

## Workshop

# “Application of Proteomics and Transcriptomics in EMF Research”

**October 30 - November 1, 2005**

STUK- Radiation and Nuclear Safety Authority,  
Laippatie 4, 00880 Helsinki, Finland

[http://www.who.int/peh-emf/meetings/proteomics\\_helsinki05/en/](http://www.who.int/peh-emf/meetings/proteomics_helsinki05/en/)

- **Workshop Chairmen:** Michael H. Repacholi & Dariusz Leszczynski
- **Workshop Rapporteurs:** Martin L. Meltz & Dariusz Leszczynski
- **Scientific Committee & Sponsoring Organizations:**
  - Dariusz Leszczynski STUK, Helsinki, Finland
  - Michael H. Repacholi WHO EMF Project, Geneva, Switzerland
  - Norbert Leitgeb EU Action Cost281
  - Gerd Friedrich Forschungsgemeinschaft Funk (FGF), Germany
  - Frank Prato URSI Commission K
  - Martin L. Meltz International Committee on Electromagnetic Safety (ICES), USA
  - Franz Adlkofer VerUm Foundation, Munich, Germany



## Scientific Program of the Workshop

# Day 1: Presentations by Experts in High-Throughput Screening Techniques

October 30, 2005, Sunday

**9:00 - 9:05 Welcome**

*Chiyoji Ohkubo & Dariusz Leszczynski*

WHO, Geneva, Switzerland & STUK, Helsinki, Finland

### SESSION 1. Chair - Dirk Koczan

**9:05 - 9:15 WHO Health Risk Assessment Process**

*Chiyoji Ohkubo*

WHO, EMF Project, Geneva, Switzerland



**9:15 - 9:55 Towards System Level Analysis of Biological Questions**

*Risto Renkonen*

Biomedicum, University of Helsinki,  
Helsinki, Finland



9:55 - 10:15 discussion

**10:15 - 10:55 High Throughput Gel-Free Mass Spectrometry-Based Proteomics**

*Timothy Griffin*

Department of Biochemistry, Molecular Biology & Biophysics,  
University of Minnesota, Minneapolis, MN, USA



10:55 - 11:15 discussion

*11:15 - 11:45 Coffee & Tea*

**11:45 - 12:25 2DE as Tool for Proteomics Studies and Protein Identification by Mass Spectrometry**

*Brigitte Wittmann-Liebold*

WITA GmbH, Teltow/Berlin, Germany

12:25 - 12:45 discussion



**12:45 - 13:25 Protein Microarrays - a Versatile Tool for Biomarker Screening**

*Dieter Stoll*

Department of Biochemistry, NMI,  
University of Tübingen, Reutlingen, Germany

13:25 - 13:45 discussion



**13:45 - 14:45 Lunch**

**SESSION 2. Chair: Dieter Stoll**

**14:45 - 15:25 Genomewide Expression Profiling using  
DNA Chip Technology - An Array of Possibilities**

*Dirk Koczan*

Proteome Center, University of Rostock, Germany

15:25 - 15:45 discussion



**15:45 - 16:25 Understanding Complex Biological Systems  
with Metabolomics: An Overview**

*Roy Goodacre*

School of Chemistry, University of Manchester, UK

16:25 - 16:45 discussion



**16:45 - 17:15 Coffee & Tea**

**17:15 - 17:55 Building a Synapse Proteome**

*J. Douglas Armstrong,*

Institute for Adaptive and Neural Computation,

School of Informatics, University of Edinburgh, Scotland, UK

17:55 - 18:15 discussion



**18:15 - 18:55 Bioinformatics and Data Mining**

*Juha Saharinen*

Department of Molecular Medicine,

National Public Health Institute, Helsinki, Finland

18:55 - 19:15 discussion



**20:30 - 23:00 Dinner for invited speakers (sponsored by NOKIA)**

## Day 2: Presentations by EMF Researchers using High-Throughput Screening Techniques

October 31, 2005, Monday

### SESSION 3. Chair: John Timms

**9:00 - 9:30 Use of Transcriptomics and Proteomics in EMF Research: Present Status and Future Needs**

*Dariusz Leszczynski*

Functional Proteomics Group, Radiation Biology Laboratory, STUK, Finland



9:30 - 9:40 discussion

**9:40 - 10:10 2D Gel-Based Proteomics: Application in EMF-Treated Human Glioma Cells and Future Directions**

*Frank Witzmann*

Department of Cellular and Integrative Physiology, Indiana University Med. Sch., Indianapolis, IN, USA



10:10 - 10:20 discussion

**10:20 - 10:50 Gene Expression Profiles in Volunteers Exposed to a 50 Hz EMF**

*James Metcalfe*,

Department of Biochemistry, University of Cambridge, Cambridge UK



10:50 - 11:00 discussion

**11:00 - 11:30 Coffee & Tea**

**11:30 - 12:00 Application of Genomic and Proteomic Approaches in Studying Bioeffects of EMF**

*Zhengping Xu*

Bioelectromagnetics Laboratory, Medical School, Zhejiang University, Hangzhou, China



12:00 - 12:10 discussion

**12:10 - 12:40 Genomic and Proteomic Assessment of 0.78 and 10 ns Pulsed Electric Fields on Human Cells: Results, Issues, and Approaches**

*Martin Meltz*

University of Texas Health Science Center, San Antonio, TX, USA



12:40 - 12:50 discussion

**12:50 - 13:50 Lunch**

**SESSION 4.** Chair: Frank Witzmann

**13:50 - 14:20 The Number of Gene-Expression Changes after Chronic Exposure to CDMA or FDMA Radiofrequency Radiation does not Change Exceed the False Positive Rate**

*Joseph Roti Roti (to be presented by Martin Meltz)*

Division of Biology & Biomedical Sciences,  
Washington University Medical School, St. Louis, MO, USA



