cyprotex
AN EVOTEC COMPANY

The development and characterisation of 3D neuronal microtissues for safety testing.

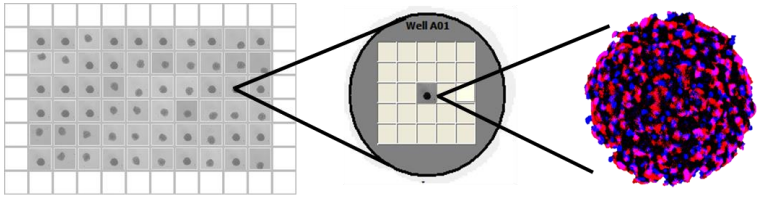
Paul Walker

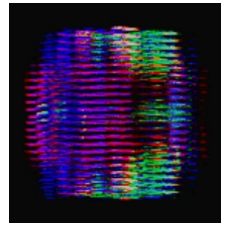
IVTS 24th November 2017

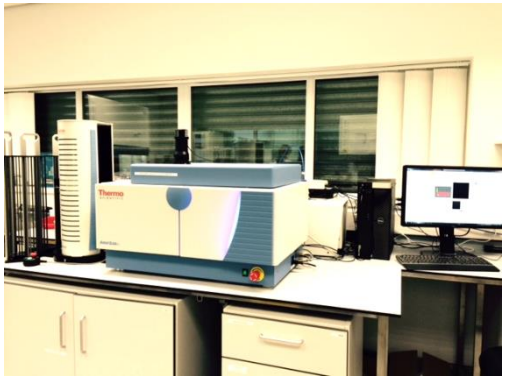
Presentation Overview

The development and characterisation of 3D neuronal microtissues for safety testing

- Brief Company overview
- 3D Organoid/Microtissue development
- Example models developed
- Confocal high content imaging
- Brain microtissue development project
- Further characterisation using Agilent whole human genome oligo microarray
- Initial HCS investigations
- Combined strategy to understand Neutoxicity potential.







Cyprotex is now part of Evotec AG

Evotec's worldwide operations

Cyprotex is an ADME-Tox CRO, based is the UK and US: Acquired by Evotec (Jan 2017)

San Francisco, Branford & Princeton, Watertown, USA

~95 employees

- Compound ID, selection and acquisition
- Compound QC, storage and distribution
- Cell & protein production
- ADME-Tox, DMPK

Abingdon, Alderley Park, UK

~400 employees

- Medicinal chemistry
- ADME-Tox, DMPK
- Structural biology
- *In vitro* & *in vivo* anti-infective platform / screening

Toulouse, France

~300 employees

- Compound management
- Hit identification
- *In vitro* & *in vivo* oncology
- Medicinal chemistry
- ADME & PK
- Early drug formulation and solid form screening
- Cell, protein & antibody production

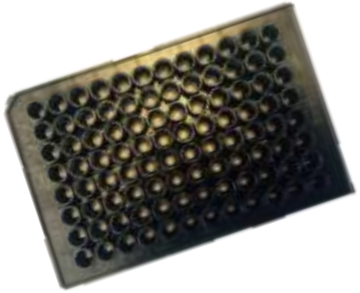
Hamburg (HQ), Göttingen and Munich, Germany

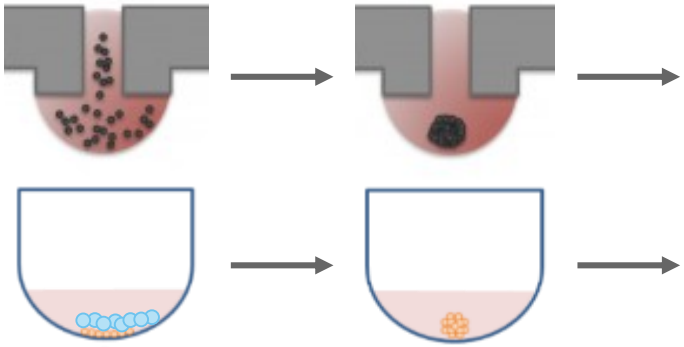
~405 employees

- Hit identification
- *In vitro* & *in vivo* biology
- Chemical proteomics & Biomarker discovery and validation
- Cell & protein production
- Antibody discovery

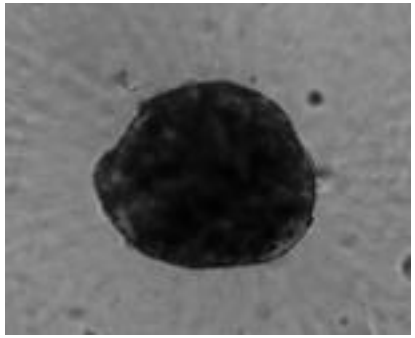
In vitro 3D Cell Culture

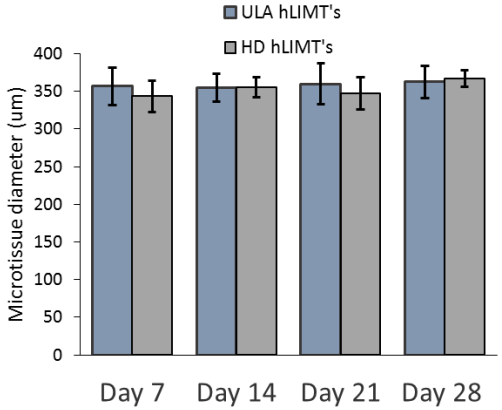
- *In vitro* three-dimensional (3D) cell cultures more accurately **reflect the complex *in vivo* microenvironment** than simple two-dimensional (2D) cell monolayers
- Spheroids are a popular 3D cell culture choice due to **cost effective** cell usage and **scaffold free**
- Spheroid formation utilised the **hanging drop technique** can be used whereby cells are suspended in droplets of medium to promote cell aggregation
- Alternatively **ultra-low adhesion microplates** can be used which are assay amenable plate formats for high content screening





