

# Abstracts



**MOFFITT CANCER CENTER**  
TAMPA, FLORIDA, OCTOBER 17-18, 2011



# Talks

**Manfred Dietel<sup>1</sup>**, Norman Zerbe<sup>1,2</sup>, Frederick Klauschen<sup>1</sup>, Kai Saeger<sup>3</sup>, Peter Hufnagl<sup>1,2</sup>

<sup>1</sup>Institute of Pathology, Charité – Universitätsmedizin Berlin, Berlin, Germany

<sup>2</sup>University of Applied Science Berlin, Germany

<sup>3</sup>VMscope GmbH, Berlin, Germany

## Virtual Microscopy on the Stony Way to Revolutionize Anatomic Pathology

Within the last decade the technical prerequisites for reliable and functional virtual microscopy (VM) have improved dramatically. For example, a wide range of novel digital slide scanners and software solutions have been developed for various virtual microscopy applications in education and research. However, a breakthrough also in diagnostic pathology is still lacking particularly in Europe. This contribution aims to analyze advantages and disadvantages of VM-functionality with respect to clinical pathology and seeks to identify important features, which will contribute to a broader acceptance of applied VM.

### *Problems*

- In daily diagnostics a pathologist will always compare slide handling of a digital microscope to that of a conventional microscope – time matters! Therefore, the first and foremost requirement for VM is speed and robustness of the loading procedure.
- Another pivotal feature is the instantaneous feedback by the virtual microscope during slide navigation. However, current virtual microscopy software often still has a little response time.
- Furthermore, a seamless integration into pathology and laboratory information systems (PLIS, AP-LIS) and a DICOM-conform connectivity to PACS systems are widely accepted as important components for virtual microscopy in routine scenarios. However PLIS-PACS integration for virtual microscopy will take time as known from RIS-PACS integration in radiology and is expensive.
- A constant and robust slide loading procedure, e.g. to scan 1000 slides over night, is lacking; likewise, there is no scanner on the market that allows integration into the laboratory workflow, such as, for

instance, the direct linking to the cover slippers used in almost all modern institutes of pathology.

- Last but by far not least no solid reimbursement system exists for conventional diagnostics or (immunohistochemical) tissue feature quantification using VM within Europe – a major obstacle for routine implementation.

### *Advantages*

- Together with specialized user interfaces such as multi-touch devices, 3D mice or eye trackers that are more and more often added to the WIMP paradigm (windows, icons, menus and pointer), an intuitive usage of digital pathology applications can be achieved.

- In this context, digital microscopes should be able to integrate accompanying services like image analysis, data mining and statistics, provided as web based services if possible. High-level extensions to digital microscopes are features like computer-assisted navigation (CAN) and computer-aided diagnosis (CAD). CAN supports a comfortable navigation and interaction within or between whole slide images in several contexts such as tracing a relevant area through serial sections. Moreover, these areas should be automatically linked across different slides or stains by maintaining the relative position. This feature would open the possibility to directly compare conventional and immunohistochemical stains of the same tissue specimen.

- CAD techniques such as decision support and content-based image retrieval (CBIR) show an emerging development activity and are already available for specific re-search contexts. But to be suitable for daily use they have to be integrated in user-friendly graphical user interfaces (GUI).

- As soon as the point is reached where all histology slides of an institute are scanned and provided digitally in the routine process, the whole laboratory and diagnostic workflow can get a far-reaching workflow integration and -automation. The laboratory processes can be supervised by a system. After slide scanning, even the diagnostic process can be automatically supervised. That can be used to organize the workload more efficiently and to even include external diagnostic personnel via Internet in case of higher workload. Furthermore laboratory processes like for example assembling the slides to a case or assigning a case to a certain pathologist can be completely automated. Those are by now laborious manual processes.

- Finally, a simple but important point, the comparison with earlier slides of the same patient can be greatly facilitated by establishing a digital patient pathology record containing all current and previous virtual slides. The development of the storage expenditure for digital archives will be reduced in the next decade, which may replace the conventional slide archive.

### *Vision*

- Automated image analysis (IA) on whole slide images is a rapidly developing research and business field. Although several vendors are currently adding image analysis capabilities to digital microscopes, most of them are proprietary and highly specialized. With regard to new concepts of targeted therapies in oncology, robust, reliable and reproducible systems to quantify immunohistochemical marker expression, e.g. estrogen receptor, HER-2, EML4-ALK, Ki-67 etc., is urgently needed.
- It is self-evident that the IA systems should be able to interact with the majority of current PLIS, instruments and several microscopy environments. Therefore, it is necessary to define standardized interfaces for digital microscopes for extending their IA capabilities.
- The IA functionality may also be offered and accessed via the internet to reduce local costs.
- A system which is on the way to fulfill the necessary criteria is called “specimen scout” for details see <http://www.specimen-scout.de/project>.
- In the joint project “HistoQuant Mamma” Definiens and the Institute of Pathology, Charité work together on the design of a virtual microscopy-based breast cancer diagnostic system dedicated to routine use. The project goal is to provide pathologists with a tool to characterize and quantify morphological and immunohistochemical tissue features and is based on over 150 cases with more than 1000 histological slides.

To summarize, we believe that several key features have to be available within the next generation of digital microscopes. Especially, CAN and CAD have a high potential since they add additional functionality and qualitative benefit for pathologists compared to conventional microscopy. Once these and the other above mentioned prerequisites are met the future of VM may become a sunny one.

**Mark Lloyd**

H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL

**Somatic Evolution of Cancer Probed by  
Multiparametric Feature Analysis of Histology**

This project uses advanced microscopy, image analysis and computational modeling to investigate how the somatic evolution of breast cancer can be characterized and measured. We have evaluated multiparametric morphological features of single cells in histology sections. Our specific hypothesis for this project proposes that single cell features will distinguish subpopulations of cells, both in the tumor and the PME, which will correlate with clues of somatic evolution including phenotypic variation, heritable changes and niche partitioning and parameterization.