14:20 - 14:30 discussion

**14:30 - 15:00 In Vitro Gene Expression Studies and Their Impact on High Content Screening Assays in EMF Research**

*Christian Maercker*

Mannheim University of Applied Sciences,  
Mannheim/Heidelberg, Germany



15:00 - 15:10 discussion

**15:10 - 15:40 The Proteomic Response of *S. Pombe* to Static and Oscillating Low-Field Strength EMFs**

*John Timms*

Ludwig Institute for Cancer Research, Proteomics Unit,  
London, UK



15:40 - 15:50 discussion

**15:50 - 16:20 Coffee & Tea**

**16:20 - 16:50 Alteration of gene expression in cultured human cells by exposure to 2.45 GHz RF fields**

*San Ming Wang*

Center for Functional Genomics, Northwestern University,  
Evanston, IL, USA



16:50 - 17:00 discussion

**17:00 - 17:20 The Proteomic Analysis of Human Endothelial Cells Exposed to Mobile Phone Radiation**

*Reetta Nylund*

Functional Proteomics Group, Radiation Biology Laboratory,  
STUK, Finland



17:20 - 17:30 discussion

**17:30 - 17:50 Proteomic Assessment of Macrophage Activation in a Plasma Bioassay**

*Roza Sypniewski*

General Dynamic, Brooks City Base, TX, USA



17:50 - 18:00 discussion

**18:10 - 19:50 Working groups preparing summary statements**

*Coffee & Tea will be served in the working rooms*

**20:00 - 22:00 Get-together event with snacks and wine (STUK's library)**

## Day 3: Summaries & rapporteurs' reports

*November 1, 2005, Tuesday*

**9:00 - 9:30** Statement proposals

**9:30 - 11:00** Discussion on statements (Moderators: *Tim Griffin & Jim Metcalfe*)

*11:00 - 11:30 Coffee & Tea*

**11:30 - 12:00** Rapporteurs' report (Rapporteurs: *Dariusz Leszczynski & Martin Meltz*)

**12:00 - 13:00** General discussion (Moderators: *Chiyoi Ohkubo & Dariusz Leszczynski*)

*13:00 - departure (on your own)*

## **The Role of Proteomics and Transcriptomics for EMF Health Risk Assessment: A WHO View**

Chiyoji Ohkubo and Michael H Repacholi

World Health Organization, Switzerland  
*[ohkuboC@who.int](mailto:ohkuboC@who.int)*



Extensive research has been conducted into possible health effects of exposure to electromagnetic fields (EMF). There is clear scientific evidence of hazardous health effects when exposure levels are sufficiently high. In such cases, when risk is quantifiable, evidence-based health risk assessment is practicable. At lower levels of exposure, such as typically found in the environment, it is the absence of data demonstrating a health risk that makes health policy difficult to establish, particularly when public concern is high. Through the International EMF Project, WHO is assessing health effects of exposure to static and time varying electric and magnetic fields in the frequency range 0 - 300 GHz.

The main objectives of the Project are to carry out reviews of the scientific literature on possible health risks from EMF exposure, identify gaps in knowledge, and establish a research agenda. The International EMF Project developed a Research Agenda in order to facilitate and coordinate research on the possible adverse health effects of non-ionizing radiation.

The RF Research Agenda defines as high priority research whose results would contribute to the WHO health risk assessment for RF exposures. The Research Agenda is ordered in separate sections by the weight each research activity carries in human health risk assessment: epidemiology, laboratory studies in humans; laboratory studies in animals and laboratory studies in tissues, cells and cell-free systems. It should also be recognized that, while epidemiological and human laboratory studies directly address health-related endpoints, cellular and animal studies are of value in assessing causality and biological plausibility. On the other hand, cellular model systems are excellent candidates for investigating the ability of RF exposures to have subtle or synergistic effects with agents of known biological activity. New research fields on phosphorylation profiling and other high-throughput assays characteristic of proteomics and related research areas may become useful tools for this purpose. The purpose of this Workshop is to provide the information needed to make an assessment of whether proteomics and transcriptomics should be promoted as a useful tool for EMF research.

## Towards System Level Analysis

Risto Renkonen

Rational Drug Design Program, Department of Bacteriology and Immunology, Haartman Institute and Biomedicum, P.O. Box 63, FIN-00014 University of Helsinki, Finland

&

MediCel Ltd, Haartmaninkatu 8, FIN-00290 Helsinki, Finland

[risto.renkonen@helsinki.fi](mailto:risto.renkonen@helsinki.fi)



Examples of system level analysis are described:

- To understand the system level role of GDP-mannose, we studied a conditional knock-out strain of the key enzyme in its synthesis; *PMI40*. The experimental procedure allowed us to study the order of mechanisms the cells launch in order to adjust to a sudden malfunction in the metabolic machinery. We collected 100 samples from the continuous cultivations for 80 hours and measured genome-wide gene expression levels, 10 enzyme activities, and concentrations of 30 intracellular metabolites. In order to carry out this magnitude of experimentations we needed to generate a bioLIMS to handle all the experimentations and generated data. Furthermore we built a sample-taking robot, which automatically took and preserved the samples. A proprietary software platform, with e.g. workflow and pathway editors, was generated for the in silico part of the work. After normalization and clustering, significantly changed genes and metabolites were searched for enrichment in biological processes, molecular functions, and macromolecular complexes. Further, gene expression levels, metabolite concentrations, and enzyme activities were searched against each other for causality over time. Overall, we focused on thorough analysis of our own data and known database data in order to reward our efforts with knowledge.
- At the transcriptome level, repression of *PMI40* activated various stress responses, such as osmotic stress, heat, oxidative stress, nutrient depletion, and DNA damage. Unexpectedly one third of the stress genes were induced even before the repression of *PMI40* had affected the corresponding enzymatic activity or GDP-mannose concentration. Genes involved in response to osmotic stress were well represented in this set, suggesting a role for the Hog1p-MAPK cascade in the initial stress response. Phd1p and Skn7p were identified as high-ranking transcription factors possibly playing a role in the initial regulatory events. Over a longer time frame the repression of *PMI40* led to starvation, as indicated by the induction of genes involved in filamentous growth and mating.

## High Throughput Gel-Free Mass Spectrometry-Based Proteomics

Timothy J. Griffin

University of Minnesota, Department of Biochemistry, Molecular Biology  
and Biophysics, Minneapolis, MN 55455, USA

[tgriffin@umn.edu](mailto:tgriffin@umn.edu)



The results generated from the Human Genome Project and other genome sequencing initiatives has revolutionized biology, catalyzing the emergence of new technologies that enable the systematic, quantitative analysis of genes and gene products. One of the most powerful of these emerging technologies has been the use of mass spectrometry to identify and also quantify expressed proteins in complex mixtures in a sensitive and high-throughput fashion. This technology has spawned the field of proteomics, with the stated goal of comprehensively characterizing protein products and their function. The combination of stable-isotope labeling of proteins and peptides, gel-free multidimensional separations, automated mass spectrometric analysis and automated sequence database searching has provided for a core methodology for large-scale identification and quantification of proteins contained in complex mixtures. This presentation will describe the state-of-the-art mass spectrometry-based gel-free technologies that have emerged to address a wide-array of biological problems that cut across the fields of biochemistry, molecular and cellular biology, and clinical research. Topics that will be discussed include: the analytical methodologies that collectively make up the current system for quantitative, gel-free mass spectrometry-based proteomics; and the application of these tools to a wide-variety of biological problems, including large-scale analysis of protein expression and abundance changes, and protein modifications.