Cell suspension seeding **Single spheroid formation**



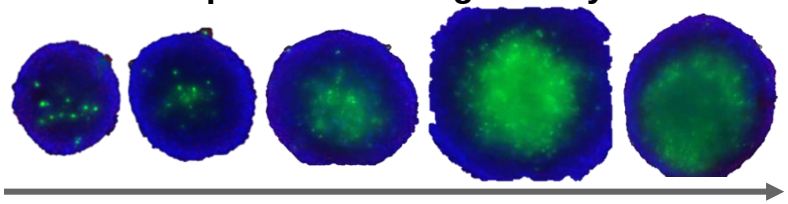


Microtissue Development Stages

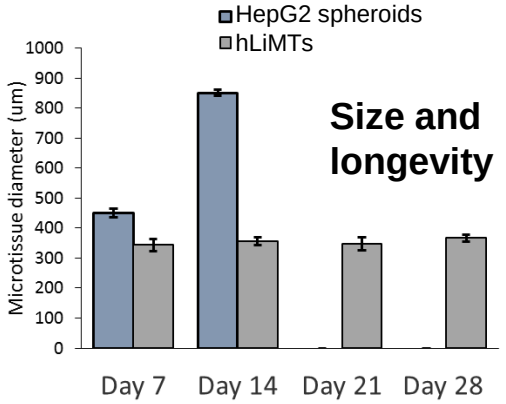
Characterisation may depend upon organoid

1. Optimisation of **seeding density** involves HCS imaging of membrane permeability (**PI**) as a marker of necrotic core combined with a measure of cellular **ATP and DNA correlation** as a marker of cell health
2. Co-cultured models re-quire **additional optimisation** of cell to cell ratios
3. Characterisation of longevity and **tissue specific functions**

Spheroid seeding density

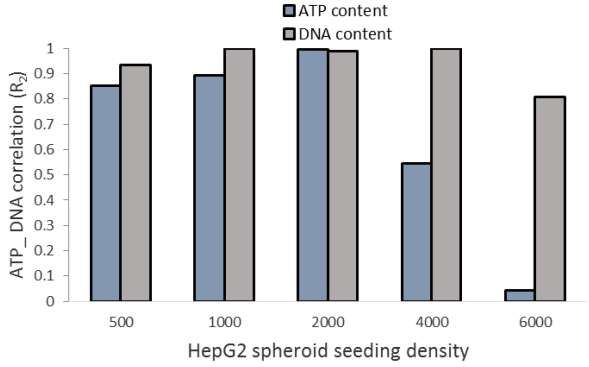


Increasing seeding density

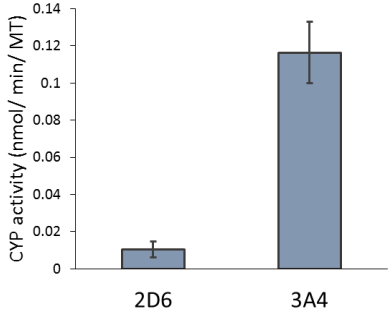


Size and longevity

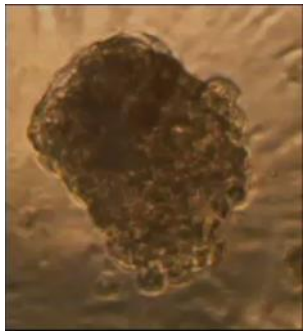
Spheroid ATP content



Tissue specific characteristics

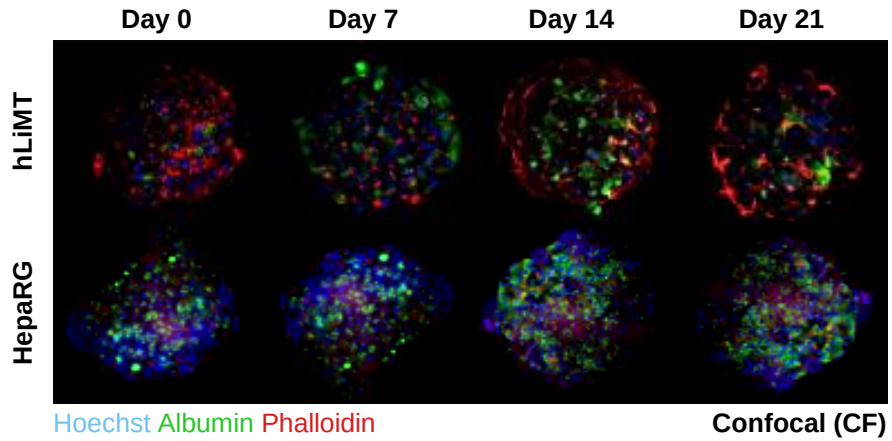


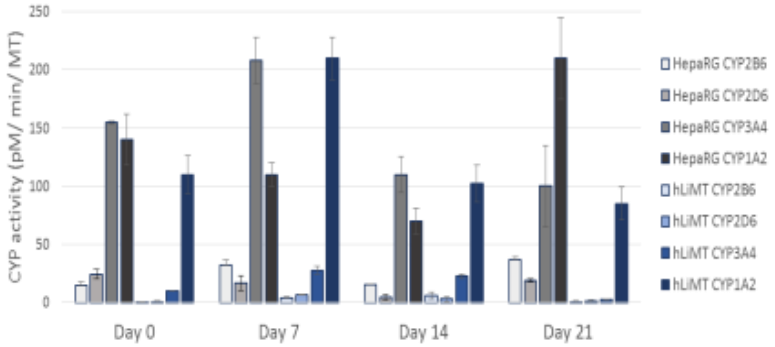
hLIMT's

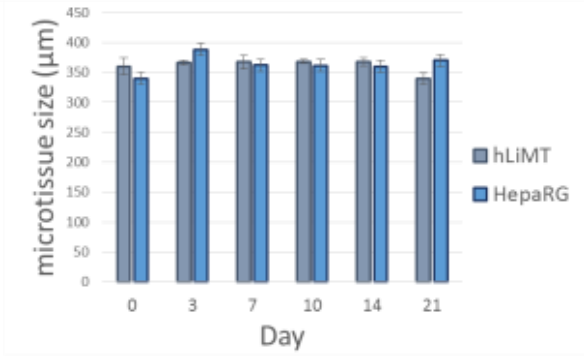


Cardiac microtissue

Human Liver Microtissues and HepaRG Spheroid Characterisation





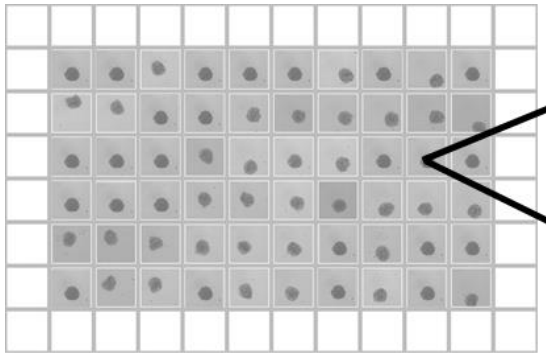


- hLiMTs and HepaRG spheroids display uniform size, shape and improved longevity
- Both models show albumin production, functional bile canaliculi and cytochrome P450 activity

