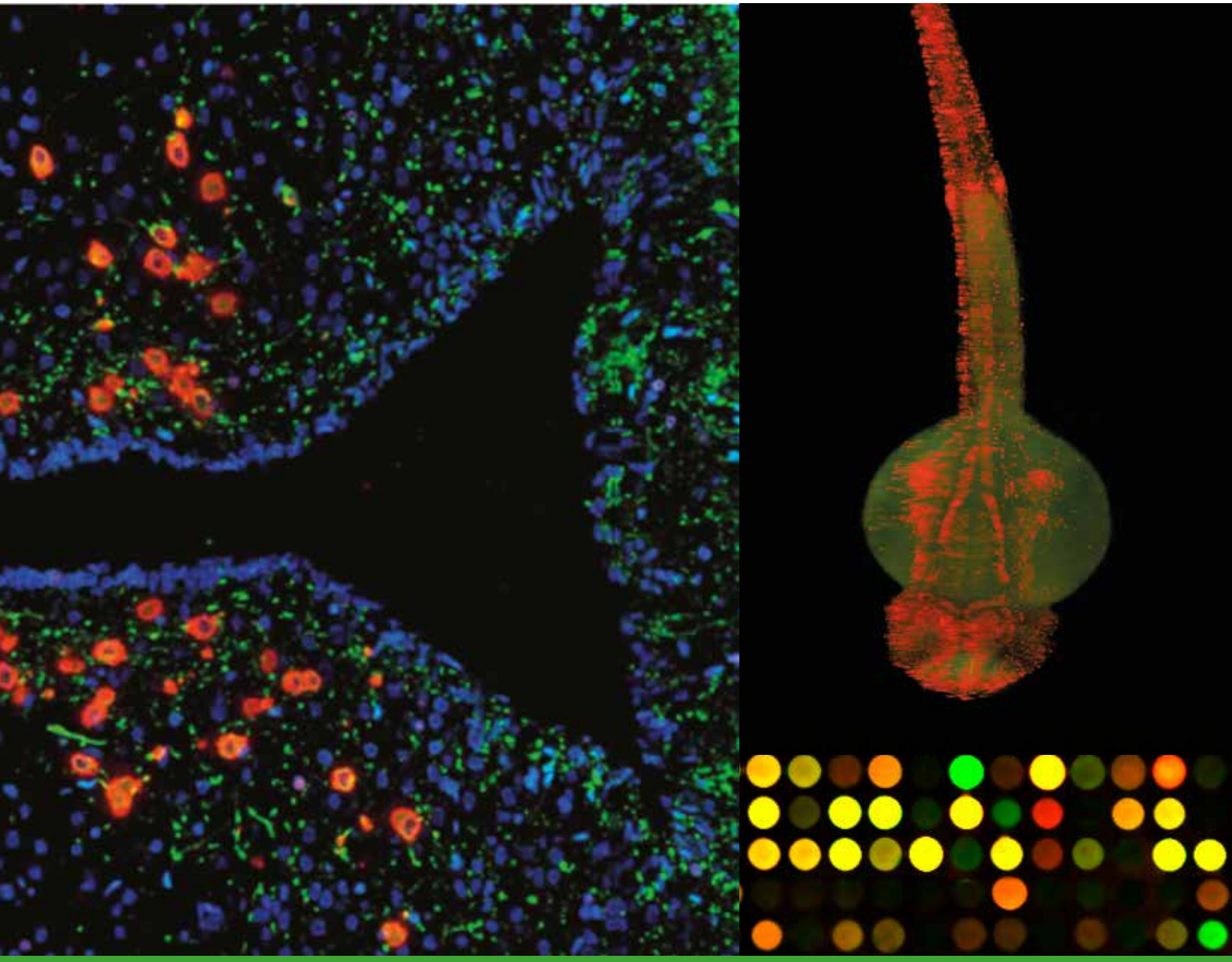
MicroMatrices®

Molecular Toxicology Services



MicroMatrices solve molecular toxicological problems using unique visualisation/localisation technologies

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Zebrafish embryo, imaged with ZEISS Lightsheet Z.1. Sample courtesy of C. Hoppe & G. Shah, Huisken Lab, MPI-CBG, Dresden, Germany.
www.zeiss.com/lightsheet<
http://www.zeiss.com/lightsheet

Frontiers, not boundaries

MicroMatrices solve molecular toxicological problems using unique visualisation/localisation technologies, adding resolving power to standard methodologies. Consisting of a highly skilled core of scientists networked to facilities of excellence across the UK, MicroMatrices is scalable, efficient and flexible. As leaders rather than followers, MicroMatrices offers innovative, collaborative, state of the art solutions for your unique efficacy and safety requirements, throughout the life cycle of your products.

