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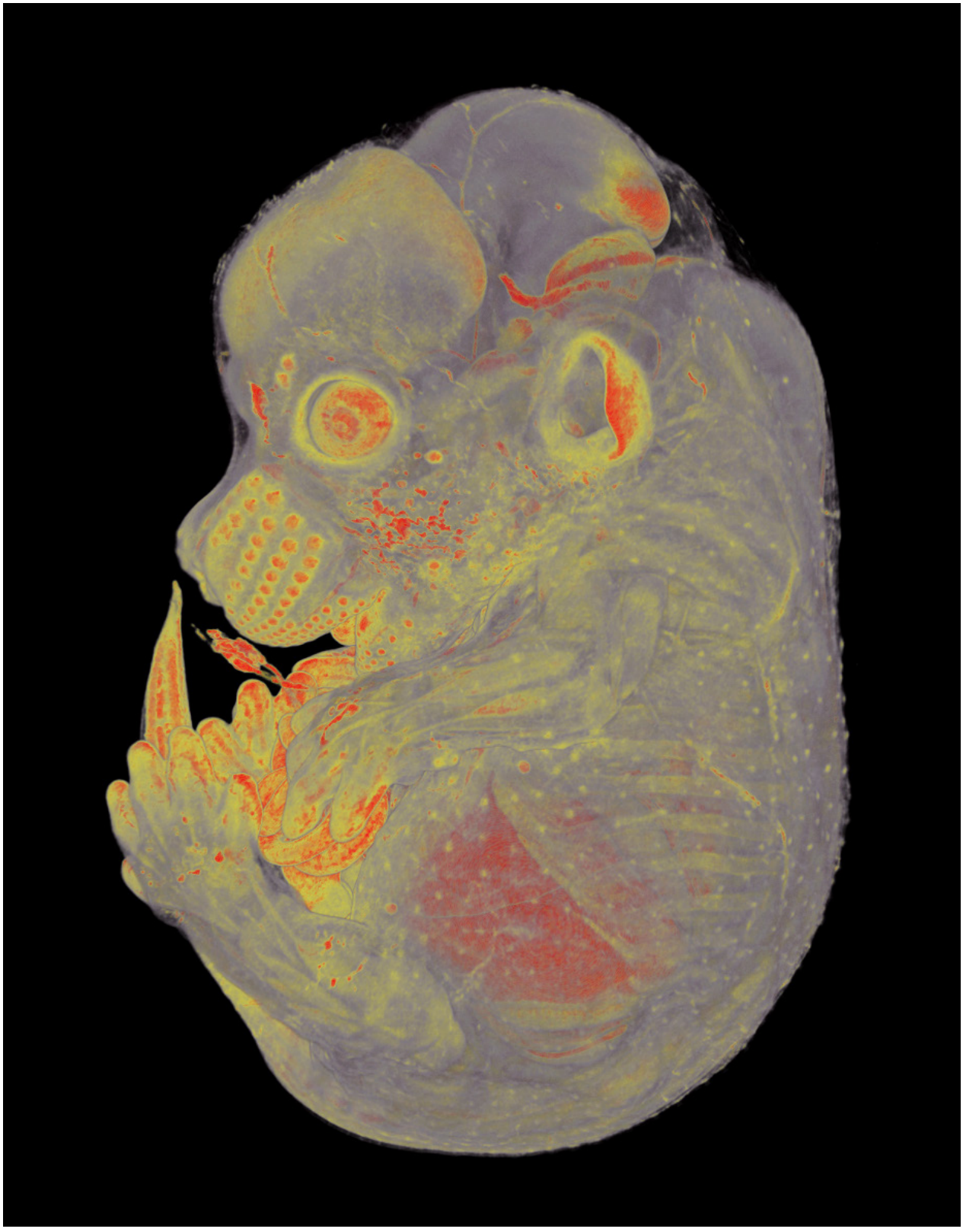
PHENOGENOMICS
NEWSLETTER



Czech Centre for Phenogenomics

The Czech Centre for Phenogenomics is hosted by the Institute for Molecular Genetics CAS, v. v. i.

IMG

PRE KLINIKY

Centre for Preclinical Testing

New services on preclinical testing

After obtaining the certificate of Good Laboratory Practice (GLP) in January 2017, the Centre for Preclinical Testing (CPT) commenced activities with the support of the Czech Academy of Science (CAS). The primary mission of the CPT is to perform preclinical testing of substances that have successfully passed through basic research, and thus to contribute towards the development of new pharmaceuticals to combat life-threatening diseases, including those currently difficult to cure.
The Institute of Physiology CAS performs a coordination role for the CPT, and testing is also performed by the Institute of Molecular Genetics CAS including National infrastructure CCP, the Institute of Animal Physiology and Genetics CAS, and the Institute of Biotechnology CAS.
The CPT offers a broad portfolio of tests under Good Laboratory Practice (GLP).

Core services:

- **Toxicity studies**, including toxicokinetic studies of promising chemical or biological agents on model animals – rodents and non-rodents (test systems: mouse, nude mouse, rat, guinea pig, rabbit, minipig) in compliance with ICH and OECD guidelines.
- **Bioanalytical, hematological and biochemical testing** of samples taken from animals during toxicity studies (determination of active substance in plasma or other biological matrices).
- **Development and validation of bioanalytical methods** for various test systems and biological matrices**.**
- **Determination of metabolites** in tissues and biological matrices (blood, plasma, urine)
- **Histopathological evaluation** of tissues from animals used in toxicity studies
- **Pharmacological studies on xenografts (nude mice)** with various cancerous cell lines, including Patient Derived Xenografts (PDX)
- **Cardiology diagnostic tests on animal models** – electrocardiogram (ECG), blood pressure measurement, cardiac imaging (Echo)
- **Synthesis, characterization and certification** of chemical substances with therapeutic potential, development of formulations for drug application

CPT facilities working under GLP regulations have an established quality system, which is regularly inspected internally by the Quality assurance unit, the National Authority and by our customers. The quality of our services is top priority for all our team members.

The CPT offers its services to customers from both the academic and commercial sectors. Testing is carried out by recognised experts with long-term experience. The great advantages are the coordinated approach, flexibility, and the comprehensive nature of the offered services. You can find out more about the scope of CPT activities on the website: www.prekliniky.cz.

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Cover Image: Iodine contrast image of bile ducts (J. Prochazka, F. Spoutil)

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Note to customers: As valued customers, we welcome your articles and feedback on the service you received. Please send all correspondence to ccc@phenogenomics.cz

The editorial team would like to thank the authors in this issue for their contribution.

Editorial

Dear Readers,

This edition of the CCP newsletter not only discusses key advancements in our research practices, most notably in our high-resolution microCT imaging protocols, mass spectrometric data analysis and electroretinography procedures but also introduces patient-derived xenographs (PDX) as a newly available service.

Also featured in this issue are articles by guest writers, Prof. Jesus Ruberte and Dr. Jan Rozman. Prof. Jesus Ruberte, an authority on mouse anatomy and pathology, presents his latest project, “Pathbio”, an advanced training program in precision mouse pathology for scientists, while the newly appointed Deputy Director of CCP, Dr. Jan Rozman, discusses uses for the metabolic data gathered by the International Mouse Phenotyping Consortium.

The editorial team

Imgene - A consortium to improve genome editing

L. Syding

IMGENE (Improving Genome Editing Efficiency) is an EU-funded consortium initiative of the Marie Sklodowska Curie foundation. It commensed on September 1st 2017 and will proceed for three years focusing on the CRISPR/Cas9 genome editing technique, in particular focusing on ways to advance the technique and the ethical concerns regarding facilitated genome editing in animals. As surely no one has failed to notice, CRISPR/Cas9 has become the genome editing tool of choice in most labs around the world due to it being highly dynamic and easy to use compared to traditional genome editing tools. However, there are still difficulties that need to be addressed, namely: increase of precise genomic editing events via the homology directed repair (HDR) mechanism and safe delivery of CRISPR/Cas9 in to the host.

IMGENE is comprised of six academic institutions, one pharmaceutical company and additional beneficiaries MilliporeSigma, Taconic and GeneticAlliance. One of the positions is hosted by the CCP/IMG facility where the PhD student is focusing primarily on delivery of CRISPR/Cas9 via in vivo electroporation and establishing transgenic spermatogonial stem cells. In addition there are seven more PhD students from six different countries working in different institutions around Europe with the same aim to develop the CRISPR/Cas9 technique but from different perspectives. One fellow focusing solely on the ethical questions regarding CRISPR/Cas9 that need to be solved in order to gain acceptance by society.

The fellows meet together with their respective supervisors for both winter and summer schools each year. The fellows train transferable skills, develop outreach activites and attend highly relevant lectures connnected to the field. The last meeting was held at the CCP/IMG facility in Vestec on 17-18th January 2019 and was lead by CCP's own assoc. Prof Radislav Sedlacek who is also the responsible PI for the PhD student in CCP/IMG.