## **2DE as Tool for Proteomics Studies and Protein Identification by Mass Spectrometry**

Brigitte Wittmann-Liebold, Thomas Pohl and Hanns-Rüdiger Graack



WITA GmbH TELTOW, Warthestr. 21, 14513 Teltow, Berlin-Brandenburg,  
Germany

[wittmannliebold@wita.de](mailto:wittmannliebold@wita.de)

New instrumentation and advanced technologies provide Proteomics studies in Cell Biology, Molecular Medicine and in drug design. Proteomics not only is essential to define different states of cell diseases, interaction of pathways on the individual protein level and in complex structures, but also assists to design new biological and diagnostic markers. Proteomics enhances our knowledge in cell differentiation, molecule transportation and signalling. New data on translation, regulation and evolution became available. Proteomics work is being applied to study antibiotic resistant strains and tissues of various lung-, brain, and heart-diseases. It cumulated in the identification of antigens for the design of new vaccines.

These advancements in Proteomics were possible through the development of two-dimensional gel electrophoresis, e.g. in the Immobilone or NEPHGE-technique, of total protein mixtures of cell lysates or tissues in combination with high sensitive mass spectrometry. For safe protein identification in complex protein mixtures it is essential to use high resolution separation methods in order to assign proteins unambiguously. WITA GmbH has contributed to these enhanced techniques by introducing new high resolution 2D-gel electrophoresis equipment that allows to separate 5.000 to 10.000 proteins per gel for Proteome analyses.

The present technological achievements are well suited for high-throughput-screening of tissues after radiation and to define biological targets of these or other electromagnetic field health effects.

## Protein Microarrays -A Versatile Tool for Biomarker Screening

D. Stoll, M.F. Templin, M. Pawlak, T.O. Joos

NMI Natural and Medical Sciences Institute at the University of Tuebingen,  
Markwiesenstr. 55, 72770 Reutlingen, Germany  
[stoll@nmi.de](mailto:stoll@nmi.de)



**INTRODUCTION:** Based on the results of the ongoing efforts in genome sequencing projects well-established DNA microarray technology and sophisticated bioinformatics platforms allow scientists to take a global view into biological systems today. But the primary actors in biological systems depicting the overall cell status in the most direct way are proteins. Therefore methods which are able to display changes in the time or stimulus dependent expression and structure of multiple proteins in biological systems are valuable tools for the “discovery-science” of biological effects on the molecular base. The highly dynamic and diverse world of protein action and interactions has been a stimulus for the development of new, highly sophisticated protein separation and analysis technologies. So far none of these technologies is able to analyse a total proteome in its challenging complexity. Only the combination of technologies and the selection of methods based on the desired throughput, the depths of information, the analytes of interest and the economical aspects enable the generation of valuable information from proteome analyses. Protein microarrays are now ready to serve as versatile tools to screen entire genomes for distinct proteins that interact with particular factors, catalyse particular reactions, act as substrates for protein-modifying enzymes and/or as targets of autoimmune responses. Therefore protein microarrays are useful tools supplementing other technologies like e.g. 2DE-PAGE / mass spectrometry or liquid chromatography / mass spectrometry.

**OBJECTIVES:** The talk will give a brief introduction showing the advantages of microspot capture assays and the prerequisites to establish and perform such assays. The basic principles and the application fields of planar microarray based systems and bead based flow cytometry approaches will be presented and discussed with respect to different applications. Normal phase and reverse phase assay principles will be compared. Advantages, expectations and limitations of such miniaturized multiplexed ligand binding assays will be discussed together with their huge potential for proteomic research, diagnostic purposes and their potential use in the elucidation of biological effects. Selected promising applications of protein microarray based assays in antibody characterization, the identification and quantification of multiple biomarkers in cancer tissues or cell samples will be presented.

## Genomewide Expression Profiling using DNA Chip Technology: An Array of Possibilities

Dirk Koczan

Proteome Center, University of Rostock, Germany  
[dirk.koczan@med.uni-rostock.de](mailto:dirk.koczan@med.uni-rostock.de)



Although we are able to generate full genome RNA profiles as an outcome of genome projects and development of high density oligonucleotide arrays new dimensions and challenges appear at the horizon. The increasing complexity of the transcriptome by alternative splicing- and processing events and the recently discovered highly complex whole genome transcriptional activity using so called “tiling arrays”(Cheng et al., 2005) are the next major goals. Not only that regulatory unknown noncoding RNA’s like microRNA’s were found, but also it appears that the dimension of dynamic regulation of the polyadenylated mRNA fraction was unknown so far. Those new findings put into question how complete our view of transcriptome regulation is. Semiconductor photolithography, such as Affymetrix GeneChip technology possesses the perspective to produce arrays with an increased density to have a deeper look in the dynamic processes occurring in different compartments of the cell. Recent technological bottlenecks are:

- The 3’ biased labeling protocols using oligo-dT primers and linear amplification by T7-Polymerase *in vitro* transcription and the question of linearity.
- Differences in specificity according to crosshybridizations and sensitivity between Affymetrix 25mer technology and longmer technologies (Agilent, Illumina, Applied Biosystems) or cDNA based microarrays (Stanford-Plattform) are one reason for a limited convergence of results of those technological platforms.
- The complexity of tissues causes overlaying expression profiles. Is the complexity too high one loses the sensitivity for the cell type of interest. It is hard to profile neurosecretorial diseases by extracting the whole brain because of its complexity. Laser microdissection equipment can help to specify the tissue specimen but leads to problems in sample amount and quality. Heterogeneity in the cell composition makes it impossible to compare the samples, even if one tries to find an appropriate normalization method. Certain miniaturization protocols were developed, but it is hard to say how those are increasing the bias.
- Therapy monitoring studies are carried out using human blood samples either as isopycnic gradient isolated monocyte fraction or as fixated full blood sample (PAXgene). The first method is introducing a systematic error by carbohydrate mediated surface receptor activation and generates a general logistic problem. The second method extracts a totally different cell population by lysing the granulocyte fraction and leads to an overflowing of the sample with globin mRNA derived from the erythrocytes. However blocking- or removal protocols are introducing yet another bias.
- One colour microarray systems are more and more overcoming the dual-colour systems, because of advantages in the experimental design and project logistics, but require higher quality standards and preferably an automatization in the washing and staining procedures.
- Bioinformatic tools are required for handling the huge amount of data, starting from primary probe level analysis (for example MAS5, Affymetrix) to interaction exploring softwares (for example PathwayAssist, Iobion) which enables us to integrate our profiling results into our current world knowledge. Less conservative probe level analysis algorithms can produce clear differences starting with the same raw data.