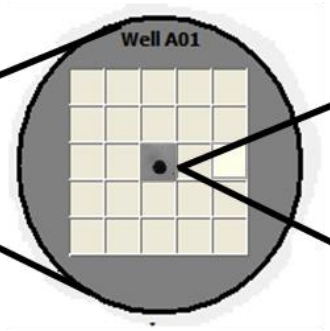
Confocal Imaging of Microtissues

Confocal High Content Imaging (using ArrayScan XTI)

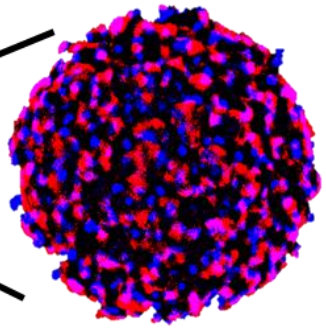
- Confocal imaging to allow us to **analyse more complicated tissue** models using high content screening techniques
- 3D models represent more ***in vivo* relevant** *in vitro* tissue model
- Combined with HCS to determine **sensitive and mechanistic cell health parameters**
- Microtissue in **ULA or transwell** imaging possible



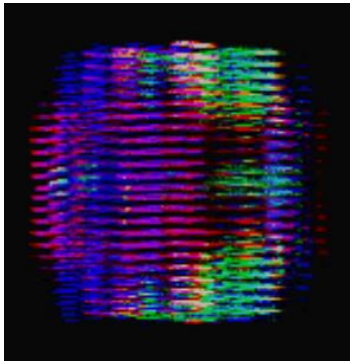
Single MT per well



Single field of view

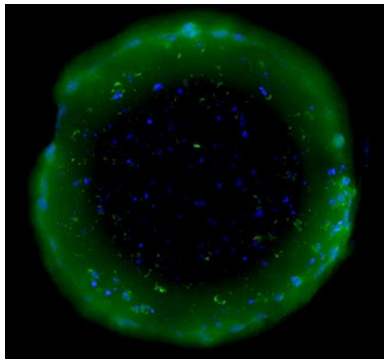
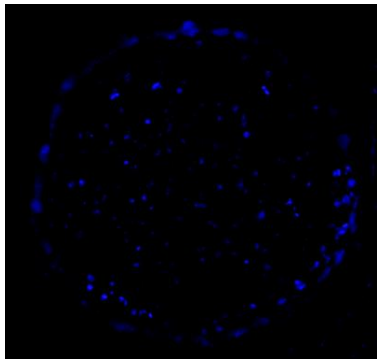
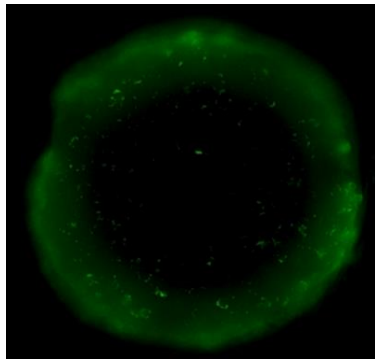
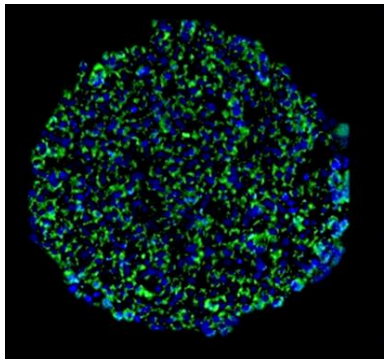
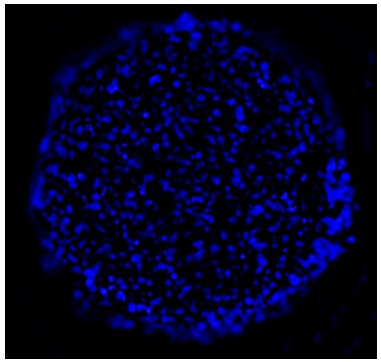
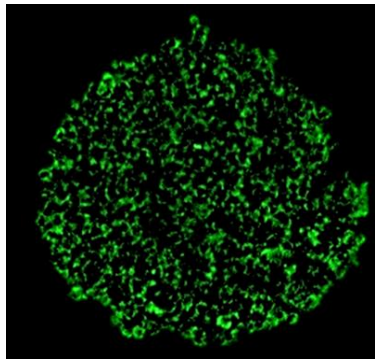


Flexibility of antibodies and dyes



Confocal and Z-Stack imaging

Confocal vs. Widefield Imaging

	Composite	Hoechst	Mitochondria
Widefield			
Confocal			

Maximum projection image capture

350µm microtissue

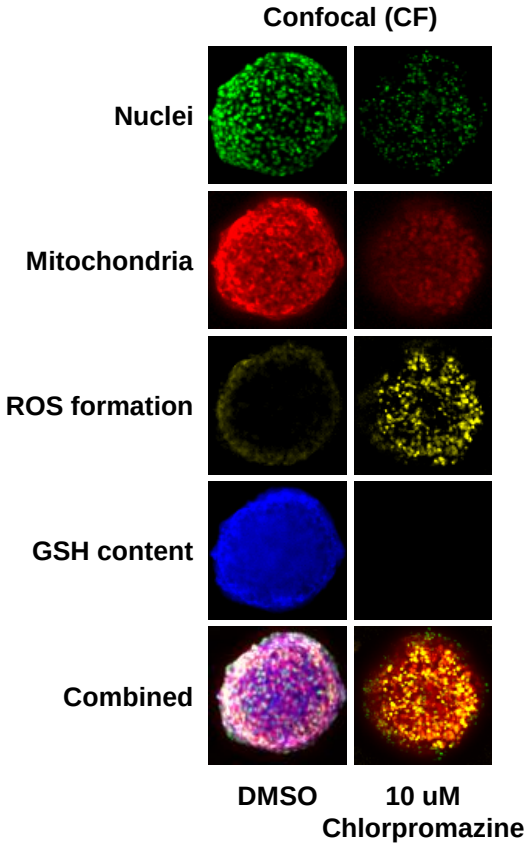
Pilot Comparison of HepaRG Spheroids and hLiMTs

Combining 3D Microtissues with HT high content imaging.