Investigators studying tumor progression as an accumulation of mutations affecting tumor fitness largely take a genetic or epigenetic approach. However, due in large part to current technical limitations, genetic heterogeneity is poorly understood on the cellular level where many clues to evolution are likely to occur. Our group hypothesizes that genetic abnormalities are manifested in subtle phenotypic variations which, with careful multiparametric feature analysis, can be identified and leveraged to specifically investigate the questions:

- 1) Does phenotypic variation suggest a progressive evolutionary trajectory?
- 2) Do progressive stages of higher grade cancer manifest distinct heritable changes or phenotypic plasticity?
- 3) Do changes in the physical microenvironment spur tumor cell evolution along adaptive landscapes as adaptive opportunities promote niche filling and diversification which then directs further tumor progression?

This novel approach has the potential to link the observation of tumor progression with its underlying evolutionary explanation. Furthermore, it is an opportunity to evaluate the translation of multiparametric feature analysis to the pathologist's toolbox, which would directly affect patient prognosis and influence therapeutic decision-making in the clinic.

**Andrew Beck**

Beth Israel Deaconess Medical Center, Boston, MA

**Image Analysis to Discover New Prognostic Features in Breast Cancer**

The morphological interpretation of histologic sections forms the basis of diagnosis and prognostication for cancer. In the diagnosis of carcinomas, pathologists perform a semi-quantitative analysis of a small set of morphological features to determine the cancer's histologic grade. However, the determination of grade in breast cancer examines only a small set of morphological features of the breast cancer epithelial cells, which has been largely unchanged since the 1920s. A comprehensive analysis of automatically quantitated morphological features could identify characteristics of prognostic relevance and provide an accurate and reproducible means for assessing prognosis from microscopic image data. We developed the C-Path system to measure a rich quantitative feature set from the breast cancer epithelium and stroma. We apply machine learning techniques to build image-feature based prognostic models and to learn biological pathways driving clinically significant morphologic phenotypes.

**Jared Schwartz**

Aperio, Vista, CA

**Digital Pathology and Image Analysis: The Window to Personalized Medicine**

The medical media is awash in reports of how personalized medicine will revolutionize the practice of medicine and that genomics may replace traditional diagnostic pathology and cytology. The reality as more scientific knowledge becomes available is the real promise in personalized medicine will have as its' foundation tools the rapidly evolving technologies of digital pathology and image analysis. Genotype and phenotype are not the same and the "gold standard" as much of diagnostics particularly in neoplastic specimens is based on over 100 years of tissue based study. In recent years the addition of specific biomarkers and image analysis algorithms has added significant new methods that leverage the information we have gained from the historical tissue diagnosis. The critical role of well documented biobanks in "omics" research is also renewed evidence of the role traditional histological specimens will play if personalized medicine is to bear fruit. To identify the "right" area of many specimens for high yield information in "omics" studies and in addition to get more new information from the tissue itself will require the power of image analysis of digitized tissue.

**Tom Nifong**

Metamark Genetics, Cambridge, MA

**Multiplex Fluorescence *in situ* Cancer Prognosis Testing in a Clinical Laboratory using Definiens Developer**

Cancer prognostication depends on both biomarker discovery and accurate quantification of the appropriate biomarkers within the region of interest. *In situ* biomarker analysis is optimally performed on the fewest number of tissue sections to minimize inter-section heterogeneity. We identified a set of protein biomarkers proven to be drivers of metastasis and lethal outcome. We used multiplex fluorescence immunohistochemistry for *in situ* tumor mask and biomarker detection, and then developed a feature-based algorithm in Definiens Developer to identify the cancerous regions of interest and quantify multiple biomarkers. We are applying our multi-biomarker model to segregate aggressive from indolent cases.

## Yuling Luo

Advanced Cell Diagnostics, Hayward, CA

### ***In situ* Detection and Quantification of Single RNA Transcripts by RNAscope**

*In situ* analysis of biomarkers is highly desirable in molecular pathology, because it allows the examination of biomarker status within the histopathological context in clinical specimens. While IHC and FISH are widely used in the clinic to assess protein and DNA biomarkers, respectively, *in situ* RNA analysis is rarely utilized in the clinical setting due to low sensitivity and specificity of current methodologies. As a result, RNA biomarkers are analyzed by RT-PCR at the expense of destroying the tissue context, which is often highly heterogeneous. This is a large gap considering the abundance of RNA biomarkers discovered through whole-genome expression profiling. ACD has developed RNAscope®, the first RNA *in situ* hybridization platform with single molecule detection sensitivity, enabling rapid development of RNA-based biomarker assays. *In situ* RNA biomarker expression can also be quantified by counting the spots of individual RNA molecules using advanced imaging analysis algorithms, making quantitative *in situ* single cell expression profiling feasible for the first time.

**Melvyn S. Tockman, Mark Lloyd**

H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL

**A Look at the Tumor's Edge**

During mitosis, the cell is required to duplicate both DNA and the centrosome that forms the spindle poles for accurate DNA segregation. These processes are linked through CDK2/cyclin E. This link may be assisted by the tumor suppressor gene, DLC1. Disruption of this link while the cell is being held in G2/M arrest allows amplification of centrosomes relative to DNA. These additional spindle poles are a major cause of aneuploidy (chromosomal instability). Aneuploidy provides the young cancer cell with a palette of potential genomes from which to select for optimal growth in its microenvironment. Although additional centrosomes are advantageous during early tumor development, once the genome is optimized for growth, additional centrosomes are no longer advantageous. This process has been termed “Karyotypic Convergence”.

We have measured centrosome features in confocal images of cultured cells to distinguish immortalized bronchial epithelial cells from A549 lung cancer, and we've restored normal centrosome appearance after MUC-1 transient transfection of A549 cells. Centrosome features are able to distinguish aggressively growing PC-9 adenocarcinoma cells (which have lost the p53-dependent mechanism of G2/M arrest) from less aggressive A549 cells (p53-dependent G2/M checkpoint intact). Centrosome features of stage I lung tumors at the time of resection were able to distinguish patients who died (within 4 years of resection) from survivors (lived >9 years after resection).

To validate these observations in a larger sample, we used Definiens Developer XD v1.5 software to select centrosomes and their associated nuclei as Regions of Interest from confocal images of resected lung tumor. Algorithm development required successive iterations from the toolbox to approach physician-selected centrosome frequencies. We took 3 confocal images from each of 5 tumor zones, each more proximal from the tumor's edge by three high power fields. Physician-selected ROIs within each zone were ultimately matched by the Definiens algorithm. Numbers of centrosomes measured by both techniques support the hypothesis of Karyotypic Convergence and distinguish tumors from living v dead lung cancer patients.