Every client project is a unique proposition, hence the requirement for a collaborative approach. MicroMatrices possesses an evolving array of applications. Many of these support **Mode of Action** understanding, which can be used to generate hypotheses and design bespoke investigatory studies, to help develop a **Threshold Based Risk Assessment** and **Human Relevance** argument.

Description of Services

1. Archival FFPE Studies for Hypothesis Generation, Mode of Action Understanding, Threshold Based Risk Assessment and Non Human Relevance.

MicroMatrices offer retrospective study analyses via the extraction of RNA from archival FFPE tissue blocks for use in integrated miRNA/mRNA array investigations and unbiased and focused pathways analysis. With these results, we further explore molecular pathology using immunohistochemistry (IHC), RNAscope in situ hybridisation (ISH) colocalisation, image analysis, etc. These techniques reduce the need for new animal experiments, unlocking the potential of historic samples and aiding the design of bespoke prospective investigative studies. *An example of this approach can be found in Case Study 1.*

2. Laser Dissection Targeted (LDT) Micro Array & Pathways Analysis.

LDT facilitates focused exploration of both archival and ongoing tissue from investigative studies. The combination of laser microdissection, microarray, and molecular pathology techniques results in a powerful approach to tissue analysis for exploring molecular markers and pathway perturbations.

The technique yields sufficient mRNA to enable a tissue region-specific unbiased pathways analysis for the identification of several candidate genes, and gene expression pathways for hypothesis generation and testing. *A demonstration of the application of the technique is found within Case Study 2.*

3. RNAscope in situ Hybridisation (ISH)/immunostaining (IHC) and Image Analysis and colocalisation.

MicroMatrices are expert in the use of RNAscope technology, capable of detecting and visualising a single RNA transcript within a cell with high sensitivity/specificity and little to no background, in combination with immunohistochemistry staining and colocalisation to offer clients a unique target visualisation capacity which can guide and/or corroborate mechanistic and mode of action studies.
See Case Studies 1 and 3 for examples of the application of this technique.

4. Zebrafish Array analysis.

Zebrafish toxicity profiles are strikingly similar to those found in mammals. MicroMatrices employ this model system in combination with microarray and pathways analysis for the detection of potential biomarkers of developmental toxicity as well as the investigation of the mode of action of toxicants.

5. Biomarker Discovery/ Characterisation.

MicroMatrices possess a varied and adaptable toolbox of molecular profiling techniques for the discovery of novel biomarkers for toxicity; our strength is our versatility and we will custom design assays to address the unique requirements of our clients. *Case Study 2 provides an example of the approaches which can be used in the identification of mechanistic biomarkers within target cells.*

6. Drug Target Tissue Mapping.

MicroMatrices' array of technologies are well suited to exploring and demonstrating xenobiotic targets, using both in situ hybridisation (ISH) and immune staining (IHC). The unique sensitivity of RNAscope, in particular, enables highly specific localisation. *An example of the application of this approach is contained in Case Study 3, where we demonstrate the mapping of GABA isoforms.*

7. Product Rescue/Repositioning

We can support in early discovery, development, and product life cycle maintenance. Should registration concerns arise as a consequence of regulatory tests with model species, MicroMatrices can assist. We will undertake mechanism-based risk assessment analyses to investigate hypotheses of toxicity and determine whether a case for non-human relevance can be demonstrated, potentially extending the commercial life of the product.

8. Reports and Publications.

MicroMatrices will assist in the preparation and review of manuscripts for publication, study reports, SOPs, and other technical documents; please enquire.

Case Study 1.

Carcinogenesis

A. Evaluation of Delayed Senescence of Hypothalamic Tuberoinfundibular (TIDA) Neurons in Rats - association with uterine carcinogenic potential

We quantified tyrosine hydroxylase (TH) expressing TIDA neurons in ageing rats (2 year Bioassay), in order to explore the possibility that delayed senescence of these neurons may be associated with the development of uterine tumours, as a result of dopaminergic inhibition of prolactin secretion via the hypothalamopituitary axis.

1. Dual staining for TH RNA (ISH) and protein (IHC) Formalin Fixed Paraffin Embedded tissue and subsequent quantification using Image Analysis.