- Genetic heterogeneity especially in humans, but as well in/between inbred mouse- and rat strains makes it nearly impossible to validate the data according to statistical axioms. Applying the Bonferroni correction to an increasing amount of data isn't an appropriate method to recognize the real result. Statistical significance does not reveal biological relevance.

#### Reference

Cheng J, Kapranov P, Drenkow J, Dike S, Brubaker S, Patel S, Long J, Stern D, Tammana H, Helt G, Sementchenko V, Piccolboni A, Bekiranov S, Bailey DK, Ganesh M, Ghosh S, Bell I, Gerhard DS, Gingeras TR. Transcriptional maps of 10 human chromosomes at 5-nucleotide resolution. *Science* 308, 2005, 1149-1154

## Understanding Complex Biological Systems with Metabolomics: An Overview

Royston Goodacre  
School of Chemistry, University of Manchester, PO Box 88,  
Sackville Street, Manchester M60 1QD, U.K.  
[roy.goodacre@manchester.ac.uk](mailto:roy.goodacre@manchester.ac.uk)



Recently, the completion of the human genome has accelerated demand for determining the biochemical function of orphan genes and for validating them as molecular targets for therapeutic intervention. The search for biomarkers that can serve as indicators of disease progression or response to therapeutic intervention has also increased, as has the search for prognostic markers. There is thus a great deal of interest in discovering protein or metabolite markers within this context without the need for any *a priori* knowledge (because in some cases there simply is not any). In this hypothesis generating mode, the discovery of key proteins or metabolites that may change due to disease or environmental stress may lead to novel biochemical findings. This presentation will give an overview of metabolomics and how this can be used for knowledge discovery.

However, a typical metabolomics experiment is expected to generate thousands of data points (samples *times* metabolites) of which only a handful might be needed to identify a disease state or perform some other categorisation. Thus it is obvious that current informatic approaches need to adapt and grow in order to make the most of these data. One especially needs good robust databases, very good data, excellent visualisation methods and even better algorithms, with which to turn data into knowledge. These aspects will also be discussed.

## Building a Synapse Proteome

J. Douglas Armstrong

Institute for Adaptive and Neural Computation, School of Informatics,  
University of Edinburgh, Scotland, UK  
[jda@inf.ed.ac.uk](mailto:jda@inf.ed.ac.uk)



Detailed models of the molecular machinery underlying cellular function and information processing can now be constructed using information from proteomic studies. Given such a model, the challenge we face is to use it to make functional predictions while acknowledging the limitations and errors inherent in the data used to construct the model. Here we will discuss the construction of a network model of a signalling complex in the central brain that underlies cognitive processes and its application in predicting new molecules important for cognitive illnesses.

Once a network is constructed we can then map onto it known associations or indications. In our study we focus on a protein complex known as MASC (MAGUK Associated Signalling Complex) found in the post-synaptic density of the mammalian hippocampus. MASC is enriched with proteins that in humans are linked to cognitive disorders (e.g. Schizophrenia, Bipolar Disorder). In rodents, MASC proteins are associated with phenotypes in adult behaviour and electrophysiological learning processes (LTP or long term potentiation).

How can we then use the model to predict further molecules that are important in illnesses or processes? There are two types of existing methods, both of which examine the network as a whole. The first looks at the role each protein plays in the shortest paths between other proteins and assigns a score based on that property. The assumption is that high scoring molecules are associated with global network function. The second method uses the nearness of molecules that carry a functional label. The idea here being that molecules closely linked to each other are more likely to share functional roles.

However these networks are functionally and architecturally subdivided into sub-networks, or pathways, and any prediction method should capture this property. Here we present a method that utilises the network topology to guide functional predictions. The key factor is that molecules likely to play critical roles in connecting sub-networks of functionally important molecules can be identified. We demonstrate its sensitivity and accuracy using the MASC complex as a well-defined example.

Further, we look at the effect of noise in these networks on the stability of predictions by comparing networks constructed using a combination of public domain interaction databases, commercially available datasets and our own interaction dataset curated from the literature. Perhaps not unsurprisingly the confidence one can place on the predictions is a function of the accuracy of the interaction data.

## **Bioinformatics and Data Mining**

Juha Saharinen

Department of Molecular Medicine, National Public Health Institute,  
Helsinki, Finland  
*[juha.saharinen@ktl.fi](mailto:juha.saharinen@ktl.fi)*



## Use of Transcriptomics and Proteomics in EMF Research: Present Status and Future Needs

Dariusz Leszczynski

Functional Proteomics Group,  
STUK-Radiation and Nuclear Safety Authority, Helsinki, Finland  
[dariusz.leszczynski@stuk.fi](mailto:dariusz.leszczynski@stuk.fi)



Induction of biological and health effects by low-intensity electromagnetic fields remains a controversial issue. In spite of years of research, there is still uncertainty about the biophysical mechanism that could be behind the observed biological effects. The vast majority of the biological research in this area has focused on the possibility of induction of genotoxicity, mutations, cancer, impairments in embryonic development. At the same time, there continues discussion on whether or not EMF could induce some weak effects that, although not able to induce life-threatening disease, could induce effects that could be detrimental to the quality of life. This life-non-threatening effects could include such symptoms as e.g. sleep-disorders, headaches etc. There is also the possibility that the effects could lead to health benefits. Even when biological effects have been reported, the biophysical mechanisms behind their occurrence are unknown. Thus far, the hypothesis driven studies have been unable to reliably determine the possible effects of EMF. Epidemiological studies are often expected to provide the answer whether EMF exposure might be hazardous to human population. However, finding and scientific validation of any potential health hazard using epidemiological approach alone might be not possible. This, because the “low sensitivity” of epidemiological methodology might be insufficient to reliably detect health impact of the weak biological effects caused by low doses of EMF. Furthermore, the presently conducted epidemiological studies are focused solely on the induction of cancer. Therefore, independently of their outcome, these studies will provide information only about the cancer. These studies will not provide information about the other potential effects of EMF exposure. Thus, the presently conducted epidemiological studies will be unable, because of their focus on only one aspect of the health issue, to give EMF “a clean bill of health”. We have earlier proposed that the use of the high-throughput screening techniques (HTST) of proteomics and transcriptomics (the so called “Discovery Science”) will be a useful approach to determine possible biological targets of EMF on the sub-cellular level. Search through databases shows that only few studies have been published where HTST methods were used to determine gene or protein responses to EMF exposures. Part of the studies has presented data obtained using transcriptomics approach and part of the studies has used proteomics approach. In two proteomics studies, besides examining protein expression changes, also protein activity was examined through determination of the level of protein phosphorylation. Here, we broaden the “Discovery Science” approach and we propose that besides the combined use of data from gene expression screening and protein expression screening, also methods screening for changes in the protein activity (like e.g. phosphorylation level) should be included in the analyses. Use of only one of the above screening approaches may, and will, lead to false negative results. Our published studies have shown that 900 MHz GSM signal exposure of human endothelial cell line EA.hy926 affects stress response pathway regulated by protein Hsp27. It was determined that the exposure had very modest effect on protein expression (25-30% increase) but that the effect on protein phosphorylation was most pronounced and the phosphorylation level of Hsp27 has increased by over 4-folds. Importantly, further studies have shown that the increased Hsp27 phosphorylation, which was induced by mobile phone radiation exposure, is of sufficient magnitude to significantly alter cell physiology and to lead to the stabilization of F-actin stress fibers and to shrinking of the cells. In conclusion, the analysis of cell response to the weak signal, such as EMF radiation, using either only gene or only protein expression assay will lead to false negative effects. Therefore, only the combination of results from three areas of HTST: obtained with transcriptomics (gene expression) and proteomics (protein expression and activity) should be used in determining whether and how cells respond to EMF.