**Scott Webster**

Dako, Carpinteria, CA

**Integration of Image Analysis in the Development of Biomarker Assays and Companion Diagnostics**

Digital image analysis has gained a measure of acceptance as a tool for the interpretation of immunohistochemical (IHC) stains in the pathology laboratory, especially for those requiring more complex or quantitative interpretation. It appears inevitable that the day will come when many more clinical IHC stains include image-assisted analysis, especially those with predictive or prognostic clinical claims such as Companion Diagnostics.

This increasing acceptance of image analysis can also be seen as a broadening role in the development cycle of clinical biomarker IHC assays. Typically, the participation of imaging has been limited to the generation of algorithms to match already developed IHC assays. While this scenario has yielded successes in the past (e.g. a range of validated commercial HercepTest™ image analysis algorithms) it is likely that it will give way to a more integrated approach. Just as image analysis can bring consistency and objectivity to the interpretation of biomarker stains currently used in the clinical setting, it can provide the same benefit to the development of companion diagnostic assays prior to their validation in clinical studies.

Image analysis can be applied to various stages of IHC assay optimization. This presentation will review how imaging can a) facilitate accurate assessment of antibody titration curves and b) afford improvement in assay dynamic range through comparisons with data obtained from techniques such as western blot or ELISA.

**Vikas Prasad<sup>1</sup>**, M. Athelougou<sup>2</sup>, D. Kaemmerer<sup>3</sup>, L. Peter<sup>3</sup>, A. Lupp<sup>4</sup>, J. Schulz<sup>4</sup>, J. Saenger<sup>5</sup>, G. Binnig<sup>2</sup>, R. P. Baum<sup>1</sup>

<sup>1</sup>Dept. of Nuclear Medicine and Centre for PET/CT, Zentralklinik Bad Berka, Germany. Current Address: Dept. of Nuclear Medicine and Centre for PET/CT. Charité University Hospital, Berlin, Germany

<sup>2</sup>Definiens, Munich, Germany

<sup>3</sup>Dept. of General and Visceral Surgery, Zentralklinik Bad Berka, Germany

<sup>4</sup>Institute of Pharmacology and Toxicology, Friedrich Schiller University Medical Center, Jena, Germany

<sup>5</sup>Institute of Pathology, Zentralklinik Bad Berka, Germany

## Translation of Histopathological Information to *in vivo* Molecular Imaging using PET/CT and Therapeutic Management

*Aim* Somatostatin receptors (SSTR) have five different receptor subtypes (SSTR1-5) and are expressed in varying degrees on neuroendocrine tumors; SSTR2 being overexpressed in most of the good to moderately differentiated gastroenteropancreatic NET (GEP-NET). Until now most of the therapeutic somatostatin analogues used either in cold form or coupled with radionuclides are targeted towards the subtype 2. However recent results have suggested that in some tumors there is relatively low SSTR2 and predominance of other subtypes. Immunohistochemistry is currently the routine standard for mapping different SSTR subtypes expression in NET cells, however as of yet there is no validated imaging tool which quantifies the receptor density. Initial results have suggested the maximum Standardised Uptake Value (SUVmax) a semiquantitative parameter derived from static somatostatin-receptor PET/CT as an indirect method for receptor density mapping. Keeping these in mind, we tried to validate the in-vitro SSTR2 density measurement using a digitalized pathology (Definiens XD) with in-vivo Ga-68 DOTANOC PET/CT.

*Methods* We used image analysis and data mining algorithms applying Definiens XD, an image analysis platform which is based on the Definiens Cognition Network Technology which is object based, knowledge driven and uses context which allows also to analyze image data from different data modalities at once. First we obtained PET/CT imaging data in neuroendocrine tumor patients after injection of Ga-68 DOTANOC and DOTATATE and calculated (by Esoft) SUVmax, SUVmean, and molecular tumor volume (MTV) of each single tumor

lesion and normal liver SUVmax. Patients were then operated and each single tumor lesion removed was marked and correlated to the lesions seen of the Ga-68 SMS PET/CT before. Then, immunohistochemical analysis of tumor tissue was performed by using specific polyclonal and monoclonal antibodies for the staining of SSTR1-5. At least five tissue slides for each patient were analyzed. Visual analysis (HER2 score and IRS) of such slides shows “qualitative” correlations between staining intensity and PET/CT uptake parameters. This engaged us to use Definiens XD for the analysis of these tissue slides in order to quantify our hypothesis. We developed an automated image analysis solution for the single cell analysis and quantification of the tissue slides. This image analysis solution, i.e. the Definiens XD Rule Set, separates tumor from non-tumor regions, calculates SSTR1-SSTR5 stain intensity in the tumor regions, calculates areas of tumor and non-tumor regions, extracts nuclei in tumor and in non-tumor regions, counts nuclei in both tumor and non-tumor regions, calculates/quantifies morphological properties of the single nuclei (area, symmetry, stain intensity in the nuclei etc.), calculates relations between tumor and non-tumor regions and calculates user defined features in analogy to HER2 and IRS scores. In a next step, we used data mining methods in order to find correlations between PET/CT and SSTR2 image analysis results and between HER2-score and IRS and SSTR2 image analysis results.

*Results* Correlation coefficients for SUVmax, SUVmax\_PVG, SUVmean, SUVmean\_PVG, MTV ranged from 0.83 to 0.99 ( $p < 0.005$ ). The tumor SUVmax showed a significant correlation with HER2 and IRS scoring. In the context of a feasibility study we calculated such correlations for 9 patients (out of 30 patients overall). The preliminary results show a significant correlation between immunohistochemistry for SSTR2 staining and the PET/CT uptake values. A correlation was also found between SSTR2 staining and the corresponding HER2 and IRS grading. Such correlations show both positive and negative correlation coefficients and  $p$ -values  $< 0.005$ . The values for the correlation coefficient are between -0.824 and 0.75.

*Conclusions* The Definiens XD image analysis software allows analysis of all the different image data modalities we use in this study. PET/CT image guided receptor density mapping was found to have significant correlation with digitalized immunohistopathology using Definiens XD. These first results are very promising and could potentially have a wide clinical application in direct transfer of in-vitro information to in-vivo imaging and therapy.