Additionally, to strengthen the exchange and collaboration between the fellows, each PhD student will spend a couple of months in another laboratory or with a beneficiary.

Precision pathobiology for disease models

J. Ruberte and G. Gracia

Mouse-based studies are essential for all Precision Medicine Initiatives (PMIs), which aim to transform current medical practice to personalized healthcare. Global PMIs will combine tens-of-thousands of human individual genetic profiles, deep phenotyping, and environmental exposures and lifestyle behaviours to guide therapies more targeted and cost-effective than current "one-size-fits-all" strategies. Mouse models will be needed to provide efficient and accurate assessment of the scientific validity of such gene-environment correlations.

Mice represent over the 60% of laboratory animals used in Europe for pharmaceutical and biotechnology companies, as well as research institutions (Fig. 1).

The transgenic and genetic manipulation technologies applied to the mouse have provided experimental proof that a significant proportion of inherited diseases in all species share a common genomic source. A genetic mutation associated with human disease, when inserted into the mouse genome, always induce some or all of the human disease phenotypes in the mouse. Accordingly, almost 95% of the human genes have a mouse homolog. As a direct consequence, loss-of-function mutations or disruption of the sequence within a gene in a mouse model is an important starting point for discovering and understanding normal gene function. The recent revolution in gene editing, the CRISPR-Cas9 technology, has also provided an additional and powerful approach to design and produce mouse models with the same insertion or deletion missense mutations that occur in the human genome, which are responsible for a significant proportion of inherited or acquired human disease.

Furthermore, the success of using genetically engineered mice to evaluate genetic disease hypotheses has encouraged the development of large-scale European and global projects, making the mouse the most used animal model. However, the scientific community lacks sufficient human resources and expertise in mouse pathology to effectively and reproducibly characterize and validate these animal models; in particular the ability to confirm causation of identified human disease variants as pathogenic. This is obvious when scientific publications are analysed (Fig. 2). Despite the increasing number of scientific papers involving the use of mouse models, pathology is employed as an analytical tool in only half of these and in only one out of 25 articles pathology and imaging are used to validate the mouse model. Thus, it is clear that pathobiology is a critical tool in the analytic armamentarium for informative mouse phenotyping (1, 2, 3).

Due to the lack of adequately trained experts to accurately and reproducibly characterize the morphological phenotype of mouse models at the organ and tissue level, many researchers are forced to rely on their own DIY (Do-it-Yourself) pathology. As a result, the scientific literature contains numerous examples of phenotypic misinterpretations (4). The mouse has unique anatomical, histological, and pathological features, along with spontaneous and expected strain-associated pathologies that must be recognized as significant findings. Preputial glands that humans and other mammalian species do not possess are only one example of misidentification of normal mouse organs (5). More important, is the fact that DIY pathology, the interpretation of histopathology by untrained investigators, is generating a

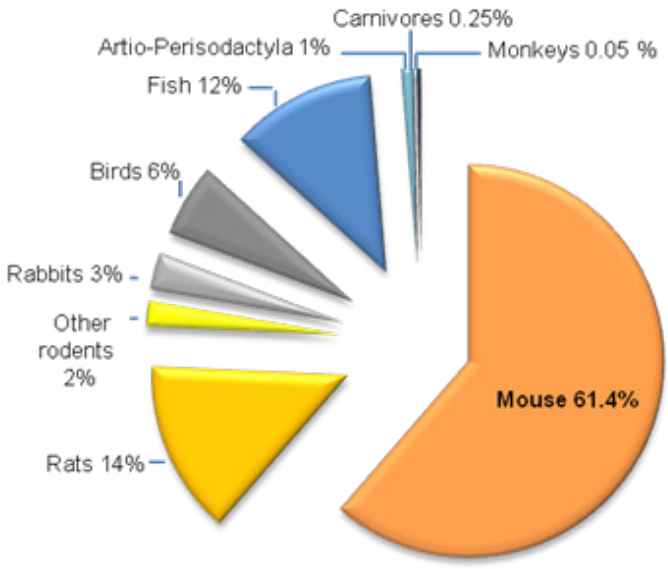

Figure 1: Seventh Report from the Commission Council and the European Parliament on the Statistics on the number of animals used for experimental and other scientific purposes. COM (2013) 859 final.

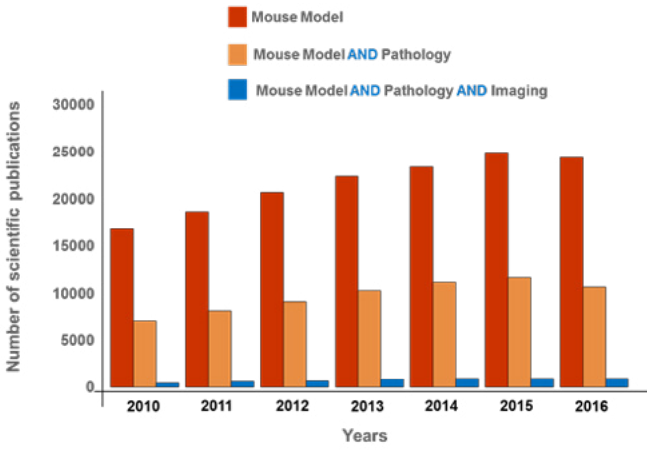

Figure 2: Number of publications on mouse models involving pathological and imaging analysis. (Source PubMed)

lack of reproducibility of results obtained using mice in studies of biology, disease and toxicology (6, 7). In addition, we find widespread failure to report key features of an investigation and phenotypic interpretation; a problem with all investigators, whether trained pathologists or not (8, 9). An analysis of past preclinical studies indicates that the cumulative prevalence of irreproducible research exceeds 50%, resulting in approximately US$28,000,000,000/year spent on preclinical research that is not reproducible. This is very counter-productive, hinders advances in our understanding of human disease and the development of novel therapies, increases cost and redundancy in research, and uses animals unnecessarily (10, 11). Moreover, lack of expertise in identifying and interpreting experimental findings in mouse models can miss important findings, as well as report spurious findings leading to unfounded criticism of potentially valuable mouse models (12, 13).

Figure 3: The innovative concept of Mouse Precision Pathobiology launched by the PATHBIO Consortium

Despite this increasing demand for mouse pathobiology experts, there is a proven significant deficiency of specialised training opportunities for veterinary and medical pathologists and biomedical researchers to acquire the necessary expertise, especially formalized and recognized programs in Higher Education. In this scenario, no single European University has all the expertise, resources and personnel required to design and establish a strong educational program in Mouse Precision Pathobiology, which integrates pathology, anatomy, embryology, imaging, ontologies and informatics (Fig. 3).

Mouse Precision Pathobiology will break the classic boundaries between anatomical and histological laboratories integrating mouse morphology and functional anatomy passing through its different levels from the organic, the histological level, to the cellular and subcellular levels (Fig. 4). The integration of specific anatomical techniques such as the osteological preparations or blood and lymphatic vessels injection together with classic and new miniaturized microscopic techniques will allow a more complete phenotyping of mouse disease models.

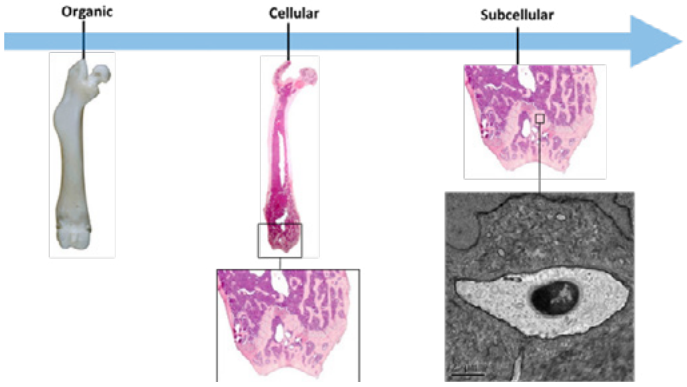

Figure 4: Mouse Precision Pathobiology integrates morphology through its different levels

Traditionally, imaging is considered an independent discipline in medical sciences. However, there is a growing appreciation of imaging in mouse phenotyping (14). The argument for that is that if we are interested in studying mouse mutants as models of human diseases, we should have the same diagnostic capabilities for mice that we have for humans. Furthermore, unlike what happens in humans, mouse images obtained by X-ray, Computed Tomography (CT), and Magnetic resonance (MRI) are morphologically comparable with their equivalent histopathological images (Fig. 5). Thus, the combination and integration of in vivo imaging technologies and gross and histologic pathological post-mortem techniques will increase the accuracy of morphological mouse phenotyping.

In this scenario several universities, research institutions and companies, aka. the three sides of the "knowledge triangle", have come together to establish PATHBIO, a Knowledge Alliance initiative awarded by the European Commission (600803-EPP-1-2018-ES-EPPKA2-KA), to develop a Virtual Learning Platform with innovative teaching materials and to organize and deliver intensive specialized summer courses in Mouse Precision Pathobiology. The PATHBIO project also aims to design a Joint European Master Degree. The new degree program aims to become a benchmark Master qualification internationally in Mouse Precision Pathobiology.