## 2D Gel-Based Proteomics: Application in EMF-Treated Human Glioma Cells and Future Directions

Frank Witzmann

Department of Cellular and Integrative Physiology,  
Indiana University Medical School, Indianapolis, IN, USA

[fwitzman@iupui.edu](mailto:fwitzman@iupui.edu)



Most if not all of the toxic effects of chemical exposure involve the alteration of protein expression in target cells and tissues. It is reasonable to assume that the effects of exposure to electromagnetic energies might be exerted in a similar fashion, despite conflicting reports on the ability of ELF-MF to affect gene expression. Whether these alterations are the result of altered gene regulation, altered cell signaling processes (post-translational modifications), or other chemical modification of expressed proteins, it is possible to detect and analyze them more thoroughly than ever before. We have applied a large-scale, highly parallel two-dimensional electrophoretic (2-DE) gel platform to the analysis of differential protein expression in magnetic field (MF)-treated human glioma cells. Triplicate sets of human glioma SF767 cells were maintained in 10% FCS-DMEM and exposed for 3 h to a 60-Hz magnetic field (sham or 1.2  $\mu$ T). Proteins from 2 million cells/sample ( $\sim$ 150  $\mu$ g) were then separated on large-format 2D gels, the gels stained and image analyzed, and quantitative differences in expression determined statistically. In this preliminary study, we demonstrated altered expression of 10 proteins (3 increasing in abundance with 7 decreasing in abundance) along with the altered regulation of 30 genes (5 up regulated and 25 down regulated). Despite this moderate EMF effect, a functional relationship between the identities of affected genes and the altered proteins was absent. This presentation will address the utility of the 2-D gel-based proteomic approach in studying the effects EMF, describe its strengths and weakness, and suggest technical strategies for improved protein expression analysis.

Supported by AFOSR Grant F49620-03-1-0089.

## **Gene Expression Profiles in Volunteers Exposed to a 50 Hz EMF**

James Metcalfe

Department of Biochemistry, University of Cambridge, Cambridge UK

[j.c.metcalfe@bioc.cam.ac.uk](mailto:j.c.metcalfe@bioc.cam.ac.uk)



## Application of Genomic and Proteomic Approaches in Studying Bioeffects of EMF



Zhengping Xu\*,

Guangdi Chen, Han Li, Qunli Zeng, Yu Weng, Deqiang Lu, Huai Chiang

*Bioelectromagnetics Laboratory, Zhejiang University School of Medicine, 353 Yan-an Road, Hangzhou 310031, China; zpxu@zju.edu.cn*

**INTRODUCTION:** Many hypothesis-based studies have reported that certain gene and protein expressions could be altered in response to EMF exposure. However, the results are inconsistent and no clear mechanism is available. As neutral scientific discovery approaches, high-throughput screening techniques (HTST), including transcriptomic and proteomic techniques, offer feasible tools to compare large-scale gene and protein expression patterns in different biological systems after exposure to EMF. Here we used gene chip and 2-dimensional electrophoresis to investigate the global gene and protein responses to 50 Hz and 1,800 MHz EMF in breast cancer cell line MCF-7 cells.

**METHODS:** MCF-7 cells were exposed to 50 Hz 0.4 mT magnetic fields, or 1,800 MHz EMF at 2 W/kg or 3.5 W/kg of time-averaged SAR for 24 hours or less. Total RNA was isolated by Trizol method and then purified by using QIAGEN's RNeasy mini Kit. Affymetrix Genome U133A gene chips (2 chips for sham and 2 for exposure) were applied to detect the gene expression profiling following the manufacturer's instruction. Data was analyzed by Affymetrix microarray suite version 5.0 (MAS 5.0) and Affymetrix Data Mining Tool 3.0 (DMT 3.0). To conduct 2-dimensional electrophoresis, total cellular proteins were extracted and 200 $\mu$ g protein was subjected to analysis. 17 cm pH 3-10 or pH 4-7 linear IPG strips were selected in the first-dimension electrophoresis and the second-dimension electrophoresis was run in 12% uniform SDS-PAGE. Three to nine repetitions were carried out for all experiments. The silver stained images of the gels were analyzed with PDQuest analysis software 7.1. After determining differentially expressed proteins, the potential category and function of these proteins were described and certain EMF responsive protein candidates were identified by LC-IT Tandem MS.

**RESULTS:** MAS 5.0 software analysis showed that there were more than 1,000 differentially expressed genes. Among them, reproducible and consistent analyses with DMT 3.0 software revealed that 30 genes were affected by 50 Hz EMF exposure with 100% consistency in 4 pairwise comparisons, including 6 down-regulated and 24 up-regulated genes. 5 up-regulated genes were found after exposure to 1,800 MHz EMF at 3.5 W/kg of SAR, including protein phosphatase 1 regulatory (inhibitor) subunit 12A (PPP1R12A), transducin (beta)-like 1X-linked (TBL1X), ephrin-B2 (EFNB2), topoisomerase (DNA) I (TOP1) and a function unknown gene.

Around one thousand proteins were detected using pH 3-10 IPG strip in 50 Hz MF or sham exposed group. Up to 6 spots have statistically significantly altered (at least 5 fold up or down) compared with sham exposed group. 19 ones were only detected in exposed group while 19 ones were missing. These proteins fell into five categories, such as cytosolic transport proteins and regulators of certain protein phosphorylase. Three spots were selected to be identified by LC-IT Tandem MS as RNA Binding Protein Regulatory Subunit, Proteasome Subunit Beta Type 7 Precursor and Translationally Controlled Tumor Protein. As to 1,800 MHz EMF, pH 4-7 IPG strips were used to separate the proteins. Several proteins were significantly changed comparing with sham-exposed, and the detailed differential protein spots will be presented.