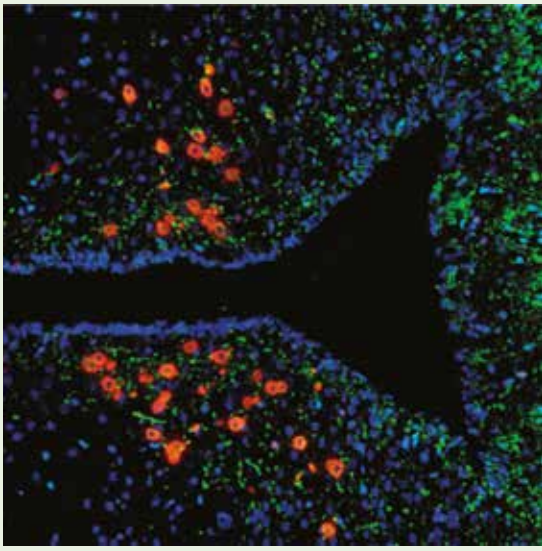
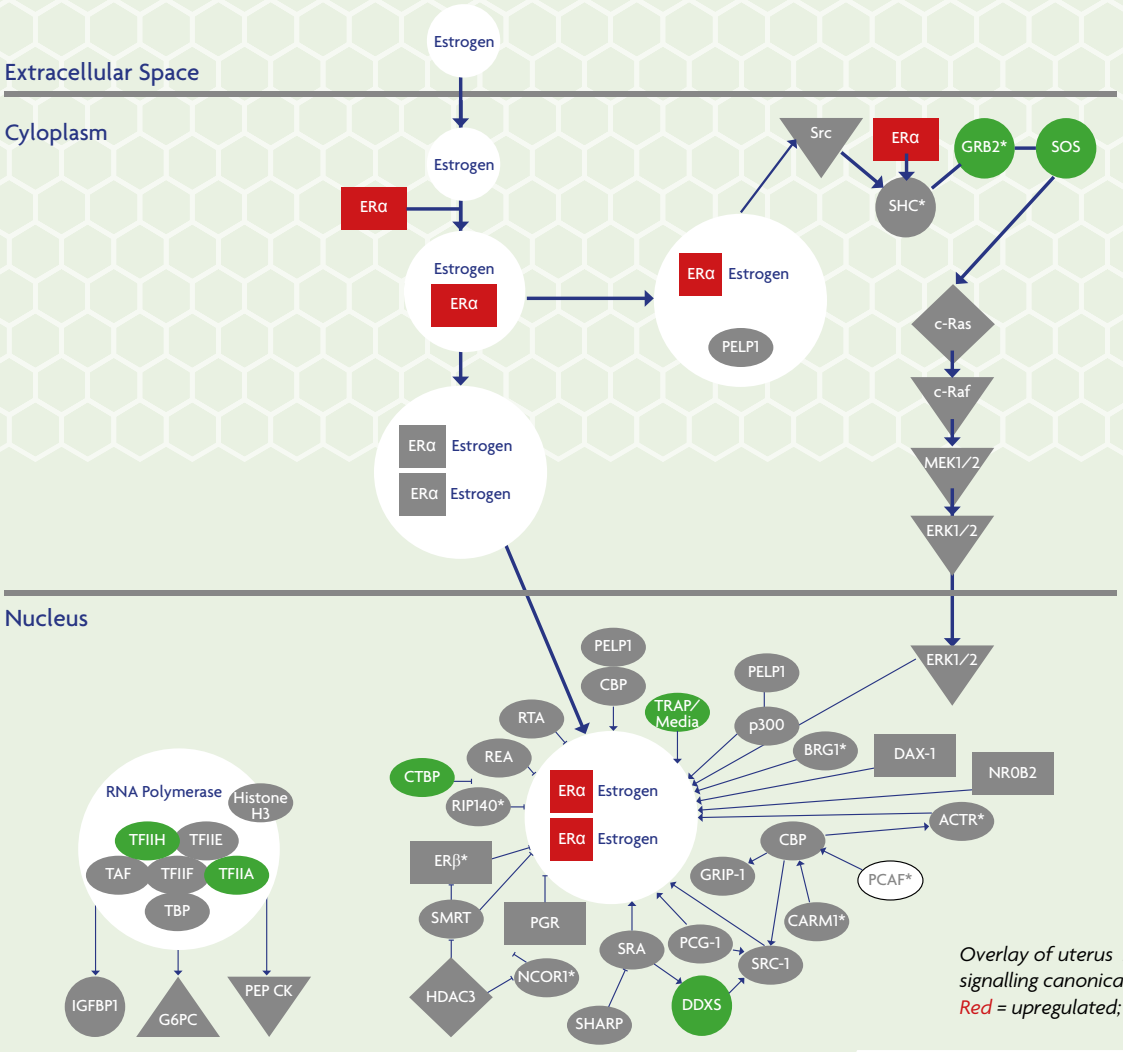


Fig. 1a, TH RNA by ISH (red) and TH protein IHC (green) dual staining – a more accurate way of measuring dopaminergic neurons.

2. Microarray and pathways analysis of laser dissected FFPE tissues known to be regulated by the hypothalmo-pituitary axis, to explore signalling pathways (e.g. Estrogen Receptor signalling), that might be associated with uterine carcinogenesis.



Overlay of uterus microarray data on estrogen signalling canonical pathway
Red = upregulated; Green = down-regulated.

Fig. 1b, Overlay of uterus microarray data on estrogen signalling canonical pathway

Case Study 1. Carcinogenesis cont.