**Kristine Burke**

Millennium Pharmaceuticals, Cambridge, MA

**A Computer-aided WSI Solution for Tissue-based Biomarker Assessment in Preclinical and Clinical Programs in Oncology**

Cellular imaging technologies facilitate the understanding of drug activity in the physiological context of a cell or tissue by enabling the quantitative analysis of cellular and sub-cellular events. In early stages of oncology drug discovery, in vitro image-based assays are utilized for compound screening to determine levels of target inhibition in cells. As a program advances from in vitro to in vivo models, image-based assays are utilized, as part of a preclinical biomarker strategy, to determine the pharmacodynamic response in mouse xenograft models. And finally, as the program enters clinical trials, image-based assays are used as part of an overall biomarker strategy to assess target inhibition in humans.

At Millennium, we use a variety of imaging technologies, including Definiens Tissue Studio/Composer/Developer, to quantitatively assess compound effects in both cells and tissue. We are leveraging these technologies to improve both the quantitative accuracy and the overall workflow of our biomarker assays; developing tools to integrate multiple platforms and using machine learning approaches for ROI detection. We are also working with Definiens and other external companies to develop and run routine assays, allowing in-house scientists to focus on novel assay development.

This presentation will provide examples to highlight how these developments have impacted our biomarker programs through the early and late stages of drug development..

**Glyn Colebrooke**  
Philips, Eindhoven, NL

## **Digital Histopathology, Stratified Medicine and Robust Companion Diagnostics**

The role of Histopathology in translational medicine is undergoing a renaissance. A new analytical objectivity is being achieved by combining digital systems with image analysis algorithms and high quality chemistry and staining systems. A new very high throughput scanner and image management system from Philips is set take this to the next level. Histopathology testing methods are well integrated into the cancer care cycle and the association with tumor morphology increases the specificity of predictive and prognostic biomarkers. This places the histopathologist at the center of patient stratification and yet the granularity of this stratification is compromised by the inherent subjectivity of the analysis. There is evidence to show that digital histopathology can re-empower these widely used biomarkers. A more objective analysis of tissue images brings the opportunity to accurately grade and stage tumors together with quantifying molecular biomarkers for prognosis and prediction. Philips has introduced a very high speed digital imaging system for histopathology and with Definiens and Dako offers a heritage of excellence in digital image analysis, chemistry development and companion program management to provide continuity from discovery to the clinic.

**Joseph Krueger**

Director of Biology, Flagship Biosciences, Flagstaff, AZ

**Digital Pathology and IVDMIAs: The Shortest Distance Between Two Points**

The recent co-approval of drugs and their companion diagnostics to detect critical mutations in lung cancer and melanoma demonstrate the clinical and strategic value of being able to identify patients who will respond to a targeted therapy. However, most types of cancer are rarely driven by a single mutation, but rather epigenetic processes which rely on multiple signaling pathways. The activation of these signaling pathways is dependent on the microenvironment of the tumor cells, which is modulated by tumor stroma, vasculature, immune response, hypoxia, and other biological processes. The measurement of multiple variables is necessary order to evaluate the significance of these processes on a targeted therapy, requiring the use of an IVDMIA (In-Vitro Diagnostic Multivariate Index Assay) as a companion diagnostic. IVDMIA refers to a panel of quantified biomarkers in combination with an algorithm that is used to form a prognostic score “that predicts a person’s risk of developing a disease or condition”. The FDA has described one suitable form of an IVDMIA as “a device that integrates quantitative results from multiple immunoassays to obtain a qualitative score”, as long as it relies on the analysis of several variables in a completely objective manner. This description encompasses immunohistochemical (IHC) approaches, and thus creates the opportunity for IVDMIAs to be created using IHC analysis of FFPE (formalin fixed) sections. Specifically, digital pathology based quantification techniques provide the best means to determine these context-specific events and objectively score these measurements. Additionally, the reliance on IHC from FFPE sections is contiguous with the current use of pathology as the “gold standard” of determining prognostic indicators (such as ER/PR/HER2 in breast cancer). Thus, the pursuit of an IVDMIA based on IHC of FFPE sections is the most straight-forward approach to generate IVDMIA companion diagnostics. However, this approach is not without its own hurdles, as a methodology to analyze multiple independent IHC markers from FFPE sections has not been cleared by the FDA. Success in this endeavor will require the elegant use of modern digital imaging techniques to assess multiple IHC markers from a single patient sample to fulfill the FDA’s definition of an IVDMIA..

**Robert Gillies**, Virendra Kumar, Yuhua Gu, Mark Lloyd, Marilyn Bui  
H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL

## **Keynote Lecture:** **Multiscalar Image Analysis: A Window onto Tumor Heterogeneity**

“Anatomics” is the process of extracting large numbers of quantitative descriptive features from biomedical anatomical images. A hypothesis driving this work is that cell and tissue anatomy reflect underlying gene expression and that molecular events can be inferred (modeled) via profound analyses of anatomic features. These image features are thus mineable data that can be used to parse against, e.g. medical outcomes or gene expression data. A long term goal of this work is to develop models that will enable improved prognostics, prediction and understanding of underlying cancer biology using multiscalar imaging.

Our current projects in this area include: non small cell lung cancer, NSCLC, macroscopically imaged with computed tomography, CT (aka “radiomics”), and breast cancer imaged microscopically with histology and immunohistochemistry. Future plans include radiographic analyses of breast cancers and histological analyses of lung cancers to develop two coordinated datasets. For the radiomic analyses of NSCLC, we have developed automated lesion segmentation algorithms using Defnians LuTA and extracted 219 3-dimensional and 102 2-dimensional features from each image of 276 lung cancer patients who underwent biopsy for gene expression profiling. Included in this feature set are 119 features related to tumor heterogeneity: e.g. texture features. For the histological analyses, we have developed robust cell segmentation algorithms and extracted 37 single cell features from 25 patient’s histological images comprised of about 50,000 cells, that, in ensemble, are accurate descriptors of cellular heterogeneity in tumors. At both scales, feature sets are analyzed for covariance, which will allow a reduction in the dimensionality of feature space; feature reproducibility using test-retest data; and biological range. Informative (prognostic) features are expected to be those that are not redundant, have low test-retest variance and a large biological range.