The PATHBIO summer courses will be held during the three

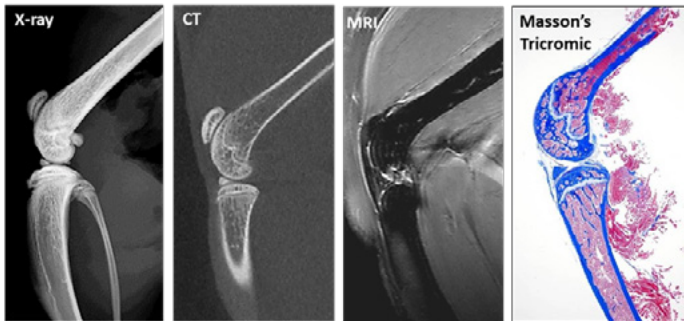

Figure 5: Mouse Precision Pathobiology integrates pathology and imaging. Equivalent images of the mouse knee using imaging and histological techniques.

years of the project. They will take place over one month and comprise of three modules (Fig. 6). Module 1 will be dedicated to anatomy, embryology, and histology; Module 2 to Imaging (micro-CT, MRI, and Ultrasound Biomicroscopy); and Module 3 will be devoted to pathobiology, genetics, and bioinformatics.

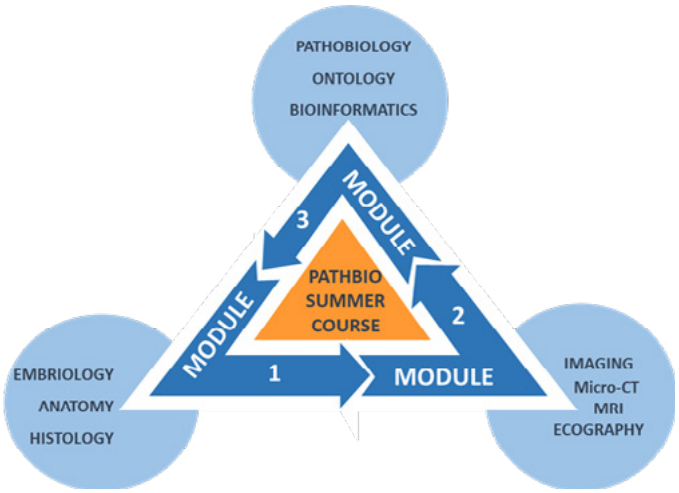

Figure 6: Modular Organization of the PATHBIO summer courses

The PATHBIO project and its engaged partners are ideally constituted at the European level to develop a strong educational program for Mouse Precision Pathobiology. PATHBIO includes leading universities (UAB, UCAM, UCPH, FMV-ULisboa, Unina, and USFD) in the field. Furthermore, five major European mouse clinics (CERBM-ICS, MRC Harwell, HMGU, IMG, and CNR) equipped with integrated facilities for production and characterization of genetically engineered mouse models are also part of PATHBIO. These mouse clinics are developing and using state of the art mouse imaging technologies and have long standing expertise in pathology phenotyping along with the data-capture, annotation, and bioinformatic analysis capabilities to interpret the resulting datasets. Three of the largest producers and suppliers of mouse models worldwide heavily involved in drug research (CHARLES RIVER, JAX and TCP Inc.) and a company leader in the field of preclinical imaging (FUJIFILM VISUALSONICS) are also essential partners of PATHBIO. FELASA and ICLAS are two important European and global animal science associations, representing common interests in all aspects of laboratory animal science in Europe and beyond, which are also integrated within the PATHBIO Consortium. Finally, the interaction with the Asian, Australian, South American and African Phenogenomics centres as associate partners, provides PATHBIO with a unique and pre-eminent global position to define a common and fundamental skill set required.

If you are interested in applying for a PATHBIO summer course please contact: ggarnau@gmail.com

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Electroretinography

J. Lindovsky

Introduction
Electroretinography (ERG) is a useful tool for objective, non-invasive assessment of retinal function both in the clinic and the laboratory. ERG measures electrical responses of retina evoked by light stimulation. The recorded signals obtained under various light conditions, stimulation intensities and timing protocols allow to individually assess the function of different retinal cells, such as photoreceptors, bipolar cells, as well as other types of neurons present in the retina. It is widely used in human medicine for the diagnosis of retinal disorders and in the laboratory for description of disease models or characterization of mutant mouse or rat strains. The method is non-invasive and it is used in laboratory rodents in a way similar to human patients. However, unlike in human, it requires the use of general anesthesia in animals.

Electroretinography is carried out in the Czech Centre for Phenogenomics as a secondary screening method, which means that ERG is used in selected cases to further elaborate on eye phenotypes and complement eye morphology findings with functional data. The experimental protocols can be adapted to answer specific questions raised by individual investigators. In addition, visual evoked potentials from the visual cortex can be recorded alongside the classical ERG.

The Mouse Eye
In the anterior part of the eye, the optical system allows the light to enter and focus at the rear inner surface of the eye ball. Here the photons are detected by a highly specialized light-sensitive nervous tissue, the retina. The retina is organized in functionally and histologically distinct layers. It contains five types of neurons: photoreceptors (rods and cones), horizontal cells, bipolar cells, amacrine cells and ganglion cells. Müller cells play the role of glia in the retina. The outer surface of the retina is enclosed by cells of the pigment epithelium. This fundamental organization of the eye is shared among all vertebrates, including humans. Nevertheless, the mouse eye differs from the human eye in several aspects.

The mouse lens has a ball shape and it occupies a substantial volume of the eye, nearly 75%. People are trichromats - their retina contains cones sensitive to three different light colors, or light wave lengths: long-wave (L) cones most sensitive to red color, medium-wave (M) cones sensitive to green color and short-wave (S) cones sensitive to blue color. In contrast, mouse and rats are dichromats, where only two types of cones have been discovered, M-cones with the highest sensitivity to the light of 510 nm, green, and ultra-violet sensitive S-cones with the sensitivity peak near 350 nm. The highest concentration of cones in the human eye is in the focus of the optical apparatus, in the spot called fovea centralis, which does not possess any rods. Such a specialized place is missing from the mouse eye. Alos, there are markedly more rods than cones in the mouse retina, the proportion of number of rods to cones is approximately 35:1, whereas in human it is around 15:1. In contrast to human patients, the electroretinography in animals is usually performed under the general anesthesia, which is necessary to calm the movements of the animal and allow placement of the electrodes. It can be considered as a non-invasive experimental procedure, since the electrodes are only placed on the eye surface and under the skin, but the vascular and nervous systems, including the retina, are certainly affected by the anesthetics and the ERG signal has been shown to differ in particular features between anesthetized and awake animals. Last but not least, it is important to point out the physical size of the mouse eye. The eye ball diameter in mice is only 4 mm, therefore, all experimental techniques, including the electrophysiological, must be adapted to the small size of the object of interest.

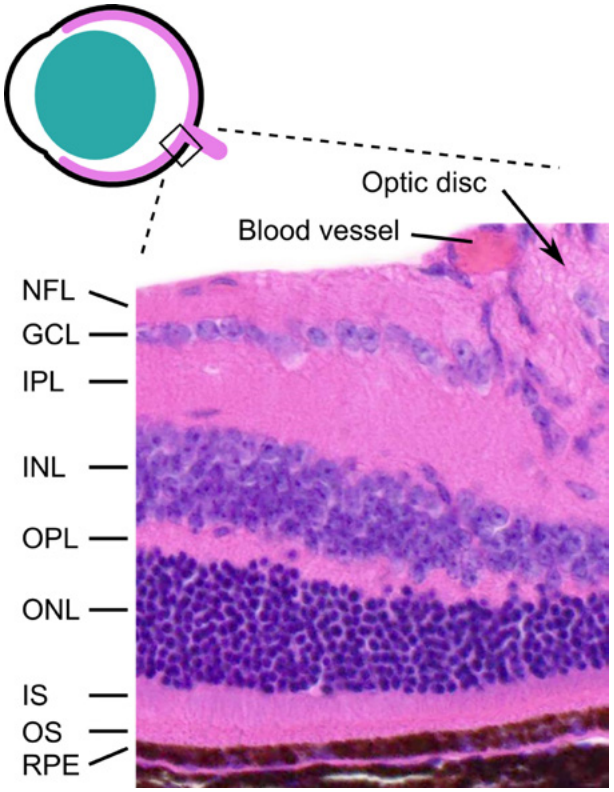

Figure 1: Cross-section of the mouse retina. Typical histological view of the retina near the optic disc. In hematoxylin-eosin staining all the fundamental layers of the retina can be nicely distinguished. The arrow marks the optic disc where all nerve fibers leave the retina to continue toward the brain as the optic nerve. The optic disc is also the spot through which blood vessels enter the retina. NFL nerve fiber layer, GCL ganglion cell layer, IPL inner plexiform layer , INL inner nuclear layer, OPL outer plexiform layer, ONL outer nuclear layer, IS inner segments of photoreceptors, OS outer segments of photoreceptors, RPE retinal pigment epithelium.