In this small set of reference compounds with a 5x C_{max} cut-off HepaRG spheroids are slightly more sensitive to DILI compounds than hLiMTs

Drug	C_{max} (µM)	DILI category	Cyprotex hLiMT DILI prediction using MEC (µM)	Cyprotex hLiMT DILI prediction using ATP MEC (µM)	Most Sensitive Feature	Cyprotex 3D HepaRG spheroid DILI prediction using MEC (µM)	Cyprotex 3D HepaRG DILI prediction using ATP MEC (µM)	Most Sensitive Feature
Amiodarone	5.3	P	6.51	15.4	ROS	2.41	5.12	DNA
Trovafloxacin	19.7	P	45.2	54.4	GSH	7.27	7.27	ATP
Diclofenac	10.1	P	50	78.1	DNA	30.5	38.8	DNA
Flutamide	5.4	P	3.63	8.72	ROS	7.43	7.75	SIZE
Lapatinib	19.2	P	1.79	12.6	ROS	0.77	1.21	GSH
Nitrofurantoin	21	P	24.7	51.3	ROS	4.89	9.27	SIZE
Carbamazepine	50.8	P	49.2	73.6	DNA	81.5	81.5	ATP
Sunitinib	0.25	P	0.24	0.417	MMP	0.28	1.1	GSH
Troglitazone	6.29	P	0.99	1.55	MMP	1.69	25	DNA
Fialuridine	1	P	11.5	11.5	ATP	1.41	1.41	ATP
Nefazodone	4.3	P	13.7	13.7	ATP	11.6	11.6	DNA
Perhexiline	2.16	P	1.03	1.49	ROS	1.69	1.76	DNA
Tolcapone	21.96	P	21.9	21.9	ATP	18.2	20.5	MMP
Acetaminophen	165.4	P	302	302	ATP	240	342	SIZE
Bosentan	4.7	P	12.3	29.4	DNA	10.4	35.2	DNA
Ticlopidine	8.1	P	17.5	27.2	DNA	34	36.2	MitoMass
Azathioprine	2.22	P	2.48	2.48	ATP	0.28	0.278	ATP
Chlorpromazine	0.94	P	0.34	0.347	ROS	1.07	3.48	SIZE
Tamoxifen	1.18	P	1.54	1.98	ROS	3.52	10.8	SIZE
Buspirone	0.01	N	3.12	3.12	ATP	NR	-	-
Entacapone	3.276	N	40.2	40.2	ATP	45.4	45.5	GSH

≤ 1x Cmax
≤ 5x Cmax
>5x Cmax



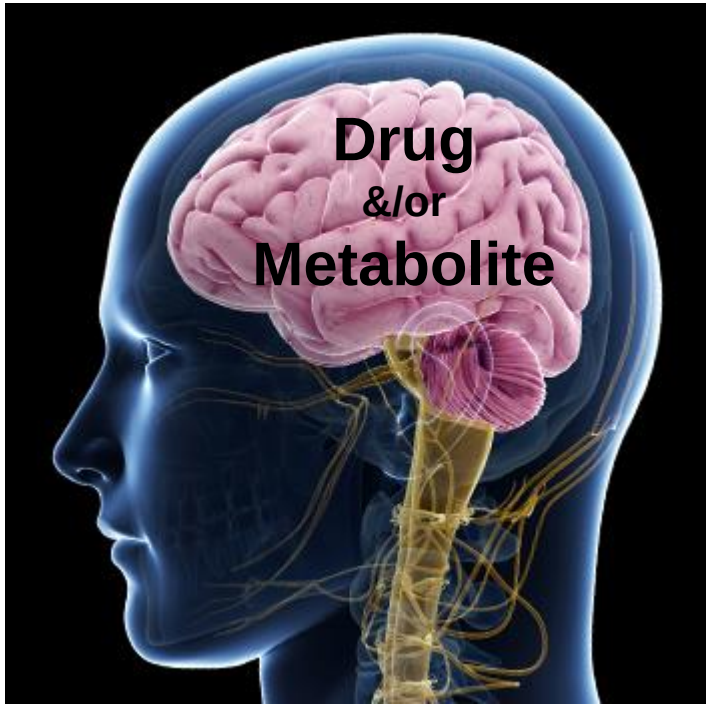
See posters for more details and examples

Drug-induced Brain Neurotoxicity

Current pre-clinical *in vitro* neurotoxicity models often focus on neurons alone, in a restrictive 2D environment with acute compound exposures

Complicating factors:

- Blood-brain barrier deregulation
- Neurogenesis impairment
- Neuroinflammation
  e.g. astrocyte swelling
- Neuronal dysfunction
  - Chemical perturbment
  - Cell death
- Vascular dysregulation