**Jörn Hopke**

Sanofi Oncology, Cambridge, MA

## **Applications of High Content Screening in Oncology Drug Discovery Using Object-based Image Analysis**

In recent years high content screening (HCS) has developed into a well established platform within drug discovery. Numerous commercially available high content imaging systems provide software packages for image analysis. However, the utility of these tools is often confined to a narrow set of predefined assays and customized solutions, such as Definiens Developer Rulesets must be implemented, taking advantage of the increased sophistication of object-based image analysis. In particular, in the context of Oncology Drug Discovery, automated analysis of drug-induced disruption of colony formation is often hampered by difficulties in brightfield image analysis, widely varying phenotypes, and frequent occurrence of artifacts. These issues were addressed by a ruleset exploiting the map concept and image layer operations as implemented in Developer XD. Wound healing assays are another mainstay of oncology drug discovery. Traditionally, cell migration is evaluated by measuring the area of cells encroaching into an artificially created wound. This approach, however, does not differentiate between migration and proliferation. A measure of the nuclear-to-wound edge-distance (NED) is introduced – implemented within a Developer ruleset - that provides a more realistic assessment of the migratory component of wound healing. Finally, Endothelial-to-mesenchymal transition (EMT) is a cellular process implicated in fibrosis and metastasis. We have developed a high content phenotypic screen to identify small-molecules that can prevent or reverse TGF- $\beta$ 1-induced EMT. An Architect solution and a Developer ruleset were built to quantify nuclear and cellular morphology as well as reliably identify the ZO-1 tight junction marker. Of the multiple parameters that were generated as part of the image analysis an optimal subset was selected by minimizing the cross-validated error. The selected features were used in a supervised classifier to call positives.

Esther Aix<sup>1</sup>, Hind Azegrouz<sup>1</sup>, Johannes Zimmermann<sup>2</sup>, **Ignacio Flores<sup>1</sup>**

<sup>1</sup>CNIC, Madrid, Spain

<sup>2</sup>Definiens, Munich, Germany

## **High Content Image Analysis in Heart Regeneration, Stem Cells and Telomere Biology**

How an organ develops and persists during adult life is a fundamental question in biology. One hypothesis of organ maintenance is that stem cell functionality determines the ability of tissues to replace worn-out or injured parts. However, for most tissues the nature of the organ-forming cells and stem cells is poorly defined. We recently showed that within tissues there is a gradient in the length of telomeres, the physical chromosome ends. Given that telomeres shorten with each cell division, we hypothesize that the most primitive cells will be those cells harboring the longest telomeres.

We are currently conducting a high-content telomere length analysis to study the location, prevalence and status of putative cardiac stem cells and their progeny during organogenesis and aging. We are also examining the relationship between telomere length and the ability of cardiac cells to generate new cardiac tissue. Finally, we are investigating the factors that regulate telomere length, with the aim of defining their contribution to cell differentiation. Our more recent results taking these approaches will be presented at the 2nd International Definiens Symposium.

**Alexander Heidrich**

HKI, Jena, Germany

## **Automated Feature Extraction from microPET/CT Images of Live Chick Embryos *in ovo*: Incubating a Versatile Model for Preclinical Imaging**

The chick embryo *in ovo* is an excellent *in vivo* model system that has a long history of application in almost any field of biomedical research.

Consequently the embryonated chicken egg is also a highly interesting subject for preclinical imaging particularly with the emerging availability of high resolution microPET/CT scanners. The research of the microPET/CT imaging group at the HKI Jena is focused on establishing the chick embryo *in ovo* as model system especially for but not limited to the *in vivo* stability evaluation of newly synthesized tracers and the imaging of infectious diseases. However, besides technical challenges, such as anesthesia and tracer injection methodology, also in our lab the dominant bottleneck remains the efficient and reproducible analysis of acquired image data.

In this talk we present an approach for the automatic analysis of 3D <sup>18</sup>F-fluoride microPET images of live chick embryos *in ovo*. <sup>18</sup>F-fluoride alone is a well known bone tracer but it can be also bound to other biomolecules targeting different processes of interest. The stability of these complexes can be evaluated by quantifying the enrichment of dissociated or separated <sup>18</sup>F-fluoride in the bones.

The complete process of image loading, preprocessing as well as segmentation and classification of body structures is realized by employing Definiens Developer XD, CNT and a sophisticated anatomical model.

**Günter Schmidt**

Definiens, Munich, Germany

## **The Power of Numbers – Cell by Cell: Definiens Tissue Studio 3**

Definiens Tissue Studio is the leading image analysis solution to extract fully-automatic meaningful quantitative data, from tissue slides. The latest version of the software comprises even higher performance and scalability by supporting 64-bit operating systems natively, while increasing the robustness and accuracy of the automated region of interest detection with Composer technology. Integration into the image management systems from Aperio, Leica and Philips, streamlines workflow resulting in shorter times from image acquisition to image quantification.

To illustrate the power of Definiens Tissue Studio 3, we show the automated analysis of HER2 immunostained tissue microarrays of esophagogastric cancer. The Definiens image analysis is based on a true cell-by-cell scoring according to the ASCO/CAP guideline recommendations for HER2. The image analysis results are compared with pathologists' scoring. Additionally, Definiens Image Miner is used to correlate the quantitative, numerical results from Tissue Studio analysis with clinical endpoint data, such as disease-free survival time. The result backs the assumption that HER2 overexpression is a negative prognostic factor for cancer recurrence.

**Johannes Zimmermann**  
Definiens, Munich, Germany

## Extending the Limits: Definiens Developer XD 2

Over the last years, Definiens' Cognition Network Technology became the standard for the automated image analysis addressing complex questions in Digital Pathology, and other fields of biomedical research, such as High Content Analysis or Radiology. The development environment making this technology accessible to the scientific end user - even without having necessarily a background in programming or image analysis - is the Definiens Developer. Offering the graphical metalanguage CNL (Cognition Network Language) for the highly interactive creation of analysis protocols, it bridges the gap between the functional knowledge of the domain expert and the practically limitless freedom of advanced image analysis.

A new era for the international Developer community started in 2008 with the advent of Definiens Developer XD, opening up the third dimension, enabling time lapse analyses and offering novel concepts like maps, facilitating complex workflows for example in the analysis of tissue slides.