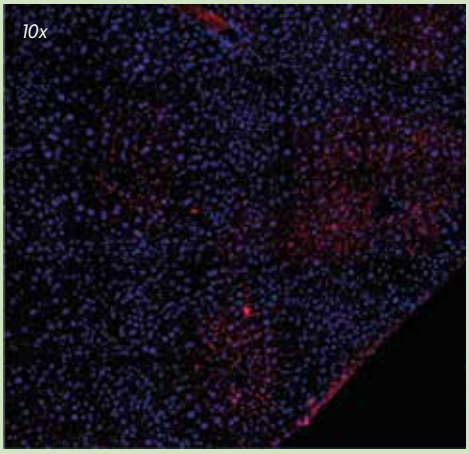


Fig. 2a, CAR nuclear translocation in mouse liver.

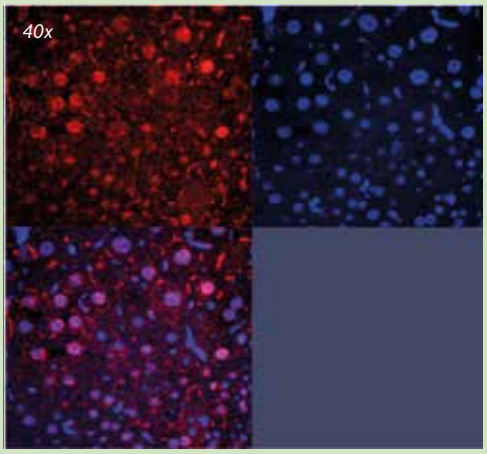
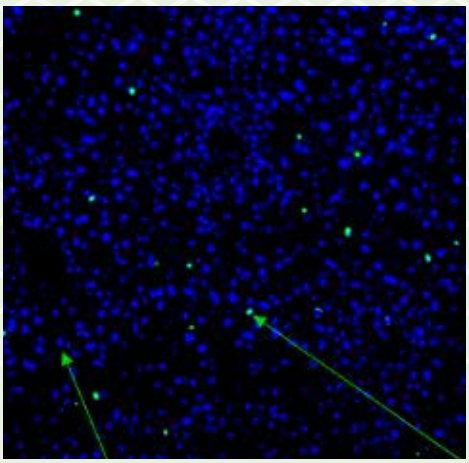


Fig. 2b, CAR immunostaining (red) and TOPRO nuclear counterstain (blue) in phenobarbital treated mouse liver.

B. Evaluation of CAR activation and cell proliferation – key events in rat and mouse liver carcinogenesis

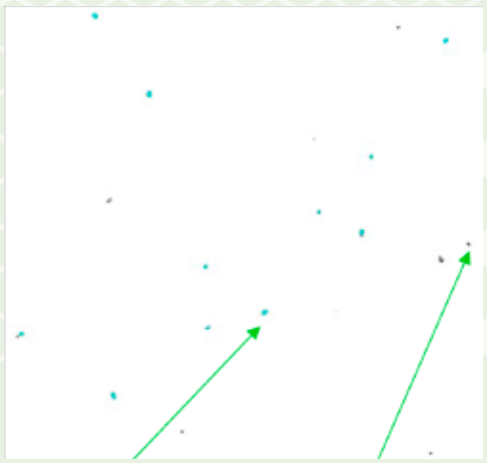
In vivo assays for CAR activation and cell proliferation in mouse liver using fluorescence immunostaining.

Fig. 3a, BrudUGreen/Torpo. Counterstain Blue



Non labelled Hepatocyte

Fig. 3b, Threshold Nuclei (Cyan) Fragments (Black)



Labelled Hepatocytes

Kuppfer cells and fragments (not counted)

Anti-BrDU fluorescence immunostaining quantified by automated semi-automated image analysis

Labelling index calculation:

$$LI(\%) = 100 \times \frac{\sum(\text{Labelled Nuclei})}{\sum(\text{Total Nuclei})}$$

The results supported a mode of action consistent with non human relevance.
Plummer et al 2013, 2014,

Case Study 2.

Developmental Reprotoxicity

Identification of a Toxicity Biomarker in Target Cells

Laser Dissection Targeted (LDT) arrays - a tool for mechanistic hypothesis generation focused on toxicity in 'target' cells. For example in fetal testes certain phthalate plasticisers cause anti-androgenic effects by inhibiting testosterone synthesis. We used LDT expression microarrays to characterise the mechanism at the level of a steroidogenesis and testes development pathway and showed it was localised to Leydig cells (Plummer et al 2007), Figure 4. All the key features of testes mal-development could be accounted for by the effects observed on this pathway.

Next, we used focused ChIP microarrays targeted to test the hypothesis that phthalates repressed the promoters of steroidogenic and testes development genes by interfering with the binding of transcription factors SF-1 and PPARα, *Figure 5.*

Fig. 5, ChIP microarray identifies transcription factor binding peaks in target gene promoters.

Fig. 6, ChIP microarray analysis showing inhibition of SF-1 binding by phthalate treatment.