**CONCLUSION:** 50 Hz EMF exposure may induce differential gene and protein expression in MCF-7 cells, however, this cell line seems to be not so sensitive in response to EMF exposure. The “EMF-sensitive” cell line and the applicability of HTST need to be discussed, and HTPT manipulations and judgement standards are required.

**ACKNOWLEDGEMENT:** This work was supported by the National Natural Science Foundation of China (Nos. 50137010 & 30170792), the Key Project of Chinese Ministry of Education (No. 104092), Zhejiang Provincial Key Projects for Science & Technology (No. 021106135), Zhejiang Provincial Natural Science Foundation (No. M303807), and Key Projects of Health Bureau of Zhejiang Province (No. 2004ZD006).

## Genomic and Proteomic Assessment of 0.78 and 10 ns Pulsed Electric Fields on Human Cells: Results, Issues and Approaches

Martin L. Meltz

University of Texas Health Science Center at San Antonio, TX, USA

[meltz@uthscsa.edu](mailto:meltz@uthscsa.edu)



The application of genomics, proteomics, and hypothesis driven studies of families of genes or categories of proteins in coordination with one another is essential for any reasonable understanding of what is occurring upon electromagnetic field exposure of mammalian cells. The clear advantage of hypothesis driven approaches, such as the RNase Protection Assay and Real Time PCR to study specific families of genes and westerns to study specific proteins, is in allowing the study of multiple exposure conditions at multiple assay times post exposure at a reasonable cost. The clear advantage of the application of genomic microarrays and 2-DE gel and mass spec analysis of proteins (proteomics) is their ability to alert the investigator to cellular events that were not previously hypothesized. However, both of these latter approaches suffer from high cost, requiring careful and limited selection of exposure conditions and times of assay post exposure. In our investigations, in the area of proteomics after exposure to extremely high average peak power 10 ns pulsed electric fields, we have found, by comparing two independent experiments, that there are both similar and dissimilar results upon examination of nuclear and cytoplasmic protein fractions, for one exposure condition and one assay time post exposure. In a recent investigation, 2 hrs after exposure of human lymphoblastoid 244B cells to 25 pulses (1.5 pulses/sec) of a 10ns pulsed electric field, with an average peak power of 200 kV/cm, 61 2DE gel protein spots were observed to change commonly in the cytosolic fraction, and 21 spots were observed to change commonly in the nuclear fraction, within the two independent experiments. A larger number of spots were observed to change within the individual experiments, but not commonly within the two independent experiments. As of the date of submittal of this abstract, 12 of the cytosolic spots and 7 of the nuclear spots have been identified using MALDI-TOF Mass Spec and ESI LC/MS. Using microarray technology, changes in RNA transcription have also been observed after the same exposure conditions and the same 2 hr post-exposure time. The RNA and protein changes remain to be correlated, keeping in mind that protein synthesis will follow RNA changes. In studies with a 0.78 ns ultrawideband pulse (250 pulses per sec, 30 minutes total exposure), with a peak average power of 1 kV/cm, the number of spots matched across all gels for the 12 UWB gels was 304 (lower than we now expect). For the 30 min UWB exposed samples, out of 304 spots matched across all 12 gels, no believable differential expression changes were observed. The 6 spots that PDQuest identified as changing (at the 99% significance level using the Student's t-test) were all found to be miss-matched spots or low intensity spots with Sypro Ruby "specks" that skewed the spot intensity. A series of biological and methodological issues and problems, related to using genomic and proteomic approaches, will be discussed.

This research has been supported by the U.S. Air Force Office of Scientific Research (AFOSR) Grant No. F49620-02-10320 (MURI, Old Dominion University), and by AFOSR Grant No. FA9550-05-1-0021.

## **The Number of Gene-Expression Changes after Chronic Exposure to CDMA or FDMA Radiofrequency Radiation does not Change Exceed the False Positive Rate**



Whitehead, T. D., Moros, E. G., Brownstein, B. H., Roti Roti, J. L.

Department of Radiation Oncology, Washington University School of  
Medicine, St Louis, MO 63108 USA

Experiments with cultured C3H 10T  $\frac{1}{2}$  cells were performed to determine if exposure to 835.62 MHz Frequency Division Multiple Access (FDMA) or 847.74 MHz Code Division Multiple Access (CDMA) modulated radiofrequency (RF) radiations induces gene expression changes. RNA was extracted for microarray analysis from cells that had been exposed to either FDMA or CDMA or sham exposed for 24 hr. C3H 10T  $\frac{1}{2}$  cells were X-irradiated (0.68 Gy) and gene expression evaluated 4 hr post irradiation as a positive control. For all experiments, gene expression was measured as changes in mRNA levels, using Affymetrix GeneChips<sup>®</sup>. For each experimental condition, a matched sham control sample was run. Each experiment was repeated 3 times. Data were analyzed statistically using an unpaired data algorithm and compared with the results of a sham versus sham comparison. The sham versus sham comparison was used as a measure of the frequency of false positives as a function of expression level. Exposure of C3H10T  $\frac{1}{2}$  cells to FDMA or CDMA for 24 hr at 5 W/kg specific absorption In contrast the X-irradiated sample showed higher numbers of probe sets changing expression level than in the sham versus sham comparison. Analysis of the gene expression changes in the positive control were consistent with known gene expression changes induced by ionizing radiation as seen in the literature, thus confirming that the approach used in this study has the sensitivity to detect low level changes in gene expression from non-ionizing radiation had they occurred. Thus, 24 h exposure to FDMA or CDMA modulated radiofrequency (RF) radiation at 5 W/kg did not significantly alter gene expression as detected by changes in mRNA levels over the 12,488 probe sets on the Affymetrix U74Av2 Genechip<sup>®</sup>.

