and general forensic utility of the array platform. This was done by resequencing 'challenging' samples (of non western European origin) and also resolving Caucasian samples that contain the identical HV1 and HV2 region. All results were compared to full genome sequencing using traditional fluorescent sequencing techniques and capillary electrophoresis as a detection platform. Initial results have been presented at the European Mitochondrial DNA Population Database Project (EMPOP) meeting held in Innsbruck, Austria in the fall of 2006.

Impact: Forensic typing laboratories that perform mitochondrial DNA have expressed an interest in using the GeneChip array platform. The results will allow these researchers to determine if the platform is robust enough for their specific purpose. The information we have obtained will also allow the manufacturer to make improvements in future generations of the mitochondrial GeneChip.

Future Plans: Further testing of the GeneChip to determine the performance on degraded DNA materials. We are also interested in alternative PCR amplification of the mitochondrial genome that may work to increase the sensitivity of the system.

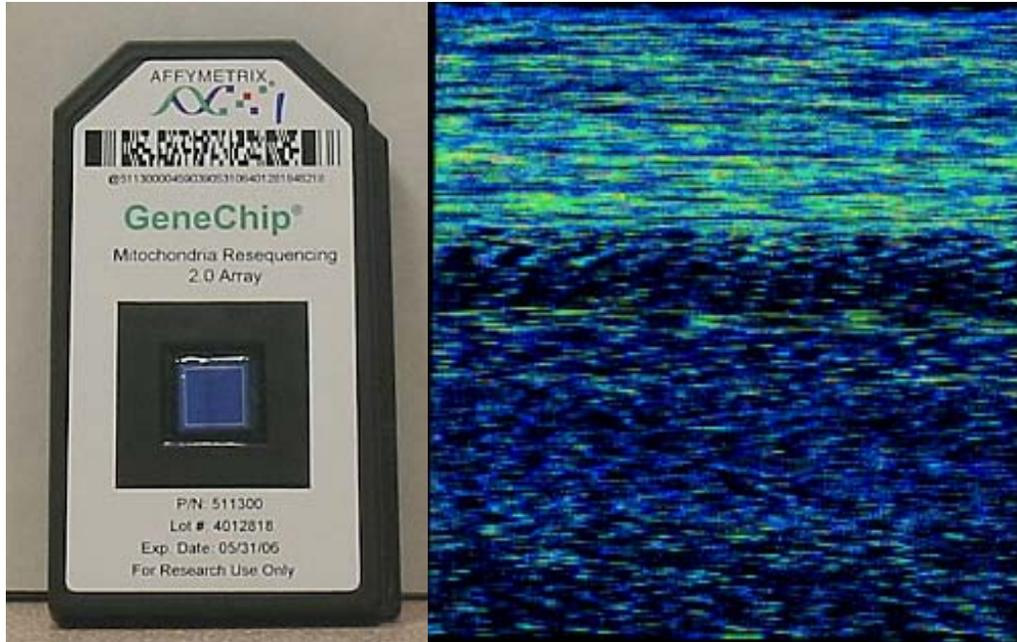


Figure: A picture of the Affymetrix GeneChip Mitochondrial Resequencing Array (left) A fluorescent scan of the array post DNA hybridization (right). Base calls are obtained from software aided analysis of the fluorescent cell intensities.

Publications:

Vallone, P.M., Jakupciak, J.P., and Coble, M.D. Forensic Application of the Affymetrix Human Mitochondrial Resequencing Array. *submitted to Forensic Science International: Genetics in 2006*

Disclaimer: This project was supported by National Institute of Justice Grant Number 2003-IJ-R-029, which is an interagency agreement between [NIJ](#) and the [NIST Office of Law Enforcement Standards](#), awarded by the National Institute of Justice, Office of Justice Programs, US Department of Justice. Points of view in this document are those of the authors and do not necessarily represent the official position or policies of the US Department of Justice. Certain commercial equipment, instruments and materials are identified in order to specify experimental procedures as completely as possible. In no case does such identification imply a recommendation or endorsement by the [National Institute of Standards and Technology](#) nor does it imply that any of the materials, instruments or equipment identified are necessarily the best available for the purpose.

Quantitative Ovalbumin Detection Using Forward-Phase Protein Microarrays and Suspension Arrays

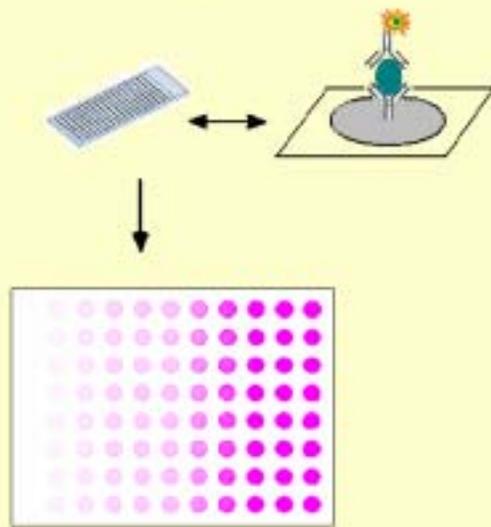
Authors: L. Wang, K.D. Cole, Hua-Jun He, D.K. Hancock, A.K. Gaigalas, Y. Zong

Program: Health and Medical Technology

Protein microarrays, one emerging class of proteomic technologies, have broad and yet unique applications for quantitative analysis and discovery. Comparing to other proteomic technologies, such as mass spectrometry, 2D gel electrophoresis, suspension arrays, micro-ELISA, and multiplexed immunoassays, protein microarrays have a unique capability for investigating inter-molecular interactions between protein-protein, protein-DNA, protein-RNA, protein-cell and therefore, become an extremely useful tool for profiling cellular signal pathways and networks of clinical samples. At the present, there are two formats of protein microarrays, forward phase and reverse phase arrays. With the forward phase arrays, high affinity antibodies are arrayed and a test samples is added to the array. Analysis of the array shows the types of analytes present in the test sample. With the reverse phase arrays, each test sample is immobilized in a single spot and each array is comprised of hundreds of different test samples. The array of test samples is incubated with one type of antibody. By screening a large number of sample arrays against a number of antibodies, the final readings are directly compared across results from multiple samples.

In the present study, we used ovalbumin, a simulant for ricin and botulinum toxins, and its high affinity, commercially available polyclonal antibody as a model system to examine the reliability, comparability, dynamic range, and linearity of the forward phase arrays with respect to the suspension arrays. It was found that protein microarrays had a dynamic range of four orders of magnitude and a sensitivity of less than 1 pg/mL, respectively. The dynamic range and sensitivity of suspension arrays were close to two orders of magnitude and 0.25 ng/mL, respectively. The sensitivity we observed for the suspension arrays is comparable to that reported for enzyme-linked immunosorbent assays (ELISA) in the literature. We used ovalbumin samples with two different purities, 38.0 % and 76.0 % (w/w), as determined by polyacrylamide gel electrophoresis (PAGE). These samples were used to evaluate the effect of impure samples on detection. The data obtained from the forward-phase protein arrays gave values that were consistent with the PAGE data. The data from the suspension arrays were not as consistent and may indicate that this format may not give as reliable data with impure samples. Knowledge of the advantages and disadvantages of the two-proteomic methods would allow their more rational use in clinical diagnosis and ultimately advance overall proteomics effort at NIST. [*J. Proteome Res*, 5, 1770-1775(2006)].

A: Forward-Phase Protein Microarray



B: Suspension Array