Results showed that phthalates inhibited the binding of SF-1 to these gene promoters and this was associated with increased binding of PPARα *(Plummer et al 2013), Figure 6.*

Fig. 4a

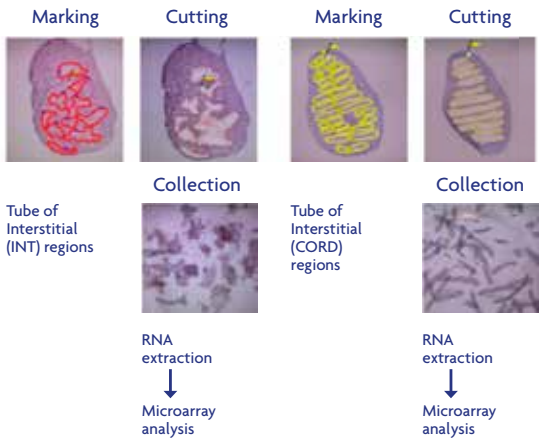


Fig. 4b

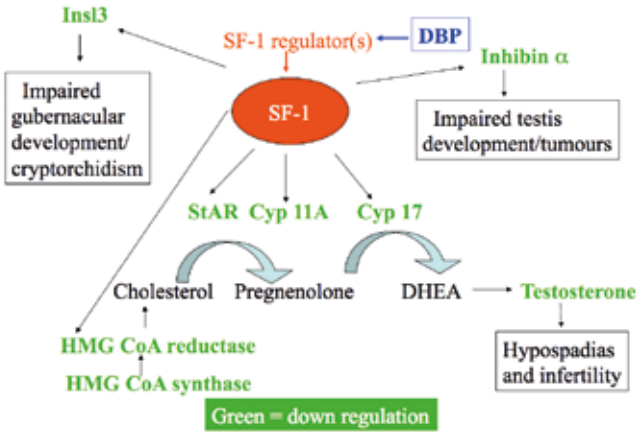


Fig. 5

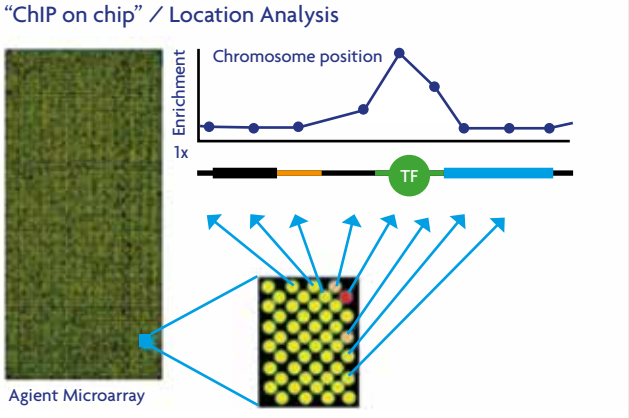
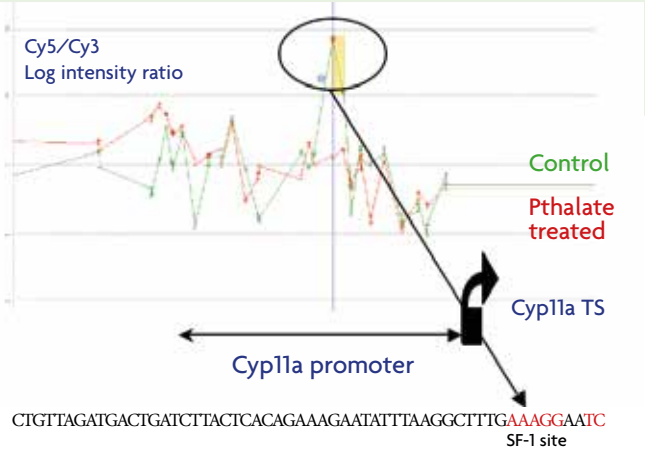


Fig. 6



Armed with this detailed mechanistic insight we used the pathway to develop a focused array-based screen for testing compounds at this level. This has proven useful in early discovery for selection of novel plasticisers with a more favourable profile i.e. lacking fetal testes anti-androgenic activity. Understanding the mechanism of the anti-androgenic effects at the level of transcription factor binding has enabled clients to better interpret data derived from the screen, *Figure 7a.*

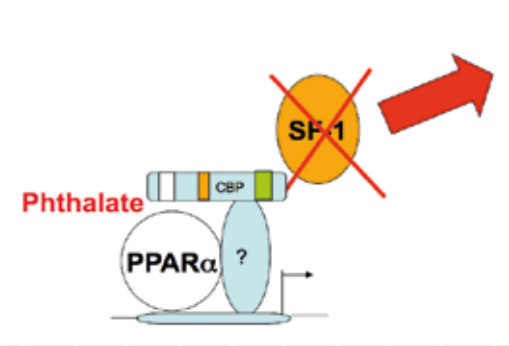


Fig. 7a, Schematic showing the application of a focused array based screen in early discovery (7a) ChIP array analysis facilitates formation of an hypothesis involving PPARα-mediated transrepression of SF-1 binding to steroidogenesis gene promoters; (7b,c,d) Schematic showing how focused array screen can be used in early discovery to select the best candidates.