The continuation is Definiens Developer XD 2: With its 64 bit processing capabilities it further extends the range of possibilities for the analysis of very large datasets and supports memory intensive operations. The artificial intelligence approach pursued in the paradigm of funneling an expert's domain knowledge into CNL rule sets is complemented by a number of machine learning tools, like Bayesian classifiers, KNN, Support Vector Machines and Trees. The Cognition Network Language is enhanced by arrays, allowing the concerted processing of items like features, image object levels, image layers etc. simultaneously. A range of new algorithms and features completes the Definiens Developer XD 2.

**Arno Schäpe**

Definiens, Munich, Germany

**Extracting the Essence: Definiens Image Miner**

Definiens Image Miner provides a comprehensive suite of statistical data analysis and visualization methods, which retain a close link to the underlying image. Users can combine images and extracted image information with clinical data, and use feedback loops to optimize results throughout the complete analysis chain. This unique feature combination enables deeper insights into the biological and clinical information contained within images, faster results, scalable workflows and better decision-making when developing image-based biomarkers.

In a live demonstration, we will present an overview of Definiens Image Miner and demonstrate its data visualization and analysis capabilities. We will show how Image Miner works with Definiens XD Developer to develop segmentations and classifiers for the analysis of H&E-stained tissue images. Using measurements extracted from the image analysis combined with clinical endpoint data – such as disease-free survival time – we will train and optimize a model for a new image-based biomarker analysis. We will then compare our results to pathologists' predictions based on Her2 quantification.

**Hind Azegrouz**, María Montoya  
CNIC, Madrid, Spain

## **Analysis of Individual Cell Data in Image Based High Content Screens**

The ever-pressing need to improve the yield of higher quality hits has led to the use of cell-based screenings that allow access to vast amounts of data not only well-based but also on individual cells. Analyzing this data on a per-cell level can be very informative, particularly when a well-based measure fails to capture the complexity of the phenotype. Dealing with such large data sets can also be challenging. We will discuss methods we have developed at CNIC for analyzing well-based and cell-based data, including phenotype classification, machine learning, and hit identification.

**Ansuman Bagchi**

Global Services, Merck & Co, Inc., Rahway, NJ

**Effective use of Definiens Technology for Image Analysis in Drug Development at Merck**

Imaging is a valuable tool extensively used towards developing effective biomarkers for the assessment of therapeutic effects across all phases of drug development. At Merck, various imaging techniques such as fluorescence, histology sectioning, microscopy, MRI, CT, US etc are being utilized to visualize tissues, compartments, and systems modulated by disease progression and/or therapy. Definiens software platform enables automated computerized analyses extracting relevant quantitative information from imaging data in reliable, efficient, and objective manner. Examples presented in this talk may include: Automated detection of abnormal bones in developmental and reproductive toxicology studies, quantification of RNAi particle accumulation using intravital imaging, analysis of aorta sections for development of vascular stiffness biomarkers, and quantification of beta cell mass from pancreas histology sections for diabetes biomarkers.

**Joe Trask**

The Hamner Institutes for Health Sciences, Research Triangle Park, NC

**Identifying and Quantifying Apoptotic Subpopulation Cells in Liver Sections Following Compound Dosing**

A small animal study design at the National Toxicology Program treated rats with 2,3,7,8-Tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD), a known developmental toxicant and probable human Group B2 carcinogen as classified by the US-EPA in 2000. At week 31, liver tissue from animals were preserved in paraffin embedded block and sectioned at 5-microns. Subsequent staining using probes for apoptosis (TUNEL, Activated Caspase-3, and DAPI) were used to determine expression level and if damage occurred in liver tissue. Aperio scanned slides were analyzed using tools from the Definiens Developer XD and Tissue Studio Image Analysis toolbox. Workflow process using Definiens tools and outcome will be discussed.

**Cleopatra Kozlowski**  
Genentech, San Francisco, CA

## **An Entirely Automatic Method to Score Crypt Loss and Infiltration from Pathology Slides in a Mouse DSS Model of Colitis**

The DSS (dextran sodium sulfate) model of colitis is a mouse model of inflammatory bowel disease, a condition that affects more than 1 million people in the US alone. Microscopic symptoms include loss of crypt cells from the gut lining, and infiltration of inflammatory cells into the colon. An experienced pathologist requires over 6h for 100 H&E slides to manually score loss of crypts in selected regions of the mouse gut. In order to increase the efficiency of scoring, we used Definiens Developer (Definiens AG, Munich) to devise an entirely automatic method to quantify loss of crypt cells in the whole slide. The method relies on a combination of morphological and intensity based features of every tissue area, as well as a neighbor-based classification method. We found an overall correlation of  $> 0.7$  between independently assigned automatic and manual scores, in approximately 800 DSS treated mice. The correlations were robust across 12 studies, in spite of large variations in slide quality and staining intensity. Though the analysis requires approximately 40min of CPU time per slide, cluster computing allows us to analyze up to 500 slides overnight without any manual intervention. Further, we developed another entirely automatic method to count infiltrating macrophages, neutrophils, and T cells in IHC of serial sections to the H&E slides. Overall, our methods greatly facilitate the scoring of mouse colon in DSS induced colitis, and enable quantitative analysis of morphological tissue changes and cell infiltration.



# Posters

**M. Athelougou<sup>1</sup>, M. Eblenkamp<sup>2</sup>, G. Schmidt<sup>1</sup>, F. Novotny<sup>2</sup>, E. Wintermantel<sup>2</sup>, G. Binnig<sup>1</sup>**

<sup>1</sup>Definiens Munich, Germany

<sup>2</sup>Institute of Medical Engineering, Technical University Munich

## **Image Analysis for the Calculation of the Toxicity Degree of Cells in Phase Contrast Microscopy Images**

Because of the very special type of contrast in phase-contrast images, it is almost impossible to perform fully automated single-cell analysis and quantification successfully. Because fluorescent dyes are highly toxic, phase-contrast images are commonly used to monitor live cells. In this poster, we present a method for the fully automated segmentation, classification and quantification of individual cell morphology in phase-contrast images. We calculate the confluence of the cell population and quantify the degree of toxic damage to each individual cell following phenol incubation. The results are then compared to standard cytotoxicity assays.