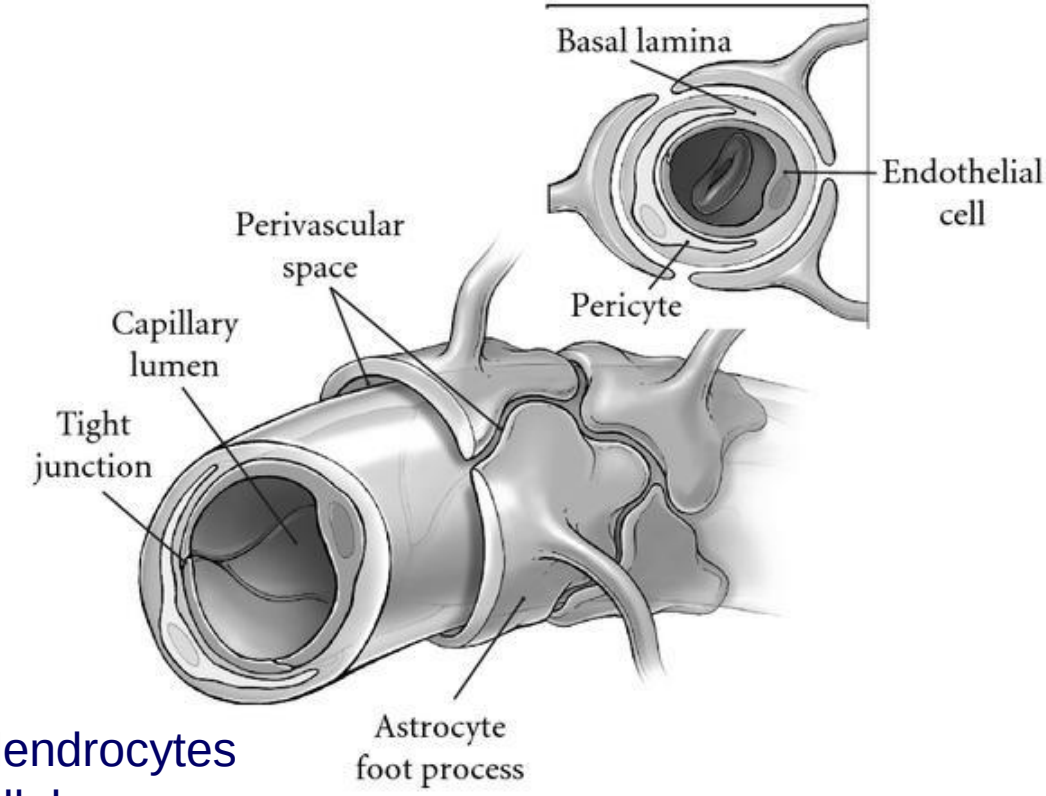


The importance of Astrocytes

No longer considered “filler cells”

Astrocyte Function

- Structural
- Glycogen fuel reserve buffer
- Metabolic support
- Blood-brain barrier
- Transmitter uptake and release
- Modulation of synaptic transmission
- Vasomodulation
- Nervous system repair
- Long-term potentiation
- Promotion of the myelinating activity of oligodendrocytes
- Regulation of ion concentration in the extracellular space



Anderson *et al*., (2011) Cardiovascular Psychiatry and Neurology

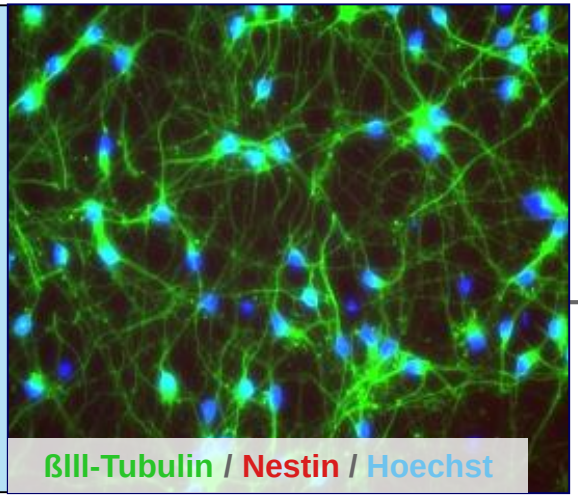
iPSC CDI Human Neural Microtissue

Collaboration with SEAC (Unilever)

iCell®

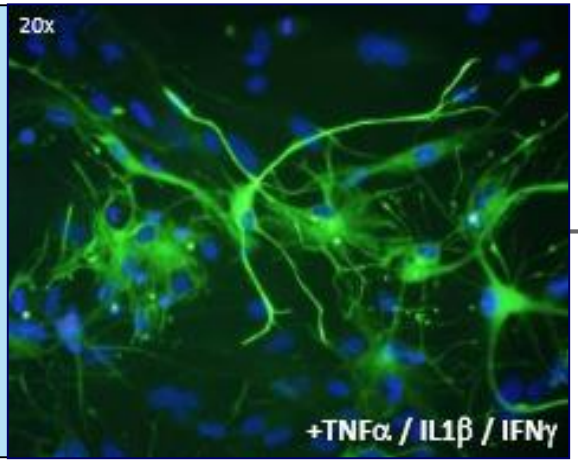
Neurons

Cellular Dynamics international a FUJIFILM company

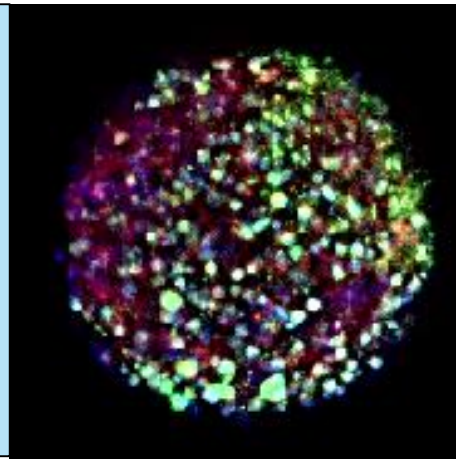


iCell Astro-cytes

Cellular Dynamics international a FUJIFILM company



Brain Micro-tissue



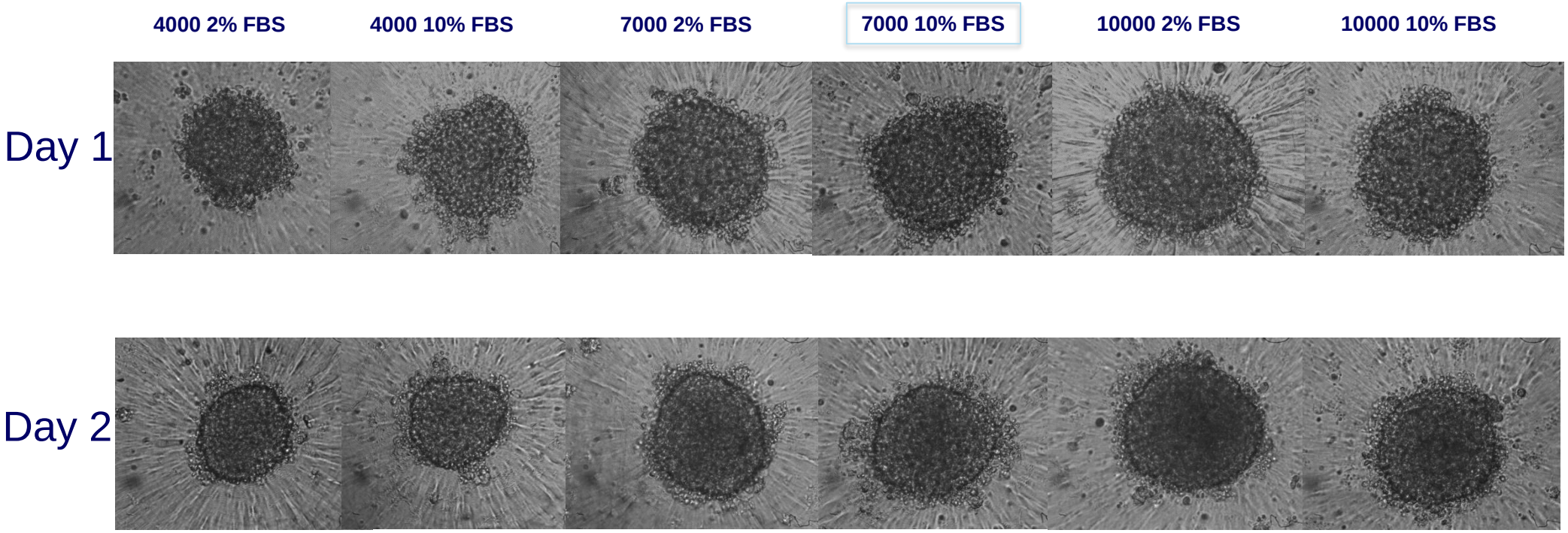
Safety & Environmental Assurance Centre

www.tt21c.org

Microtissue formation (Seeding)