TEST COMPOUND A › B › C

Fig.7b

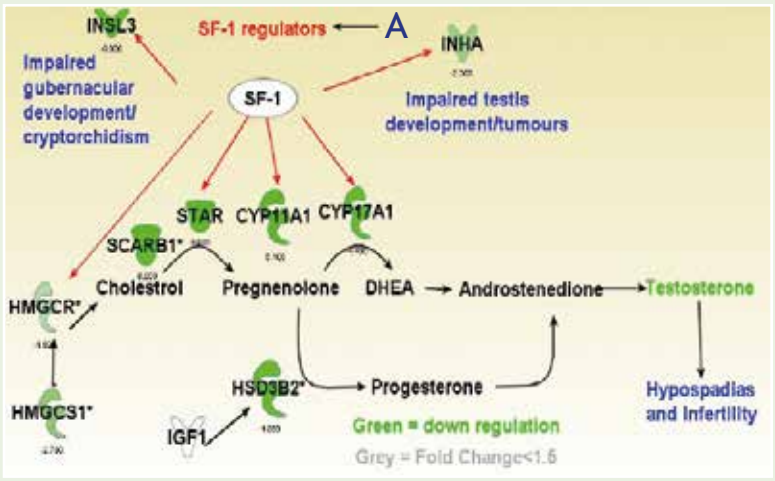


Fig.7c

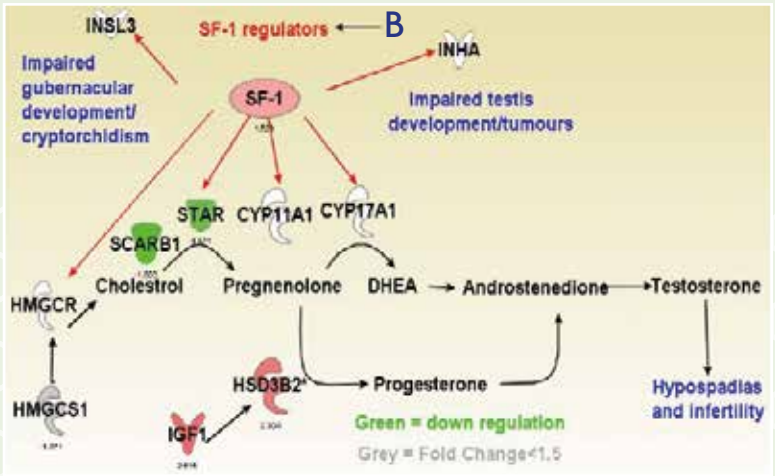
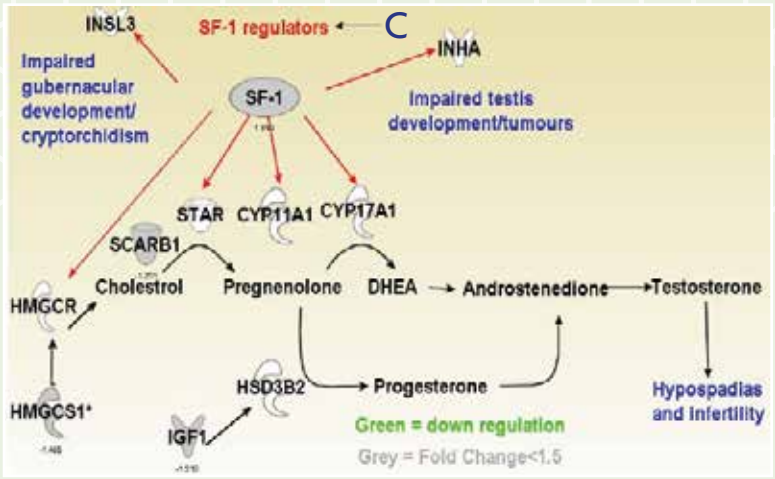


Fig.7d



Case Study 3.

Exploring the distribution of Xenobiotic Drug Targets to support Understanding Efficacy and Safety

A. Mapping Differential expression of GABA isoforms

Central and peripheral effects $GABA_A$ receptors occur in all organisms that have a nervous system. To a limited extent the receptors can be found in non-neuronal tissues. Due to their wide distribution within the nervous system of mammals they play a role in virtually all brain and many peripheral functions. There are numerous subunit isoforms for the $GABA_A$ receptor (Figure 8) which determine the receptor's agonist affinity, chance of opening, conductance, and other properties. It is important to understand isoform distribution when exploring specific disease targets. For example, anticonvulsant effects can be produced by agonists acting at any of the $GABA_A$ subtypes, but current research in this area is focused mainly on producing α2-selective agonists as anticonvulsants which lack the side effects of older drugs such as sedation and amnesia.