Cell Types in the Mouse Retina

Several layers may be distinguished in a histological cross-section of the retina (Fig. 1), containing functionally and morphologically distinct cell types. The outer layer is made of the retinal pigment epithelium (RPE), which participates in the renewal of outer segments of photoreceptors and chromophore molecules coming from the isomerisation of the rod outer segment from all-trans to 11-cis. RPE also plays a role in spatial buffering of K+ ions. Fundamental cells in the retina found just below the RPE are photoreceptors, the cones and the rods. There are 6.4 million rods and 180 thousand cones per mouse retina. Their photo-sensitive outer segments comprise the outer segment layer (OS), their inner segments (IS) contain large number of mitochondria and the cell bodies are found in the outer nuclear layer (ONL). The outer segments of photoreceptors consist of stacked membrane discs (in rods) or membrane folds (cones) with visual pigments. The response of the photoreceptors to light stimulation is slightly counter-intuitive. Rods and cones are active, their membrane potential is depolarized and their synapses release neurotransmitter glutamate in the dark, whereas presence of light brings their membrane potential to normal resting value and decreases the synaptic output. In the dark, the photoreceptors are depolarized because high concentration of cGMP in the cytoplasm activates cGMP-gated cation channels. Detection of a photon by the pigment protein rhodopsin, namely its light-absorbing moiety, 11-cis retinal, sets in motion a biochemical cascade that ultimately lowers the concentration of cGMP, thus closing the cation channels. Photoreceptors make contact with bipolar cells in the outer plexiform layer (OPL). Bipolar cells are so named because they typically have two neurites: the dendritic process, which branches out towards the rods or cones, and the axonal process whose terminal arborizes to make contacts with amacrine and ganglion cells in the inner plexiform layer (IPL). Bipolar cells may be divided into two main categories, the OFF-response type and the ON-response type. ON and OFF cells respond to glutamate at the synapse through distinct mechanisms. The OFF bipolar cells use ionotropic cation channels of the AMPA-kainate variety, the glutamate released from photoreceptors in darkness depolarizes these cells. The ON bipolar cells use metabotropic receptors and glutamate hyperpolarizes the cells in the dark. To date, fifteen different subtypes of bipolar cells have been identified in mice based on their morphology, localization, connections in the retina and transcriptomic markers. The OPL contains also horizontal cells which are involved in the lateral inhibition among photoreceptors. Horizontal cells are activated by glutamate released from photoreceptors and feed back to the photoreceptor terminals through an inhibitory synapse. Horizontal cells are also coupled with each other via gap junctions. The fourth neuron type in the retina is represented by amacrine cells. The majority of amacrine cells have their cell bodies in the inner nuclear layer (INL) and they are involved in the retinal circuitry making connections among horizontal, bipolar and ganglion cells. Most of them release the inhibitory neurotransmitters, glycine or GABA. According to the degree of the dendritic spread, the amacrine cells are categorized into narrow-field, medium-field, and wide-field types, thus far nearly forty types of amacrine cells have been identified in the mouse retina. The most inner cellular layer of the retina is the ganglion cell layer (GCL). Ganglion cells and certain types of amacrine cells are the only neurons in the retina which produce action potentials. They extend their axons in bundles that run through the nerve fiber layer (NFL) to the optic disc, where the axon bundle exits the retina as the optic nerve and projects toward the central nervous system. Within the retina the axons are unmyelinated to prevent light from scattering within the retina, but the axons in the optic nerve are myelinated to accelerate propagation of the action potentials. The total number of ganglion cells per retina in mice is around 45,000. They could be classified into almost forty different types including the intrinsically photosensitive cells which express the photopigment melanopsin. Representatives of glia in the retina are the Müller cells. Müller glial cells span the entire thickness of retina and extend many flattened branches to surround retinal neurons. They are responsible for the homeostatic and metabotropic support of neurons, isomerization of retinal to the 11-cis form, but they also guide light through the retinal tissue from the vitreous body up to the photoreceptors.

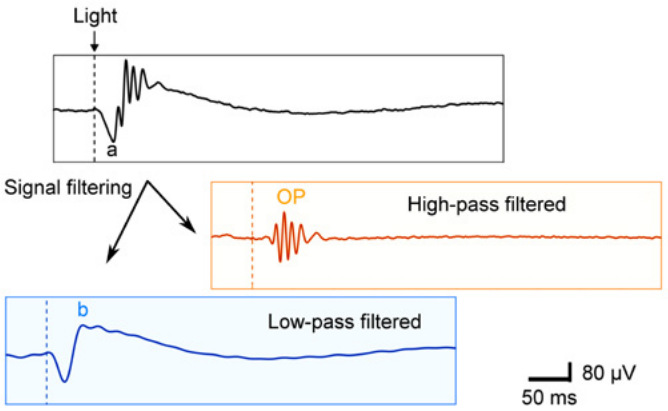

Figure 2: Example of the ERG response. Individual components of the response of the retina to a short light stimulus can be analysed separately after frequency filtering. Activation of photoreceptors (rods and cones) is manifested in the ERG as down-going sharp peak called wave a. Slower and up-going wave b, reflecting the activation of ON-bipolar cells, is better seen if the signal undergoes low-pass filtering, which cleans the response off all faster waves. In contrast, the oscillatory potential (OP) is made visible by high-pass filtering algorithm.

Electrogenesis of the Electroretinogram

The electroretinogram is an electrical potential generated by the retina in response to a change in illumination. It is the summed activity of all retinal cells, and consists of overlapping positive and negative component potentials (Fig. 2) that originate from different stages of retinal processing. Individual components of ERG response may be separated off-line with signal processing algorithms, and analyzed individually in order to estimate the function of various retinal cell types, such as photoreceptors, bipolar cells, the pigment epithelium and others. Most commonly a single flash of white light is used as stimulation, but coloured light can be used too, and the stimulation protocol can consist of quickly repeating flashes, as well as of long pulses of light, according to the specific question the ERG is to answer.

The first major negative peak visible in the ERG response to a flash stimulation is the wave a. It reflects suppression of the current flowing through the outer membrane of the photoreceptors, which is an ultimate result of light detection in the photoreceptor cell. Thus, parameters of the a-wave, such as its amplitude and latency (in the ERG community called 'implicit time'), provide information about the activity of photoreceptors. The second large wave in the electroretinogram is the positive-going wave b. It has been shown that b-wave corresponds to activation of bipolar cells of the ON type. In a mouse ERG a few

small waves, often four, can be seen on the rising edge of the b-wave. These oscillatory potentials (OP) in spite of the fact that they probably do not reflect any repetitive oscillating activation in the retinal circuitry. Rather, the individual OP peaks arise from distinct neuronal subpopulations within the retina, such as amacrine and ganglion cells. To date, the scientific community has not been able to agree on the significance of the individual OP components, however. Interestingly, the OP is much more pronounced in a mouse ERG than it is in the human. Some works show that OP appears in the ERG as a consequence of use of anesthetics, because OP is nearly absent from the awake mouse recordings. Later, around 1.5 second, another positive and slow wave may appear in the ERG recording, the wave c. Unlike other waves in the signal, wave c is not directly linked to the activity of specific population of neurons. It is an electric potential composed of negative-going deflection caused by the neural retina, and positive going peak of the same latency caused by retinal pigment epithelium (RPE). Both of the components are linked to the movement of large amounts of K+ ions released during neuronal activity to the extracellular space and buffered by RPE (positive component) and Müller glial cells (negative component). The c-wave is positive when the RPE component is larger than the Müller cells component.

In order to distinguish rod and cone function, specific experimental conditions can be engaged. Although the underlying principle of light detection is common for all photoreceptors, the cone and rod pathways differ in their sensitivity to light and in the kinetics of response. The retinal circuits linked to rods are very sensitive and can detect a single photon, but they are not capable of following higher rates of flickering stimulation. Cones are much less sensitive to weak stimuli but if the light intensity is sufficient, they can accurately represent a quickly flickering light source. In a dark-adapted eye, upon stimulation with low intensity of light, the electroretinogram is exclusively rod-driven. With use of higher light intensities the response is a blend of rod and cone pathway activity. Finally in light-adapted eyes the ERG responses represent the cone pathway activity alone, since the rods are desensitized by intensive illumination. Specific tests of rod or cone pathways in the retina can be designed if accurately selected light conditions and stimuli are used in the experiment.

ERG Experiment

In general, at the Czech Centre for Phenogenomics the ERG experimental procedures follow the international standards as they were suggested by the International Society for Clinical Electrophysiology of Vision (ISCEV), originally developed in 1989 and updated in 2015 for human patients, with respect to conditions specific to a laboratory mouse.