All mirotissues formed under each condition

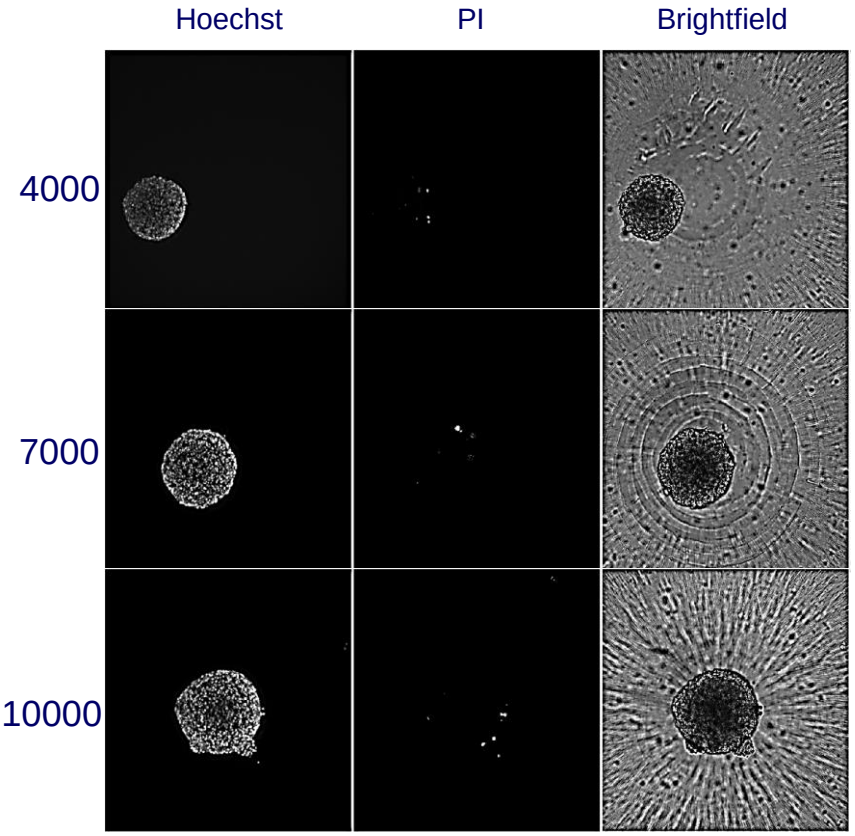
- 1:1 ratio of astrocytes and neurons
- Seeding density test with 2% or 10% FBS in media