In animal safety testing (dog/rat), depending upon exposure levels, the consequences of GABA agonists/antagonists can be widespread, impacting upon a range of tissues including the brain, adrenal, gut, liver and kidneys. The tissue effects reflect the isoform distribution, which we have uniquely demonstrated using RNAscope in situ hybridisation, ISH (Figure 9, 10).

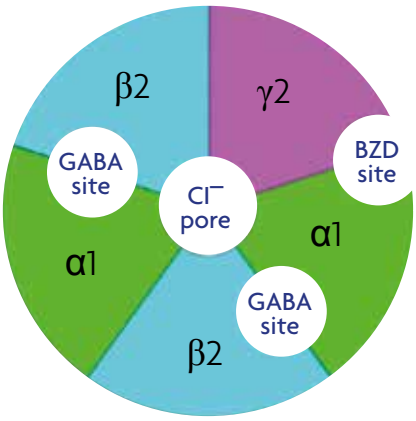
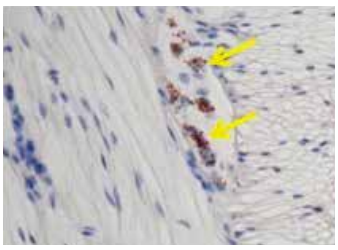
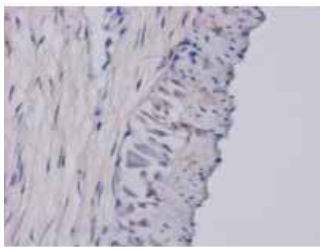


Fig. 8, Schematic diagram of a $GABA_A$ receptor protein ($(\alpha 1)_2(\beta 2)_2(\gamma 2)$) which illustrates the five combined subunits that form the protein, the chloride (Cl-) ion channel pore, the two GABA active binding sites at the α1 and β2 interfaces, and the benzodiazepine (BDZ) allosteric binding site.

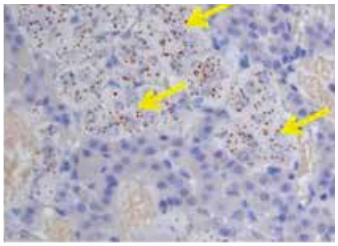
Fig. 9, Representative images of $GABA_A$ receptor isoform subunit RNAscope hybridisation in rat gut and adrenal FFPE tissue, (x40 images); positive staining, (brown spots/yellow arrows) and negative staining (no brown spots) can be observed, indicating tissue-specific and isoform-specific mRNA expression.



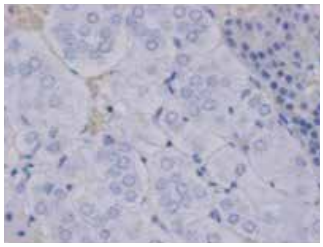
GABAB3 gut



GABAA6 gut



GABAB3 adrenal



GABAA6 adrenal

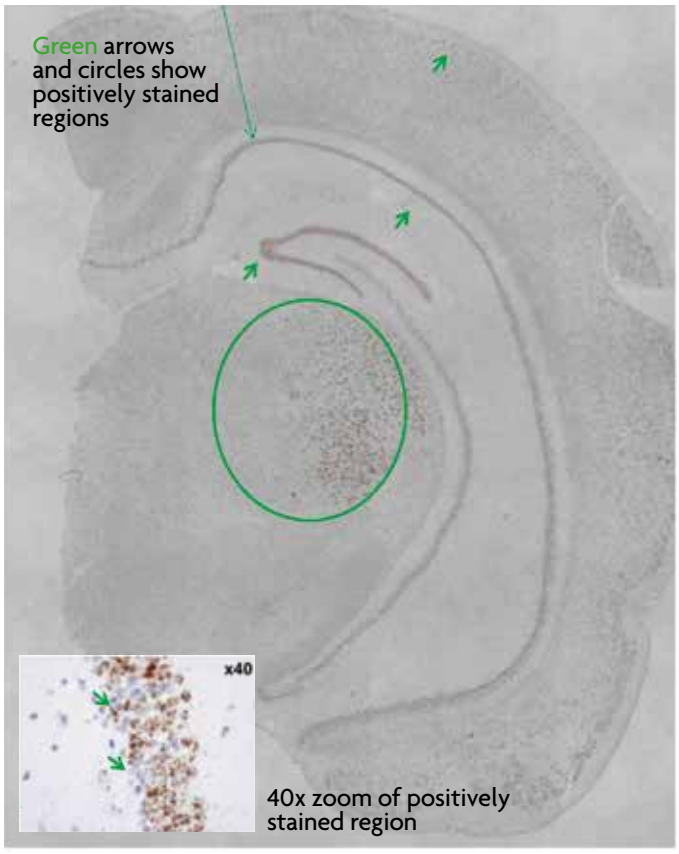


Fig. 10, Tiled (5x) images of rat brain sections staining with GABA α3 (A) and GABA α4 (B)-specific RNAscope probes, the area within the circle containing the dorsolateral geniculate nucleus, the medial geniculate nucleus, and the lateral posterior thalamic nucleus.