## Jens Brodbeck<sup>1</sup>, Felix Chu<sup>2</sup>, Melissa Gonzales Edick<sup>2</sup>, Gary Cain<sup>1</sup>

<sup>1</sup>Department for Safety Assessment

<sup>2</sup>Department of Pathology

Genentech, South San Francisco, CA

### Fluorescence Compared to Brightfield CD35 Detection in Lymphoid Follicles of the Monkey to Assess Immunomodulatory Treatment

*Aims* Lymphoid follicles of secondary lymphoid organs are dynamic structures inducible by immunization and/or inflammatory stimulation. Follicular dendritic cells (FDC) are specialized antigen-presenting cells exclusive to the germinal centers (GC) of lymphoid follicles. Within the GC, FDC's form a reticular network that sustains the architecture of lymphoid follicles and interact with the GC B cells via several mechanisms including complement receptors such as CD35. The expression of CD35 in lymphoid follicles is therefore a measure of the efficacy of immunomodulatory (IMM) treatment strategies.

*Methods* CD35 in formalin-fixed paraffin-embedded spleen sections of cynologous monkeys was detected with a mouse monoclonal antibody and visualized using a polyclonal secondary antibody coupled to either horse-radish peroxidase for DAB precipitation or the fluorophore Alexa647. Digital brightfield and fluorescence images (20x) were acquired on a Nanozoomer 2HT (Hamamatsu) and Ariol SL-50 (Genetix) whole slide scanner respectively. Images were quantified using Definiens Developer software.

*Results* Fluorescence significantly increased the dynamic range of CD35 compared to chromogen. Fluorescence signal intensities ranged from 0 – 256 compared to 0 – 0.8 using chromogen. Both detection methods showed a small but significant decrease in follicular CD35 following IMM treatment without resolving dose effects. However the increased dynamic range of fluorescent CD35 allowed a robust distinction between follicles containing germinal centers and those without. Thus a clear dose effect after IMM treatment could be resolved using fluorescence, but not chromogen for CD35 detection.

**T. Chen, V.C. Estrella, R.A. Gatenby, R.J. Gillies, M.C. Lloyd**  
H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL

## **Using Definiens Software Analyze Intracellular and Tumor Extracellular pH**

Dysregulated pH is emerging as a hallmark of cancer because cancers show a 'reversed' pH gradient with a constitutively increased intracellular pH that is higher than the extracellular pH. This gradient enables cancer progression by promoting proliferation, the evasion of apoptosis, metabolic adaptation, migration and invasion. Comprehension of mechanisms of pH regulation in tumors is of paramount importance for therapeutic implications. Several new advances, including an increased understanding of pH sensors, have provided insight into the molecular basis for pH-dependent cell behaviors that are relevant to cancer cell biology. Here we describe the use of a Olympus FV1000 MPE multiphoton laser scanning microscope in conjunction with a window chamber affixed to the dorsal skin of Severe Combined Immunodeficiency (SCID) mice for the study of the tumor pHe gradient in the WC; normal cell intercellular pH<sub>i</sub> gradient and how to analyze the pH image with using Definiens image software.

**V.C. Estrella, T. Chen, M.C. Lloyd, A.H. Ibrahim, J. Wojtkowiak,  
H. Cornell, R.J. Gillies, R.A. Gatenby**

H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL

## **Multi-Modal Imaging and Definiens Analysis of *in vivo* Tumor Invasion**

Invasion of normal tissue is a characteristic of malignant tumors and is critical for tumor progression. We have previously proposed that tumor-associated acidity is a critical factor in cancer invasion. Tumor acidity is a result of increased glucose metabolism that causes elevated H<sup>+</sup> production and excretion so that the extracellular pH of malignant tumors is typically acidic compared to the extracellular environment of normal tissue. Observations that virtually all clinical cancers demonstrate increased glucose metabolism on FdG PET scans have led to the acid-mediated invasion hypothesis. Briefly, this model proposes that H<sup>+</sup> ions flow along a concentration gradient from the tumor into adjacent normal tissue. The resulting acidic environment is toxic to normal cells, promotes a degradation of the basement membrane, increases angiogenesis, and blunts the immune response to tumor. In this study, we used a mice dorsal window chamber model in conjunction with intravital multiphoton microscopy and Definiens to dynamically observe and quantify tumor growth and invasion. In addition, *ex vivo* studies of the tumors were used to investigate the expression of metabolic tumor and angiogenic markers (GLUT-1, NA/H<sup>+</sup> exchanger, CD31), which were in turn analyzed using the Definiens software to elucidate the underlying biology of tumor invasion.

**Robert J. Gillies, M.C. Lloyd, K.A. Rejniak, M. Robertson-Tessi, M.M. Bui, V. Kumar, Y. Gu, R.A. Gatenby**

H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL

## **Multiscale Image Analysis and Modeling to Investigate Heterogeneity**

'Pathomics' is the study of quantitative pathology including molecular and morphological feature extraction. 'Radiomics' is the extraction of features from CT, PET and other radiological imaging techniques. The purpose of this study is to evaluate a combination of multiparametric features from multiple scales [micro-( $\mu\text{m}$ ), meso-(mm), and macro-(cm)] and of patient data to compose a comprehensive data group of image analysis features. Our hypothesis is that taken together, this mineable database of feature data, when correlated with outcome will off insights into cancer progression, optimized personal treatments, and ultimately improved outcomes.

Our current projects in this area include: non small cell lung cancer, NSCLC, macroscopically imaged with computed tomography, CT, and breast cancer imaged microscopically with histology and immunohistochemistry. A great deal of data is being collected including 219 3-dimensional and 102 2-dimensional features from each image of 276 lung cancer patients who underwent biopsy for gene expression profiling, as well as 37 single cell features from 25 patient's histological images comprised of about 50,000 cells.

At both scales, feature sets are being analyzed for covariance, which will allow a reduction in the dimensionality of feature space; feature reproducibility using test-retest data; and biological range. Informative (prognostic) features are expected to be those that are not redundant, have low test-retest variance and a large biological range.

In order to understand these static image data points in a more dynamic way, mathematical modeling is being employed to understand trends of growth dynamics and potential drug resistance and efficacy. Further developing the feature database and modeling components together allows for the most robust experimental validations of the models in tandem with model derived results which drive predictions which can spur new experiments and ways to evaluate the data.