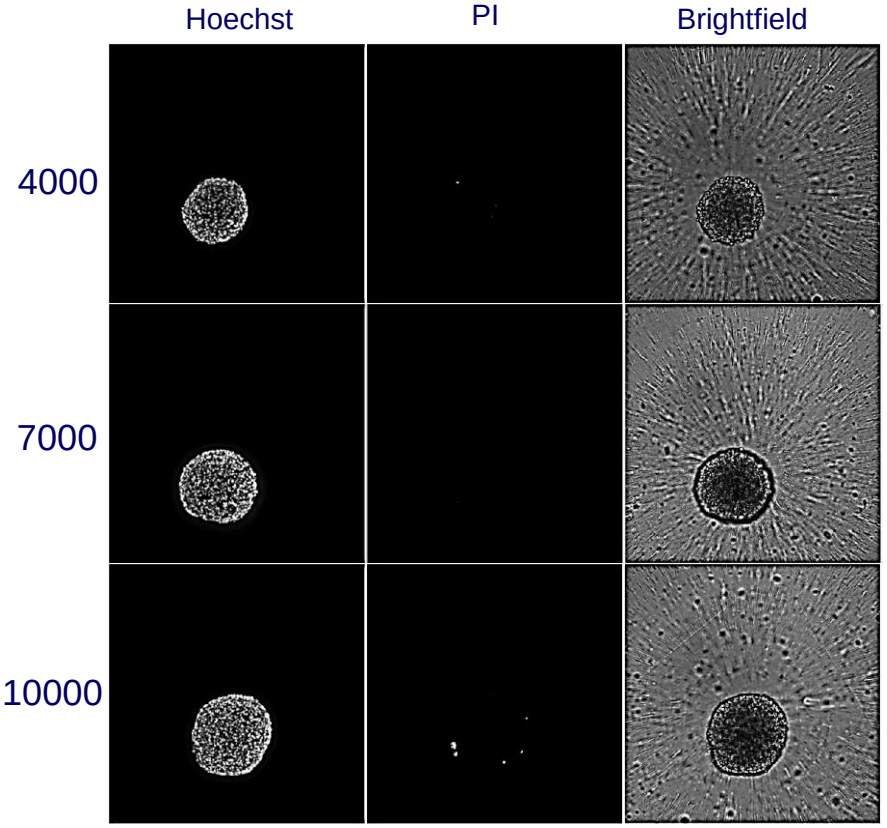
Microtissue viability

PI staining to check for necrotic core

Day 0 (Day 3 post seeding) with 2% serum



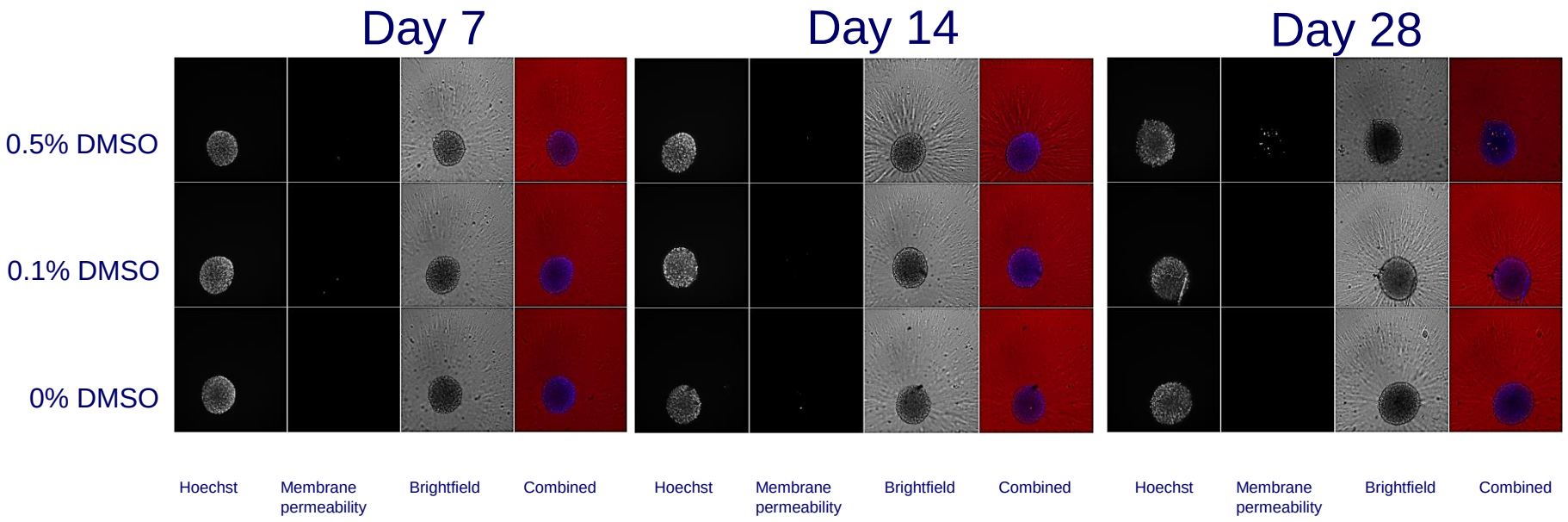
Day 0 (Day 3 post seeding) with 10% serum



- 4000 size difficult to handle (tend to float due to low density)
- 10000 size show morphological variability with irregular shapes
- Therefore 7000 size selected; optimal handling size and uniform

Viability assay over time and DMSO tolerance

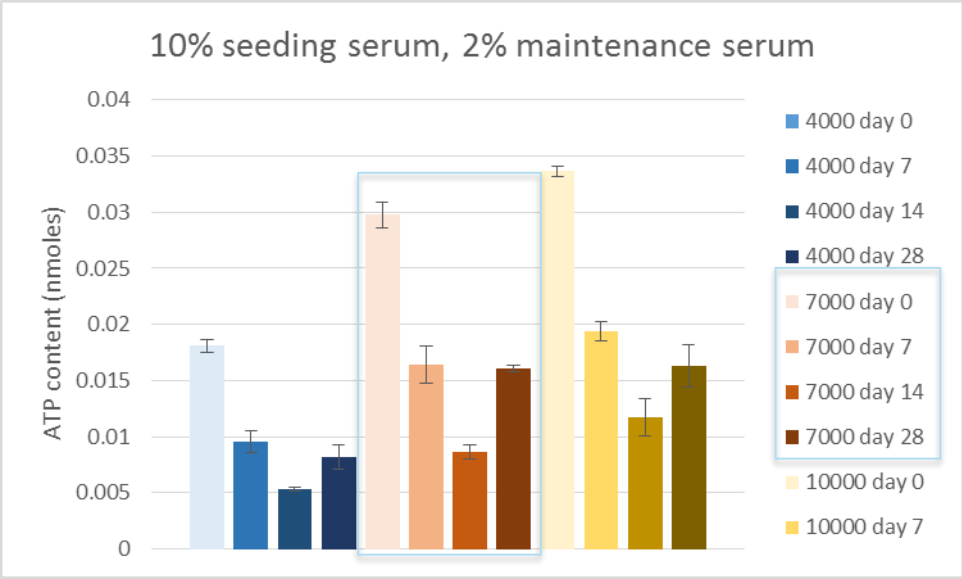
7000 cells/ MT, seeded in 10% serum, maintained in 2% serum

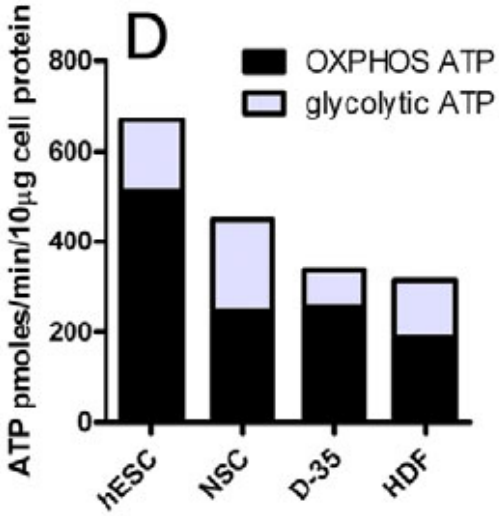


- All microtissues display an increase in cell membrane permeability following 28 days of 0.5% DMSO, no increase prior to this.
- Microtissues show no significance difference in viability across different media types

Basal ATP content over time

ATP demand linked to neural differentiation





A reduction in ATP demand and mitochondrial activity with neural differentiation of human embryonic stem cells

Matthew J. Birket, Adam L. Orr, Akos A. Gerencser, David T. Madden, Cathy Vitelli, Andrzej Swistowski, Martin D. Brand and Xianmin Zeng*
Buck Institute for Age Research, 8001 Redwood Blvd, Novato, CA 94945, USA
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Accepted 10 October 2010
Journal of Cell Science 124, 348-358