Positively stained cells/regions are indicated by green arrows/circles. Right hand panels show x40 zoomed images of the $GABA_A$ α3 (top)/α4 (bottom) receptor staining. $GABA_A$ α4 showed positive staining in the striatum (green circle), however this region was not stained by the $GABA_A$ α3 - specific probe.

B. Target identification and Tissue cross reactivity

Drug targeting using a targeted antibody linked to a toxin has been widely explored, particularly in the cancer field. One of the key hurdles is the specificity of the antibody, so that any off-target effects are minimised. MM has the capability to search the gene data bases, to design and develop antibodies (in collaboration with key partners in Dundee) for bespoke targets (Fig 11) and explore tissue cross reactivity using immunohistochemistry (IHC).

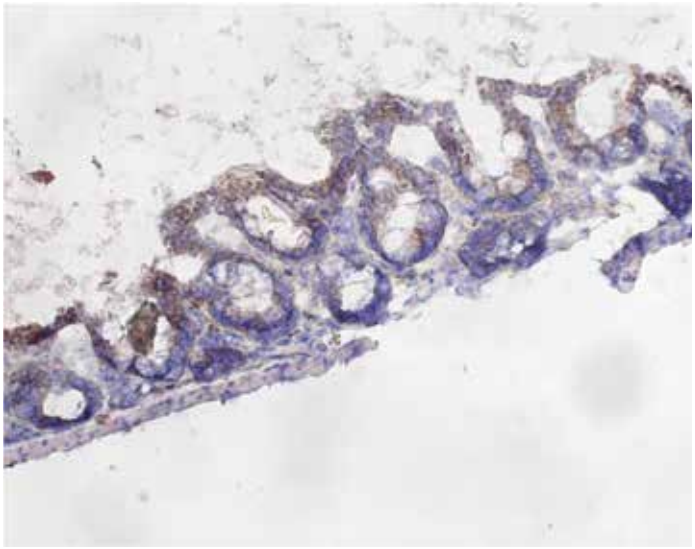


Fig. 11, Target Identification Mouse colon stained with anti-GLUT7 polyclonal antibody staining seen on villi epithelium. GLUT7 is a mucosal specific antigen screened from a whole range of bioinformatically derived antigens.

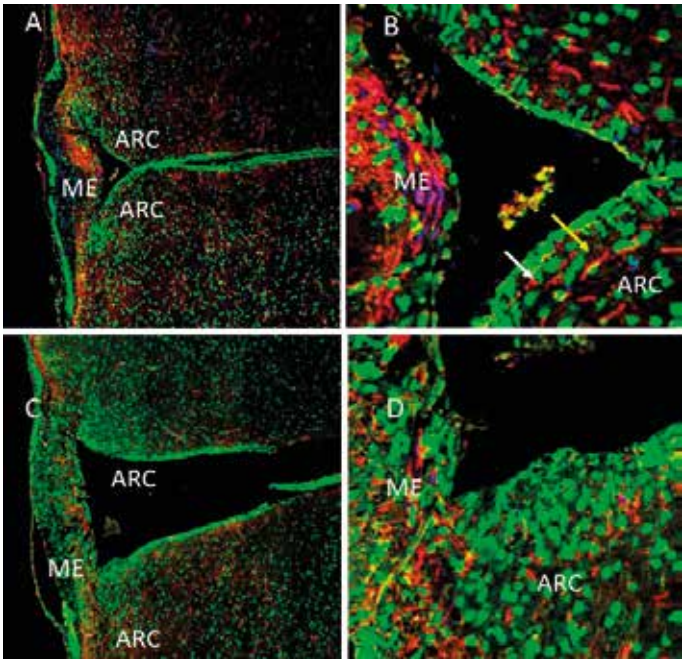


Fig. 12, Leptin R and STAT 3 dual staining no colocalisation
Red = Leptin 3, Green = STAT3

C. Dual Localisation

MM has developed a uniquely sensitive method to explore both message and protein expression in the same cell, as well as protein colocalisation/dual expression. This links into their hypothesis generation capability, using laser capture microdissection, genomics (microarrays) and pathway exploration.

2014 Plummer et al, The Toxicologist, 138, issue 1, abstract ID 1909.;
2013 Plummer et al, ToxSci 132(2), 443-457
2013 Plummer et al, The Toxicologist, 132, issue 1, abstract ID 1549
2007 Plummer et al, Toxicological Sciences 97(2), 520-532

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