## **In vitro gene expression studies and their impact on high content screening assays in EMF research**



Christian Maercker<sup>1,2</sup>,  
Daniel Remondini<sup>3</sup>, Reetta Nylund<sup>4</sup>, Hugo W Rudiger<sup>5</sup>, Dariusz  
Leszczynski<sup>4</sup>, Ferdinando Bersani<sup>3</sup>

<sup>1</sup>University of Applied Sciences Mannheim, Germany. <sup>2</sup>RZPD German  
Resource Center for Genome Research, Heidelberg, Germany,

<sup>3</sup>University of Bologna, Department of Physics, Bologna, Italy. <sup>4</sup>STUK,  
Radiation Biology Laboratory, Helsinki, Finland <sup>5</sup>University of Vienna,  
Division of Occupational Medicine, Vienna, Austria

[c.maercker@fh-mannheim.de](mailto:c.maercker@fh-mannheim.de)

Life of cells is determined by several central processes, such as growth, proliferation, death, and differentiation. By gene expression studies of different marker genes, we were asking if any of these processes are unbalanced in cultured cells by ELF-EMF or RF-EMF. In a specialized exposure setup (Kuster and Schuderer, IT'IS Zurich), primary fibroblasts ES-1 were exposed to 50 Hz, 1 mT in 5min on/10 min off cycles for 15 or 24 h, and endothelial cells EA-hy926 to 900 or 1800 MHz, GSM, SAR 2.0 W/kg for 1h, followed by transcriptome analyses on DNA microarrays. Bio-statistics included a bootstrapping procedure to generate new datasets by permutations and the selection of outlier genes for each experiment. The data of the 50 Hz exposed fibroblasts gave evidence for an increase in cellular turnover (ribosomal proteins, ATP synthase, cytochrome oxidase). Moreover, contacts to the extracellular matrix seemed to be modified (collagens, proteoglycan 2, decorin, SPARC, integrins, actin and actin interacting proteins). Similarly, some regulated genes in RF exposed endothelial cells indicated an increase in metabolism (ribosomal proteins, translation elongation factors) and change of cell contacts (actin, laminin, proteoglycan 2). To confirm these results, proteome analyses on 2D gel analysis combined with mass spectroscopy are under way. However, these studies only give a first impression of possible effects within a cell population rather than functional information. Therefore, we are working on further in vitro experiments asking if the modulations in gene expression give rise to changes in cell behavior, or if they are temporary effects with no further consequences. One promising approach with respect to this question are high content screening methods. These are advanced microcopic tools, which allow parallel testing of several parameters (cell number, cell size, apoptosis, cell shape, signaling etc.) in a high throughput manner. This not only will allow the analysis of individual cells, but also the identification of signaling networks, which is important for the understanding of molecular consequences of EMF exposure.

Parts of this work were funded by the EU (REFLEX project, co-ordinated by Franz Adlkofer) and the VERUM foundation, Munich.

## The Proteomic Response of *S. pombe* to Static and Oscillating EMFs

Mark E. Weeks<sup>1</sup>, John Sinclair<sup>1</sup>, Donald Allen<sup>2</sup>, Amna Butt<sup>2</sup>, Caroline Wilkinson<sup>2</sup>, Nic Jones<sup>2</sup>, Mike Waterfield<sup>1</sup> and John F. Timms<sup>1</sup>

<sup>1</sup>Ludwig Institute for Cancer Research, Cruciform Building, Gower St, London and <sup>2</sup>Paterson Institute for Cancer Research, Christie Hospital, Wilmslow Road, Manchester  
[jtimms@ludwig.ucl.ac.uk](mailto:jtimms@ludwig.ucl.ac.uk)



This project aims to characterise the proteomic responses of the fission yeast *Schizosaccharomyces pombe* to environmental stress, including exposure to oxidants and low field-strength electromagnetic fields (EMFs). Specifically, the combination of a quantitative 2D-gel based proteomic method (2D-DIGE) and mass spectrometry (MS) were used to identify cellular proteins whose expression and/or post-translational modification are significantly altered by these stimuli. The findings of this study will establish whether this simple model organism can detect, respond or suffer deleterious effects following short-term exposure to static and oscillating EMFs.

As a background for the EMF analysis we first conducted a global analysis of the effects of H<sub>2</sub>O<sub>2</sub> treatment on the proteomes of wild type *S. pombe* and a Sty1-mutant lacking a critical MAP kinase component of the conserved stress-activated signalling pathway. 260 protein isoforms were identified from 2D-DIGE gels by MS, which displayed significant differential expression in response to H<sub>2</sub>O<sub>2</sub> and/or loss of Sty1. A number of isoforms displayed opposite responses in the two cell lines, identifying stress-induced proteins that require Sty1-signalling. The proteins fell into many different functional classes, with stress-response proteins and enzymes involved in amino acid biosynthesis, glycolysis, glycerolipid metabolism, protein synthesis and degradation featuring highly. Comparison of observed protein changes with a genome-wide transcriptional analysis of relative mRNA abundance and NMR-based metabolomic analysis established how these protein changes were controlled and what effect they had on metabolic pathways and ultimately on cell behaviour.

The above work provided us with the background to confidently examine the potential effects on the yeast proteome of exposure to low field-strength EMF. Owing to the sensitive nature of these studies, experiments were conducted in a blind fashion with biological replicate cultures grown in a two-chambered incubator with an adjustable field-generating apparatus and different permutations of sham or EMF exposure (1mT, 60 min). Under both static and oscillating fields, significant differences in the abundance of several protein isoforms were identified, albeit with small fold-changes. However, these changes did not correlate with the EMF exposure regimen and we conclude that exposure has no significant effect on the fission yeast proteome at the sensitivity afforded by 2D-DIGE labelling and fluorescence detection. This is in agreement with microarray studies showing no effect of EMF exposure on mRNA levels. We propose that the protein isoforms that were changing must be exquisitely sensitive to subtle differences in growth conditions between replicates or in post-culture handling and lysis.

## Applying Genome Approaches to Study Biological Effects of Radiofrequency

San Ming Wang

Center for Functional Genomics, ENH Research Institute, Northwestern  
University, Evanston, IL, USA  
[swang1@northwestern.edu](mailto:swang1@northwestern.edu)



The advance of mobile phone communication has led over 2 billion people around the world exposed to RF fields. With such a large population, we need to consider seriously the potential adverse effects of RF fields on human health.

We are studying the topic by addressing two fundamental questions:

1. Could the RF radiated cells sense RF insulation by altering its gene expression pattern?
2. Could the RF radiated cells sense RF insulation by altering its genome structure?

To address the first question, we performed the experiment based on the following considerations:

- a. Use 2.45GHz as the RF resource, the frequency commonly used in telecommunication;
- b. Maintain a tight control of the thermal environment during RF exposure to minimize thermal effects on the irradiated cells;
- c. Use SAGE technique to survey gene expression at the genome-level without pre-selection of any particular genes to provide a genome-wide view of gene expression in the irradiated cells;
- d. Provide sequence and quantitative information for the detected transcripts to identify potential RF-response genes;
- e. Detect the expression from both known genes and unknown genes.

The results from this study reveal that hundreds of genes including many unknown transcripts in the RF radiated cells changed their expression patterns, including the genes involved in apoptosis and cell cycle regulators. The expression levels of all heat shock genes show no increase, indicating that the altered gene expression was not related with thermal effect. From this study, we conclude that the living cells can sense the RF insulation by altering their gene expression (Lee S, et al. 2.45 GHz Radiofrequency fields alter gene expression in cultured human cells. *FEBS Letters*, 579:4829-4836, 2005).