Whole field electroretinography (Fig. 3) is performed in general anesthesia induced by an i.p. injection of a mixture of tiletamine and zolazepam. Animals are kept on a heating pad at 37 °C with eyes protected against drying with transparent eye gel throughout the experiment. A golden ring (3 mm in diameter) gently placed around the centre of the eye bulb serves as the active electrode, a needle electrode inserted in the animal's snout and another one in the animal's back serve as the reference and grounding electrodes, respectively. Measurements are usually performed in the right eye and application of atropine is necessary to induce opening of the pupil. If needed, both eyes can be recorded simultaneously, or the visual evoked potentials may be recorded along with ERG using a needle electrode placed subcutaneously above the occipital part of the skull, approximately at the position of the visual cortex. Animals are adapted to darkness for 12 hours (over night) prior to the experiment, during this time they are kept in red individually ventilated cages in a dark room. Animals are injected and all the preparatory procedures are performed under dim red light. When the scotopic (dark-adapted) part of the stimulation protocol terminates, the mice are exposed to white background light and the experiment continues with the photopic (light-adapted) protocol. The maximal length of the experiment is determined by the length of anesthesia, usually the recording is completed in 45 minutes.

Our ERG Setup at the Czech Centre for Phenogenomics

Hardware: RETI-port for animal, Roland Consult, Germany. Whole-field stimuli are delivered by a ganzfeld globe equipped with LED diodes and Xenon lamp. Apart from white light, the ganzfeld offers several spectral colors: royal blue (455 nm), blue (470 nm), green (525 nm), orange (590 nm) and red (625 nm). Single-flash, flicker and long pulse stimuli are available as well as background illumination.

Software: stimulation and recording is done by the RETI-port/scan 21 Roland Consult software. Responses are visualized and analyzed off-line using custom-made scripts in Matlab, MathWorks.

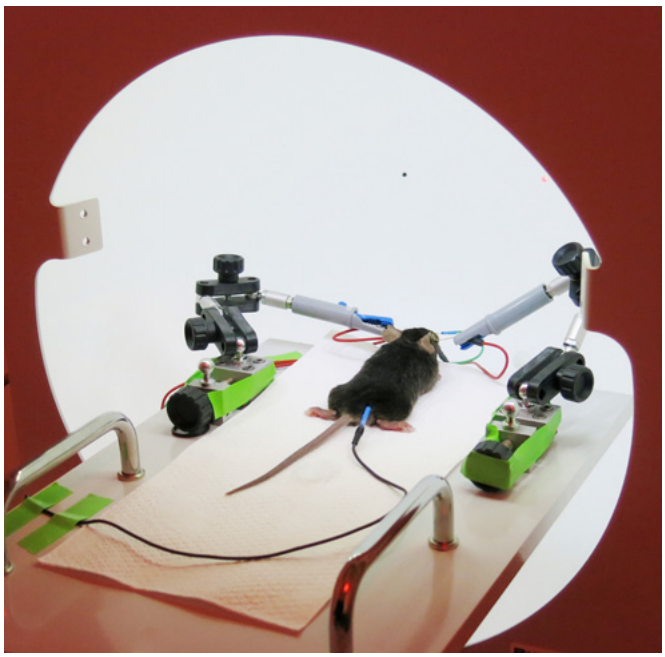

Figure 3: An anesthetized mouse inside the ganzfeld. The ganzfeld (whole-field) globe serves to disperse the light source to all directions so that the whole retina is stimulated homogenously and with the same intensity of light.

CONTINGENCY IN METABOLOMICS AND BIOINFORMATICS ANALYSIS THEREOF

Ashkan Zareie

There are various floors of uncertainty in every metabolomics experiment. The name of this article is precisely chosen to reflect the said statement. Contingency refers to a likely condition or circumstance that cannot be predicted with certainty. But more intriguing is the definition of contingency in philosophical terms which refers to propositions that are “neither true under every possible valuation and nor false under every valuation”. This exquisitely echoes the science of metabolomics, precisely the untargeted approach to the said science. Such analyses are intricate in that each and every level is rife with crucial decisions that must be made in order to optimize numerous factors and parameters so that, consequently, the best possible result can be obtained. These decisions are not only at the level of experiment and design but plague the entire analysis; post-processing, statistics, metabolite identification and profiling. However, these decisions can never lead to an absolute best-case, there is only a ‘better’ solution every time, never, or rarely, a ‘best’. The perils of such an experiment are immediately known according to and outlined in the paper Ioannidis (2005) which delineates that a research finding is more likely to be false than true when there is “great flexibility in designs and analytical modes”.

Nonetheless, what specifically causes the contingency? The decisions leading to optimization that we speak of are highly experiment- and sample-dependent and there are always compromises to be made. A setting proving highly successful for one study might turn out to be disastrous for another. To illustrate, there is usually a high level of noise in the low-intensity signals of LC–ESI-Q-TOF-MS data acquired by the high-resolution Agilent machine at our center. Said signals highly complicate the data processing step and confuse any peak detection algorithm, for it is never intuitive, even to an experienced metabolomics scientist, what constitutes an analyte and what is noise in such regions. On the other hand, filtering such low intensity signals, and in other words, cleaning up the data (which is a crucial step) leads to the removal of the signal of those compounds that are not intense by nature. In this regard, an interesting compound can be easily missed.

Setting up strict, universal standardization measurements is a task almost impossible and faced with many particularities. However, certain factors can hugely benefit the community and make it possible for metabolomics to become an exact and certain approach to the study of phenotype. The true potentials of untargeted metabolomics are yet to be exploited and discovered. But it has been shown that the said approach is a potent technique even for challenging domains, such as drug discovery and development.

An important step towards the standardization of untargeted metabolomics protocols is using an open-source file format that anyone can access and utilize. This is why at CCP, we have largely opted for converting all raw data from the proprietary format to the mzML community standard format and maintaining a copy of both the raw and mzML data. This ensures and eases cross-platform access to the data without having to rely on a specific paid software. Furthermore, using a large variety of tools for the analysis of the said data is enhanced.

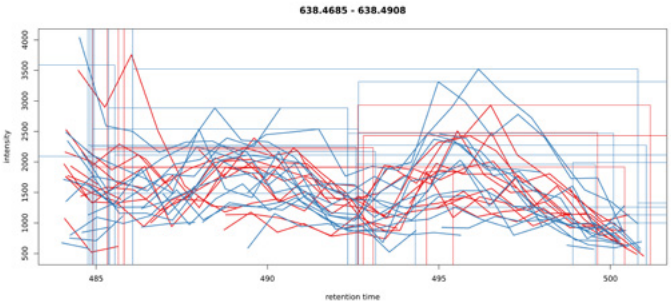

Figure 1: In this so-called region-of-interest, we observe several peaks hidden in extreme noise (which have been detected by the algorithm). It is hard to assess whether some interesting compounds are here or everything is noise.

Nonetheless, the most fundamental issue arises with the continual emergence of new software for and approaches to processing metabolomics data. Bioconductor and Github are flooded with such software, and unfortunately many of them are either abandoned projects and not maintained or not used widely by the community. This is more problematic for software that have implemented novel approaches but go unrecognized because of the aforementioned reasons. Therefore, there is an indispensable need for work towards a unified approach (i.e. software) that has the potential to become the community standard, instead of adding to the abundance of already-available software. One solution is to build on the widely-used XCMS package in R and use the data structures it offers. As an example, new peak picking approaches can be implemented directly in the said software using the already-existing and highly-cared-for backbone of XCMS. Software, such as CAMERA and MetaboAnalystR which integrate XCMS data structures, make it easy for the user to continue their analysis towards statistical analysis and compound annotation.

As each software takes a different approach to data processing, most of the time this leads to discrepancies between analyses made on a single dataset using different software. In such a case, experiments from centers that use different machines and vendor software are hardly

comparable—although use of different machines alone can account for a significant portion of challenges of comparability. Moreover, there is a palpable lack of clearcut documentation for most metabolomics software, consequently leaving numerous crucial parameters misused and mistuned most of the time. The nomenclatures of even many novel tools are non-standardized and the same term, e.g. alignment, can mean different things across different software. Hence, many powerful features in different tools are left misunderstood and unleveraged. Furthermore, there are no approaches to date, such as majority voting seen in transcriptomics tools, that would elegantly leverage the prowess of several tools at the same time for a maximum level of data exploitation and lower level of false positive detection. Such an approach

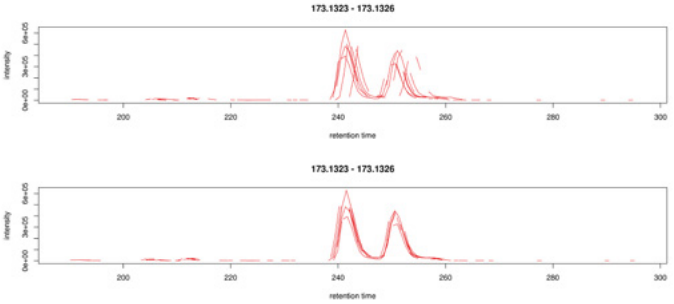

Figure 2: Identity of analytes during the pre-processing step is unknown and elution order across runs differ. This leads to what is known as retention time (rt) shift. There are several methods available for retention time correction and it is one of the most important steps in the analysis. In this figure we can see that the lower panel shows a successful rt shift correction. However, this is not always a straightforward task and peaks from different analytes across samples can be mistakenly grouped and aligned and considered as one specific analyte. Upper and lower panels of this figure show a specific LC-MS before and after retention time correction, respectively.

is worth consideration especially for noisy regions with an abundance of low-intensity signals.