**Yuhua Gu, Virendra Kumar, Lawrence Hall, Dmitry Goldgof, René Korn, Claus Bendtsen, Robert A. Gatenby, Robert Gillies**  
H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL

## **Automated Delineation of Lung Tumors from CT Images: Method and Evaluation**

An automated delineation of lung tumors from CT images is presented. Previous work utilized a “Click & Grow” algorithm which is a seed based segmentation of the lesions from the Lung field. Specifically, the lesion within the segmented lung was identified and a seed point was placed in its interior - typically at the perceived center of the lesion. Starting from the seed point, the lesion object was segmented using region growing based on similar intensity and proximity to areas with low intensity. This process commonly had to be repeated multiple times and the segmented volumes merged. In this work, an improved segmentation algorithm is presented. The new algorithm utilizes a multi-seed point approach. The multi-seed point segmentation algorithm was designed to overcome the drawbacks of the original algorithm. It uses the region growing algorithm for an automatically chosen set of multiple seed points following the initial segmentation. Thus, an ensemble segmentation is obtained from the multiple regions that are grown. Stable, accurate and automatic lung tumor delineation can be achieved as a result of the ensemble segmentation. The new method has been evaluated on a set of Moffitt Cancer Center lung tumor datasets, the similarity index (SI) was calculated, the average SI is above 93% over 129 patients with 20 different start seed points for each case. The results were also compared with 2 different readers’ and the level set algorithm, the agreements were 79.01%, 72.31% and 78.28% respectively. The agreement between two readers was 73.07%. Furthermore the multi-seed point approach is advantageous in that it only required single operator inputs.

**M.C. Lloyd, J. Proemsey, D. Song, O. Reunova, T. Zhukov, M.S. Tockman**

H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL

## **Using Definiens for Automated High-Throughput Identification of Centrosome ROIs from Stage I Lung Cancer Tissue Sections**

Centrosomal defects are detected early in various human cancers, including lung, breast, colorectal, head-neck, prostate and ovaries, but are rarely observed in normal tissues. Centrosomal defects may increase in severity during tumor progression and metastases. In this project, Definiens Developer XD v.1.5 has been utilized to define Regions of Interest prior to quantitative analysis of centrosome features as a prognostic biomarker for resected stage I lung cancers. This is being done by classification of resected tumor into long term (survivors) and short term (fatality) groups.

Specifically anti- $\lambda$ -tubulin antibodies were used to fluorescently tag centrosomes in nearly 119 lung cancer cases slides (n=15 each; 1785 total images) with nuclei counterstained with DAPI. Developer rulesets were designed to segment the nuclei and centrosomes and then re-link the two. Manually identified centrosomes were counted and locations were noted in a subset of 120 images. Challenges in image variability, contamination of non-target tissue and staining inconsistencies were found to be significant. Rulesets were developed through multiple iterations to match the manually identified gold standard. To date, 84% correlation has been achieved between the automated ruleset, and the manually derived gold standard.

In this report, our objective, quantitative algorithmic assessment of centrosome features provides the first report of quantitative measurement and analysis of centrosome features as a prognostic biomarker predictive of individual survival in stage I NSCLC.

**M.C. Lloyd, K. Rejniak, J. Johnson, T. Chen, R. Gillies, R. Gatenby, J. Brown, MM. Bui**

H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL

## **Quantitative Evaluation of the Morphological Heterogeneity in Breast Cancer Progression**

Cancer cell heterogeneity has long been accepted to be a factor of cancer progression and resistance to therapeutic intervention. To gain quantitative insights in tumor heterogeneity, many studies have been carried out at the molecular and genetic scale. However, there is little information on tumor heterogeneity at the cellular scale, i.e., the variability of individual cells with respect to phenotypic core traits like proliferation, survival, morphology, and metabolism. While genetics and signaling networks are the basis of core traits; cell variability with respect to their ability to perform core trait functions under diverse conditions within the physical microenvironment is what may decide trends in tumor growth dynamics.

**Franziska Mech<sup>1,4</sup>, Andreas Thywißen<sup>2,4</sup>, Reinhard Guthke<sup>3</sup>,  
Axel A. Brakhage<sup>2,4</sup>, Marc Thilo Figge<sup>1,4</sup>**

<sup>1</sup>Research Group Applied Systems Biology

<sup>2</sup>Department of Molecular and Applied Microbiology

<sup>3</sup>Research Group Systems Biology /Bioinformatics

Leibniz Institute for Natural Product Research and Infection Biology, HKI,  
Jena, Germany

<sup>4</sup>Friedrich Schiller University Jena, Germany

## **Automated Image Analysis of the Interaction between Phagocytes and *Aspergillus fumigatus* during Infection**

*Aspergillus fumigatus* is a ubiquitous airborne fungus and opportunistic human pathogen. In immunocompromised hosts, the fungus can cause life-threatening diseases like invasive pulmonary aspergillosis. Since the incidence of fungal systemic infections drastically increased over the last years, it is a major goal to investigate the pathobiology of *A. fumigatus*. The fungus interferes with the innate immune system. Phagocytes, especially macrophages, play an important role in detection and elimination of *A. fumigatus* conidia in the early stages of infection. These interactions are quantified using microscopy techniques. At present, microscopy images are often analysed manually, including cell counting and determination of interrelations between cells, which is very time consuming and error-prone. Automation of this process is necessary to overcome these disadvantages and to standardise the analysis. We present the application of an automated analysis to (confocal laser scanning) microscopy images from macrophages cocultured with different *A. fumigatus* strains. To perform the image analysis in an automatic fashion, we developed a ruleset [1] that generally evaluates phagocytosis assays and was processed by the software Definiens Developer XD. As a result of a complete image analysis we obtained features such as size, shape, number of cells and cell-cell contacts. The analysis reveals that different mutants of *A. fumigatus* have a major influence on the ability of macrophages to adhere and to phagocytose the respective conidia. In particular, we observe that the phagocytosis and adhesion ratio, as well as the aggregation behaviour of pksP mutant compared to wild-type conidia are significantly increased. The

obtained spatio-temporal data can further be used to set up and validate mathematical models describing host-pathogen interactions [2].

1. Mech F, Thywißen A, Guthke R, Brakhage AA, Figge MT (2011) Automated Image Analysis of the Host-Pathogen Interaction between Phagocytes and *Aspergillus fumigatus*. PLoS ONE 6: e19591.
2. Albrecht D, Kniemeyer O, Mech F, Gunzer M, Brakhage AA, et al. (2011) On the way toward systems biology of *Aspergillus fumigatus* infection. Int J Med Microbiol. doi:10.1016/j.ijmm.2011.04.014