To address the second question, we are applying a whole-genome scanning approach to determine if there are any structural changes in the radiated genome. We are performing the study with the following consideration:

- a. Use sequencing-based approaches to scan the radiated genome
- b. Provide 1-2 X coverage of the whole genome at the resolution of <5 kb across the genome
- c. Provide quantitative and qualitative information for the detected regions
- d. Determine if there is any change of genome structures in the radiated cells, including amplification, deletion, inversion, insertion and translocation.

The study is supported by Department of Defense Air Force Office of Scientific Research MURI grant (F49620-02-0337).

## **The Proteomic Analysis of Human Endothelial Cells Exposed to Mobile Phone Radiation**

Reetta Nylund

Functional Proteomics Group, Radiation Biology Laboratory, STUK, Finland

[reetta.nylund@stuk.fi](mailto:reetta.nylund@stuk.fi)



Over the past years high-throughput screening techniques have become widely used in the several different research fields. Screening at the protein expression level is usually referred as proteomics. There are various ways to screen protein expression; one of the most common at the moment is a combination of the two-dimensional gel electrophoresis (2DE) and mass spectrometry (MS). Proteins are separated by 2DE, usually by isoelectric point and molecular weight, and furthermore identified using MS. A building up a 2DE system for a specific application often requires optimization for enabling a generation of similar gels repeatedly. Repeatability is crucial for 2DE approach, since often several repeats are needed to be combined and compared against each others to obtain needed reliability. Therefore, the system needs to be built up in such manner that the similar gels are reproduced and the combination of the gels is possible. The sample preparation is a key component for successful 2DE separation and the method may vary a lot depending on the sample nature. Finding a suitable focusing method, molecular weight separation method as well as a general sample loading amount requires also some optimization. In total, the optimization of the system may take time from some weeks to months. However, after the system is on use, it is an efficient way to screen several proteins at the time and to get a general idea of the cellular level actions. Still during the analyses, it is needed also to remember the limitations of the technique. Typically staining method causes some limitations either in the detection sensitivity or in the linearity of the detection. Also protein separation from other proteins and the amount of protein is needed to be considered. 2DE pattern shows the general expression pattern, but the proteins itself are needed to be identified before finding the biological significance. This protein identification is usually done using MS. Several MS methods are used for protein identification, e.g. peptide fingerprinting and sequence tag analysis. The amount of protein has an influence on the method selection as well as on the reliability of the gained results. Common problems in MS studies are contaminants, which are in the sample, and also some non-natural protein modifications, e.g. caused by staining, may hinder the analysis. However, once in knowing hands, 2DE/MS methods provide a good overview of the general proteome appearance, in which unknown effects can be efficiently studied.

## Proteomic Assessment of Macrophage Activation in Plasma Bioassay

R.Sypniewski<sup>1,2</sup>, N.J. Millenbaugh<sup>1,2</sup>, C. Cerna<sup>3</sup>, B. Brott<sup>4</sup>, J.L. Kiel<sup>5</sup>, R.V. Blystone<sup>4</sup>, H. Coppage<sup>6</sup>, F. Witzmann<sup>6</sup>, and P.A. Mason<sup>1</sup>



<sup>1</sup>Directed Energy Bioeffects Division, Air Force Research Laboratory, Brooks City-Base, TX, 78235; <sup>2</sup>Advanced Information Engineering Services, San Antonio, TX, 78235; <sup>3</sup>Conceptual Mindworks, Inc. San Antonio, TX, 78235, Brooks City-Base, TX, 78235; <sup>4</sup>Department of Biology, Trinity University, San Antonio, TX, 78212, <sup>5</sup>Biosciences and Protection Division, Air Force Research Laboratory, <sup>6</sup>Indiana University School of Medicine, Indianapolis, IN, 46202, USA.  
[roza.sypniewski.ctr@brooks.af.mil](mailto:roza.sypniewski.ctr@brooks.af.mil)

Our previous study indicated that prolonged exposure (~50 minutes) of rats to millimeter waves (MMW) at 35-GHz at 75 mW/cm<sup>2</sup> can lead to the release of several acute phase proteins into the plasma (Radiation Research Meeting, August 2003). It has been hypothesized that a systemic response was activated. One of the fast responder cell types to circulating mediators are ubiquitous macrophages. Those cells can be stimulated and relay information through nitration, phosphorylation, or other modifications of signaling cellular proteins. Our preliminary experiments revealed that rat macrophage cells CRL-2192 cultivated with plasma from 35 GHz-exposed animals had changed morphology. To further characterize this phenomenon, the present experiment was designed to detect changes in proteins of the reporter rat macrophage cells that were stimulated with plasma from rats exposed to 35-GHz MMW at 75mW/cm<sup>2</sup> versus plasma from environmental heat (EH) treated rats or sham-exposed rats. Male Sprague-Dawley rats (350-400 g) were anesthetized with isoflurane and either sham-exposed or exposed to 35-GHz MMW at 75mW/cm<sup>2</sup> or EH at 42°C throughout the exposure period. The MMW rats were exposed over the left, shaved, lateral flank area. The body surface temperature was recorded using infrared thermography and showed an increase to 41-42°C. Following exposure, rats were allowed to awaken and plasma samples were collected at 24 hr post-exposure. Plasma from 3 representative animals of each group; 35-GHz exposed, EH exposed, and sham was added to cultures of the CRL 2192 rat macrophage cell line for 24 hr stimulation. Cells were harvested, and their protein lysates were separated on 2-D gels. Approximately 600 proteins were resolved and spots were analyzed by PD-Quest software. 2-D gel image analysis revealed significant differences in multiple proteins (sham versus MMW and sham versus EH). Spots of interest were excised, digested and identified by MALDI-TOF MS. Total screening of nitrated proteins was accomplished by transfer of 2-D gel separated CRL 2192 cell lysates to PVDF membranes on which only nitrated proteins were visualized by Western Blot protocol. Corresponding protein spots were extracted from duplicate gels for identification. Nitrated proteins were cut from the gels and identified by peptide mass fingerprinting. This accomplishment provides extended information about differential activation of the CRL-2192 macrophage by the mediators present in the plasma of 35-GHz exposed animals that was different than the proteins of the positive control activation assay (interferon gamma and LPS) or assay with plasma from EH and sham-exposed rats. In conclusion, the proteomic approach combined with a macrophage cell line bioassay provides a unique approach for identification of biomarkers of MMW overexposure.

Funded by AFRL/HE and AFRL/AFOSR