In addition to challenges that trouble bioinformatics tools, there is also a lack of consensus in experiment design in untargeted metabolomics. Meticulous randomization and use of QC samples and internal controls are often overlooked and not given the required attention. Such studies are a waste of resources as they are easily more susceptible to batch effects and bias in comparison to studies that have been carefully designed and executed. More importantly, correcting these issues (batch effects, contamination, signal drift, bias) becomes a more daunting task in the absence of a rigorous experiment design. As an example, in the absence of QC samples, randomization, and detailed experiment metadata, many powerful and advanced bioinformatics/biostatistics tools and methods become inapplicable, owing to the fact that most of the vital information that such approaches need—e.g. for batch correction, normalization, mixed model analysis, et cetera—are missing.

Standardization of metabolomics protocols will not only help scientists exploit their data and draw clear and statistically-supported conclusions, but it will also make it possible for large-scale studies that span many months to be conducted with more rigor and certainty.

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SCREENING FOR METABOLIC PHENOTYPES IN THE INTENATIONAL MOUSE PHENOTYPING CONSORTIUM

J. Rozman

Worldwide, twenty mouse phenotyping facilities from Europe, North America, Africa and Asia joined forces to build the International Mouse Phenotyping Consortium (IMPC). The Czech Center for Phenogenomics (CCP) is one of the European partners in IMPC. The IMPC aims to generate and phenotype a knockout mouse model for every protein-coding gene. In the long run, the successful completion of the IMPC program will establish the first comprehensive catalogue of mammalian gene functions. In addition to the compilation of phenotyping data, the IMPC knockout mouse models will be made available to the wider scientific community. This resource will have particular importance for the study of genotype-phenotype associations of genes linked to human disease.

The IMPC has currently generated and phenotyped knockout mouse models covering about 6,000 genes. Importantly, all phenotyping data is directly accessible free of charge via the IMPC web portal www.mousephenotype.org, and it is not surprising that this resource has already gained considerable interest from researchers across many disciplines. However, the IMPC is not a sole scientific resource, but also functions as a platform bringing IMPC experts in mouse phenogenomics , geneticists, biostatisticians, data analysts and clinicians together. Based on this collaborative work the IMPC has started to address fundamental research questions in hearing impairment, vision and eye function, metabolic disorders, and cardiovascular diseases.

The impact of collaborative IMPC work is illustrated in a research article published recently in Nature Communications (Rozman et al. 2018). In this work, metabolic function was investigated based on IMPC data from more than 2,000 genes. Specifically, glycemic level, glucose tolerance, blood triglyceride levels, body mass, metabolic rate, and metabolic substrate utilization were utilized to characterize metabolic functions. All genes that caused differences in these parameters between knock-out and control mice in the highest and lowest 5 % compared to the complete data set were considered a hit. Application of these straightforward hit criteria identified 974, that is about half of the assessed genes, as hits, thus causing a strong metabolic phenotype in the knockout mouse model. The pool of 974 genes causing strong metabolic phenotypes was the basis for all subsequent analysis presented in the paper. First, the authors evaluated whether the link of a gene to metabolic functions in mice was new and so far unpublished. It is important to note, that there is the possibility of bias towards studying genes that are well-characterized instead of genes that are less extensively studied and frequently referred to as the 'Ignorome' or the 'Dark Genome'. IMPC aims to characterize especially those genes that belong to latter part of the genome. Standardized literature and database searches revealed that 429 genes had no prior link to metabolic functions before. Strikingly, 51 of those genes had no functional annotation at all before the IMPC analysis hence these ignorome genes were for the first time successfully mapped to a biological function. This finding underpins the pioneering approach IMPC is applying to generate a comprehensive catalogue of mammalian gene functions.

The critical relevance of the IMPC data notwithstanding, its analysis bears challenges. The risk of false negative or false positive discovery is high in all large-scale biology approaches including the IMPC. The lack of standardized protocols, misleading statistical approaches, poor transparency in procedures can all potentially cause high false discovery rates. However, they generally shielded against these drawbacks because of (1) implementation of harmonized phenotyping pipelines, (2) sharing of experimental protocols, (3) rigid quality management, and (4) adherence to the ARRIVE guidelines (Kilkenny et al. 2010). To evaluate false negative discovery risks in the metabolism data set, Rozman et al. compiled a list of 666 candidate genes for diabetes and obesity from published literature. One hundred and one of the 666 genes were included in the IMPC metabolism data set. A striking majority of 100 of the 101 candidate genes caused strong or at least biologically relevant disturbances in metabolic functions suggesting that the false discovery risk is low in the IMPC metabolism data set. It is important to note, that some of the 666 candidate genes were included into the initial candidate gene list because of findings from genome wide association studies (GWAS). Here, IMPC is providing an independent line of evidence that the respective genes are involved in metabolism and – equally important – new mouse models are now available to study the involvement in more detail.

The human relevance of a mouse-derived data set such as the IMPC metabolic phenotyping described above is of considerable importance. Rozman et al. found in a meta-analysis of five genome wide association studies, that the orthologues of 23 genes of the 429 mouse genes with no prior link to metabolism showed associations to human diabetes disease terms. Patients carrying variants of these genes showed impaired insulin sensitivity, glucose intolerance or impairments in lipid metabolism. This is another key finding of the publication offering exciting perspectives not only for future research but also for patient care. The in-depth investigation of the molecular and physiological functions of metabolic candidate genes may help to identify biomarkers for early diagnosis or find new therapeutic treatments. Ultimately, this research avenue envisions the generation and characterization of humanized mouse model carrying exactly the human disease variant of the gene.

Beyond the identification of mouse and human genes controlling metabolism, Rozman and colleagues began to unravel molecular underpinnings of the observed metabolic phenotypes. Network analyses were applied to begin to understand the connections between genes on the regulatory level. Firstly, the authors mapped metabolic candidate genes to known metabolic pathways, which suggested that the phenotypic effects of whole-body knockouts of single genes

allowed the construction of network or pathway links of genes. In a second step, genetic elements comprised of specific patterns of transcription factor binding sites (so-called MORE cassettes) identified in metabolism genes allowed the construction of regulatory networks. Notably, metabolic pathway connections and the regulatory network links showed a high degree of overlap. Finally, the similarity in these genetic elements (i.e. sharing of the same MORE cassettes) helped to predict a link of the respective genes to metabolism. It seems possible, that this approach offers a new way to predict gene functions of so far unannotated genes.

In conclusion, the IMPC studies on metabolism utilized multiple-lines-of-evidence to identify new links between candidate genes and metabolic functions and finally to human disease. Since the initial analysis of the data set, the number of genes included in IMPC has significantly increased. Therefore, there are high expectations to identify an even larger set of novel genes controlling energy metabolism and glucose homeostasis in the near future. The IMPC program will remain a pivotal cornerstone in disease related phenogenomics not only for metabolic disease but also for other conditions affecting the general population such as neurodegenerative disorders or developmental impairments.

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Acknowledgment

The contribution of all researchers, lab technicians, and animal caretakers to IMPC is highly acknowledged. Special thanks to all co-authors of the publication.

High resolution micro-CT imaging for analysis of soft tissues

J. Prochazka and F. Spoutil

The use of high resolution X-ray microcomputed tomography (micro-CT) provides a non-invasive imaging tool for studying structures and tissues of various organisms. Even though this method has proven to be useful for visualizing mineralized tissue with a substantial level of detail (Figure 1). The challenge of microCT imaging is the limitation in soft tissues which have very low X-ray absorption and can be only poorly visualized. However, this limitation can be overcome by the use of special contrast compounds based on Iodine or some other heavy metal salts. The search for new contrasting agents and development application protocols is one of many missions of the Bioimaging unit at CCP in order to provide state of the art microCT imaging also for soft tissues, with specific demands on phenotyping of embryos and characterization of tumors in real 3D datasets.

The embryo phenotyping pipeline fully relies on high resolution microCT from the earliest developmental stages, where we achieved spatial voxel resolution as low as 0,7um, which allows an uncompromised view on the very early events of development.

The sufficient contrast distribution through the soft tissue allows the pseudocolor visualization of individual organs, vasculature system or somite formation in embryos. Such an approach is generating 3D data with very high emphasis on developmental system of interest and can increase the presentation potential of acquired data than the traditional grey scale microCT images.

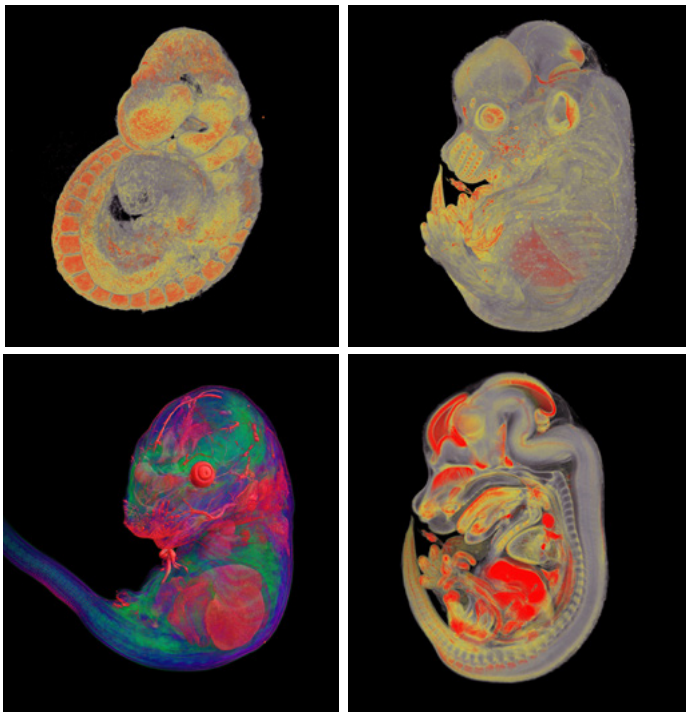

Figure 1: High resolution micro-CT images of mouse embryos with pseudo coloration to highlight tissues and structures

The established protocols and procedures for embryo phenotyping can be very easily transferred to imaging of soft tissues from adult animals. The only limitation is the volume of tissue sample. Due to detector position in the imaging chamber, the volume of sample should not be over one cubic centimeter. But even with such limitations this approach really opens new dimensions in analysis of tissues in 3D. The microCT can't beat the advanced microscopy techniques such as 2P confocal imaging or light sheet imaging, but provides very cost effective sample preparation and high throughput imaging.

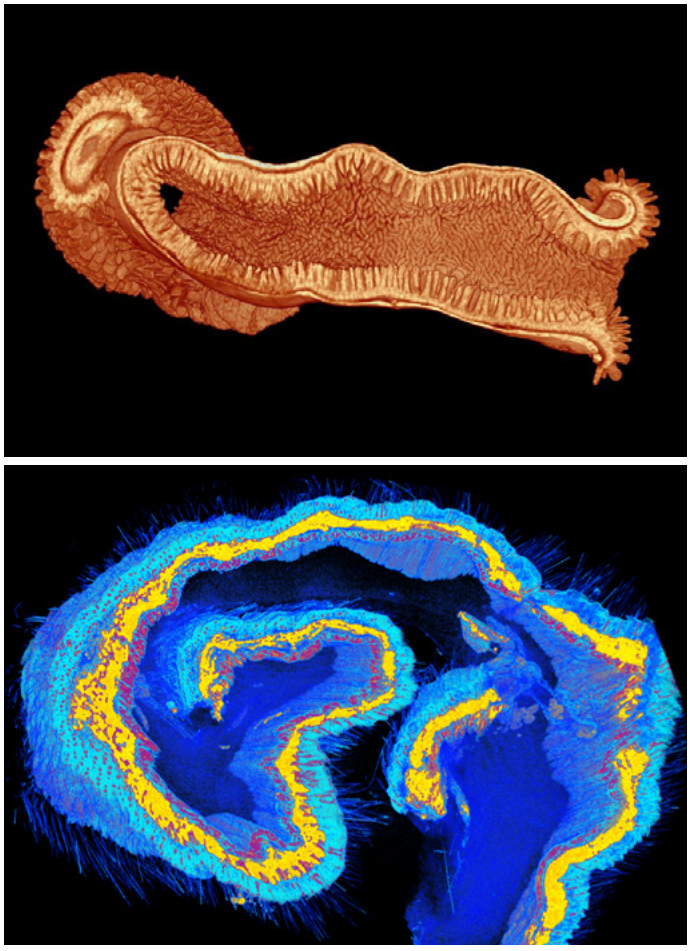

Figure 2: High resolution micro-CT images of mouse intestines with pseudo coloration to highlight structures

The advantages of contrasted tissues can be fully appreciated in analysis of tumour models in mice. The histological evaluation is critical for understanding of tumor pathobiology, however due to high labor involvement, analysis of tumor heterogeneity in 3D by classical histology is extremely cost and time challenging. The combination of microCT and histological sections can help to understand the tumor's internal structure in 3D. This is critical especially in tumors with very high internal heterogenity during their development. The ability to see the vascular network in

the tumor is also another important value, which is necessary for the understanding of tumor vascularization status when changes occur during experimental treatment.

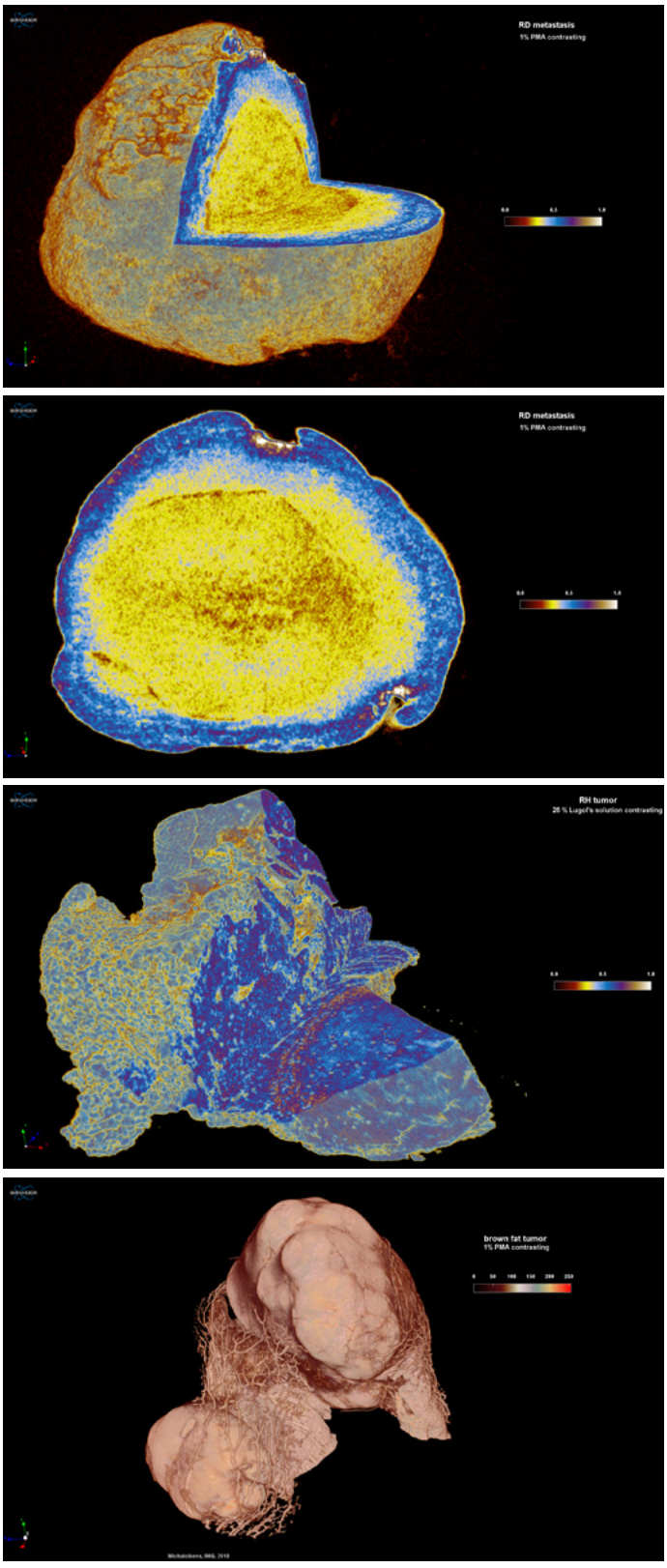

Figure 2: High resolution micro-CT images of mouse intestines with pseudo coloration to highlight structures

Currently, we have improved our imaging protocol based on Lugol's solution to avoid unwated shrinkage of tissues and specifically embryos. This provides us the possibility to develop and implement automatic and semiautomatic bioinformatic approaches for high throughput data analysis. We are also implementing new contrast protocols and testing the contrast efficiency in multiple testing samples (embryos, tumors, organs). We have already established contrasting and imaging protocols based on phospho-molybdenic acid, phospho-tungstenic acid and more new contrasts are under development. The obvious aim is to prepare new contrast agents and eventually novel combinations of multiple contrast agents to visualize structures of interests as specificaly as possible.

Future perspectives

Within the next year, we plan to establish at least 2 more novel imaging protocols with exotic contrasting agents and start to use contrast protocols in combination with bioinformatics, to achieve multi contrasted specimens with true color coding corresponding to intensities for individual contrasts analogues to fluorescence imaging in multiple channels. Use of conjugated antibodies with metal elements is another challenge we plan to pioneer in the near future to achieve comparable specificity with advanced 3D microscopy methods.

PATIENT DERIVED XENOGRAPHTS - CREATING A MOUSE AVATAR

P. Kralova Viziova

The Patient derived xenograft (PDX) department is one of the youngest department in CCP. Patient derived xenografts are models of cancer where the tissue or cells from a patient's tumor are implanted into a highly immunodeficient or humanized mouse. With focus on personalization in human cancer medicine, PDX is a very promising approach which allow us to prepare each cancer patient their own mouse avatars and test different anticancer therapies on these avatars before they are used on the original patient. The goal is to improve the drug development process in oncology and ultimately contribute to the approval of new and effective therapeutic strategies for cancer patients. Our whole network displays a wide range of expertise in technological platforms (e.g. highly immunodeficient NGS mice, orthotopic surgery, cell culture, molecular imaging, in vivo imaging, histology, microCT, Maldi, biostatistics, biobanking, metabolic cages) and the demand is high for efficient logistics with precise timings.

Often the main feature of PDXs is that the avatar has retained the genotype and phenotype of patient tumors, and shows the principal histologic characterization of their donor tumor. These models have shown to be predictive of clinical outcomes and are used for preclinical drug evaluation of safety and efficacy, biomarker identification, biological studies, and exhibit substantial infiltrative and metastatic growth to distant organs similar to which is observed in human patients. It is also a valuable platform to predict patient tumor response to certain anti-cancer agents.

In comparison, cell lines are usually established only from the more aggressive tumors and hence are not representative of complex tumor heterogeneity evident in the clinic. An actual tumor is a mixture of heterogeneous cells including cancerous cells, fibroblasts, vessel cells, and immune cells. Even cancer cells from a single patient exhibit diverse characteristics in the genomic and transcriptomic signature, contributing to the phenomenon of the intra-tumoral heterogeneity of cancer. Therefore, in many cases, drug responses cannot be fully anticipated via simple genetic profiling. For all these reasons, the establishment of cell lines is not an appropriate strategy for personalized medicine applications. Novel approaches are being developed such as short-term primary cultures or organoids, although important validation studies are still required before broad application in conventional preclinical screening projects.

How does it work? The bioptic tumor tissue from the patient can be transferred to a variety of locations inside a mouse avatar; subcutaneously, intrafemorally (for leukemic cells) or using a direct orthotopic xenograft model. Orthotopic transplantation might be done e.g. ; intraductally, into the mammary fat pad Figure 1), under the splenic or kidney capsule, into the intestine wall or into the caecum wall, intrapulmonally, intrahepatically. After the growth of the tumor, it is retransplanted again in more mice. The original patient tumor might be compared with mouse tumor after the first in vivo passage and also after other passages via microCT, histology and by imunohistochemistry. Part of the tissue is frozen and placed in the biobank. Other components tested and measured could be organoids obtained from the tissue.

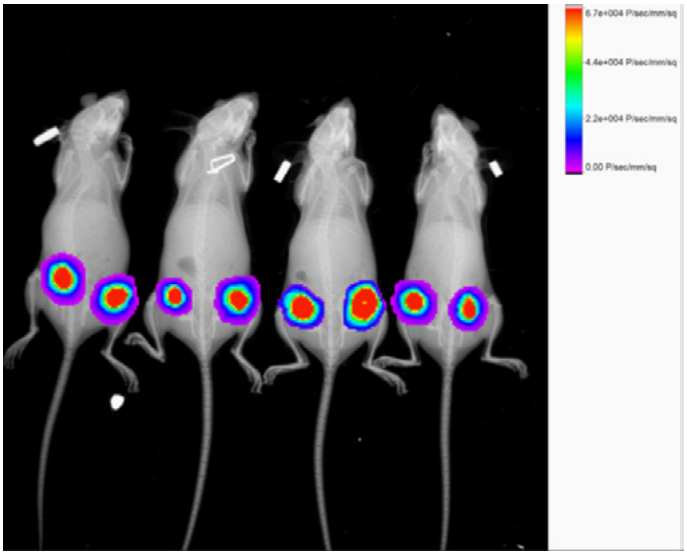

Figure 1: Development of a lumincently labelled tumor inplanted in the mammary fatpad. Image taken 14 days after injection.

The second mouse passage (2nd transplantation of tumor from donor mouse) is prepared to obtain a higher number of animals which will be used for the clinical studies and moreover, acquire more mice for orthotropic placement of the tumor which mimic the tumor environment and the patient's condition with improved accuracy. Passage samples are also kept in storage if further use is required.

During subcutaneous passages of tumors, a higher amount of copy number alterations (CNAs)2 have been observed after several mouse passages when compared with the original human tumor. This is often due to the selection of preexisting minor clones, therefore orthotopic placement and a reduced amount of mouse passages is more suitable way to keep the original and avatar tumors as genetically similar to each other as much as possible.

The obtained tumor parts frozen in biobank might be shared as well internationally via project Edirex: EurOPDX Data Portal: https://dataportal.europdx.eu Only the delivery cost is required.

Frozen tumor samples can be thawed so that they can be placed back into NSG mice. This allows repeating the study with new parameters or a new design of the study if necessary.

Our aim is to support the scientists in projects of PDX models but also in research demanding higher specialization in surgeries, such as implanting of measuring devices into mouse, orthotopic transplantation, catheterization, castration, orthotopic

placement of cancer cells lines and drugs.

The dynamics of cancer growth can be captured by modern imaging techniques from hour to hour, thanks to in vivo imaging we are able to display a wide range of distribution of drugs in the body and their effect on metastasis. As we do an observation and health check of our animals on daily basis, we are able to monitor clinically visible side effects of treatment.

We follow the highest ethical standards, combining knowledge from laboratory animals and veterinary medicine, including development of novel minimally invasive surgical techniques.

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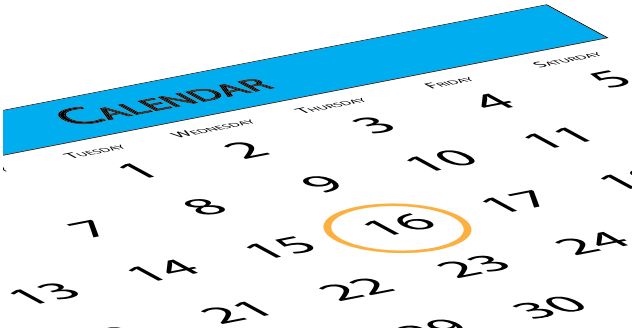
Upcoming Events

SECOND CCP PHENOGENOMICS CONFERENCE 2020

16/09-18/09/2020: Vestec, Czech Republic

More info: https://ccp-conference.cz



Past Events

CCP PHENOGENOMICS CONFERENCE 2019

12/09-13/09/2019: Vestec, Czech Republic

More info: https://www.ccp-conference.cz/2019/

8th International Symposium on Kallikreins and Kallikrein-Related Peptidases (ISK2019)

25/09-27/09/2019: Prague, Czech Republic

More info: https://www.isk2019.cz/

Journal Club

1. Tild-CRISPR Allows for Efficient and Precise Gene Knockin in Mouse and Human Cells
   Dev Cell. 2018 May 21;45(4):526-536.e5
2. CRISPResso2 provides accurate and rapid genome editing sequence analysis
   Nat Biotechnol. 2019 Mar;37(3):224-226
3. Base editing: precision chemistry on the genome and transcriptome of living cells
   Nat Rev Genet. 2018 Dec;19(12):770-788
4. A rocky road for the maturation of embryo-editing methods
   Nat Methods. 2019 Feb;16(2):147-150
5. Hypoimmunogenic derivatives of induced pluripotent stem cells evade immune rejection in fully immunocompetent allogeneic recipients
   Nat Biotechnol. 2019 Mar;37(3):252-258
6. Induced pluripotent stem cells in disease modelling and drug discovery

   Nat Rev Genet. 2019 Feb 8
7. Super-Mendelian inheritance mediated by CRISPR–Cas9 in the female mouse germline
   Nature. 2019 Feb;566(7742):105-109
8. Development of a gene-editing approach to restore vision loss in Leber congenital amaurosis type 10
   Nat Med. 2019 Feb;25(2):229-233

Czech Centre for Phenogenomics

Delivering and Characterizing Research Models

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The Czech Centre for Phenogenomics is hosted by the Institute of Molecular Genetics AVCR v.v.i.