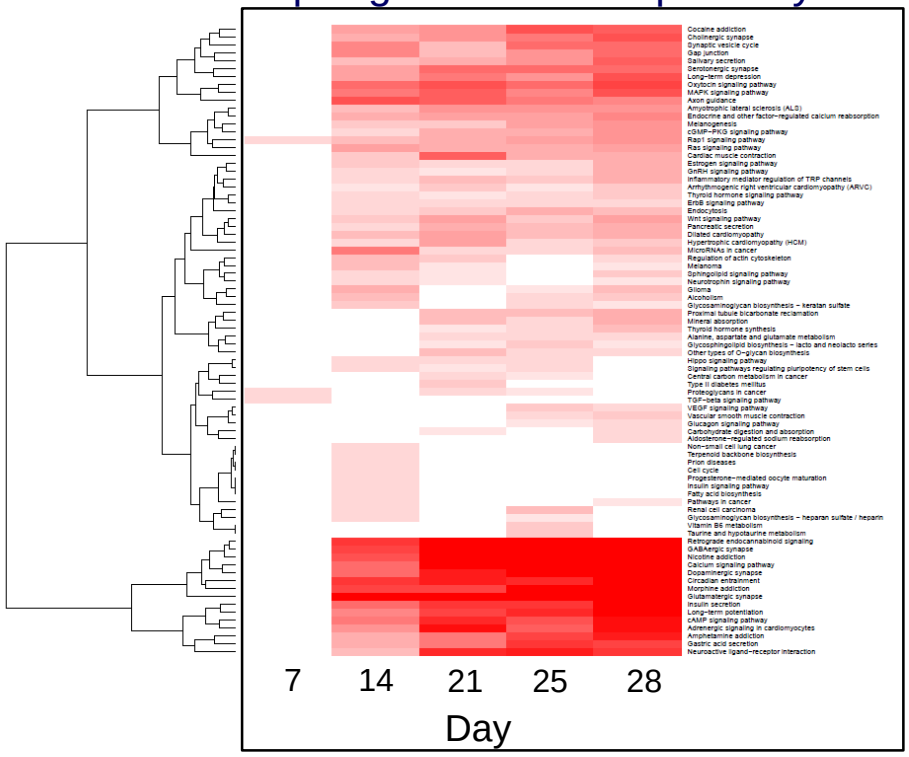
doi:10.1242/jcs.072272

- Decrease in ATP doesn't correlate with increased cell membrane permeability (PI staining) or loss in DNA structure (Hoechst staining).
- Literature suggests decrease in ATP demand with maturity of neurons.

Agilent whole human genome oligo microarray G4851B

The Brain microtissues continue to develop over the 28 day period

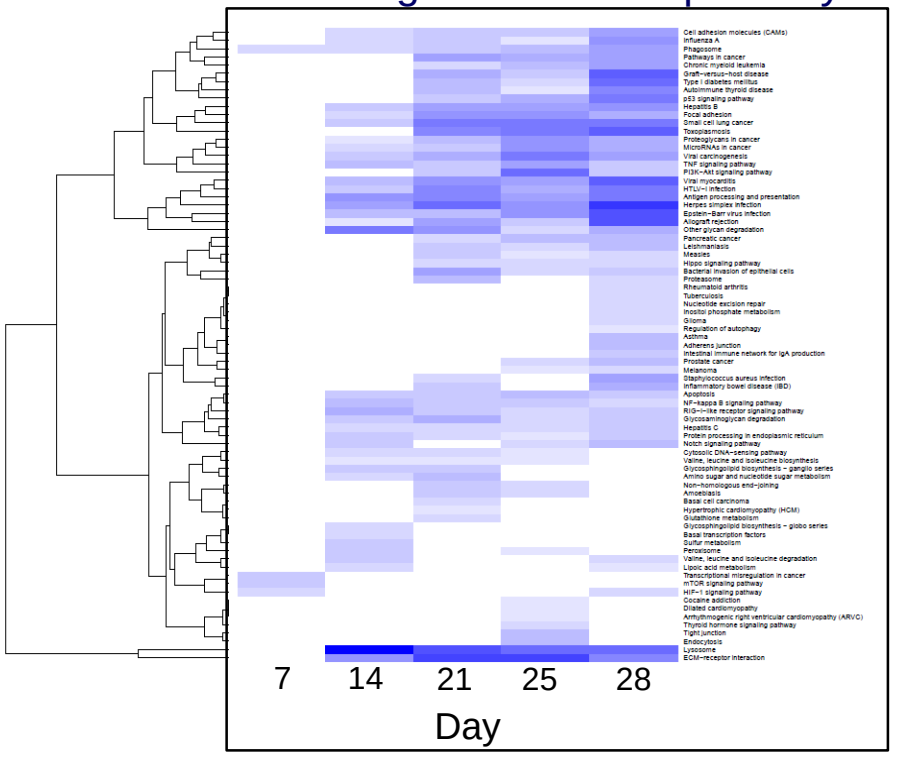
Up-regulated KEGG pathways



81 KEGG pathways

- Neuronal development e.g. synaps pathways
- Intracellular adhesion
- GAP junctions (Astrocyte/neuronal linking?)
- Axon guidance peaks at day 14.

Down-regulated KEGG pathways



78 KEGG pathways

- Cell adhesion (CAMs) – remodelling?
- Focal adhesions
- ECM−receptor interaction

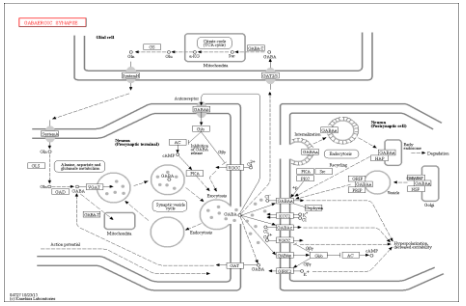
Neurotransmitter pathways

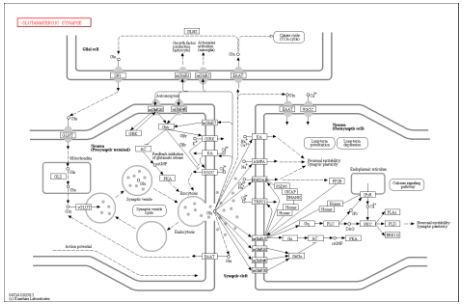
Pathway associated with all 5 types of neurotransmitter increase up to 28 days

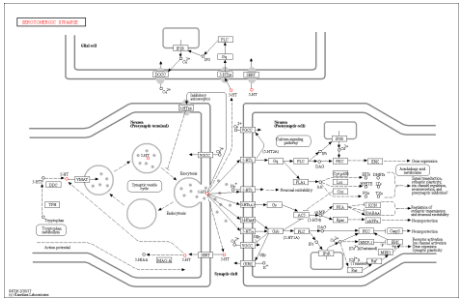
Classification by neurotransmitter production:

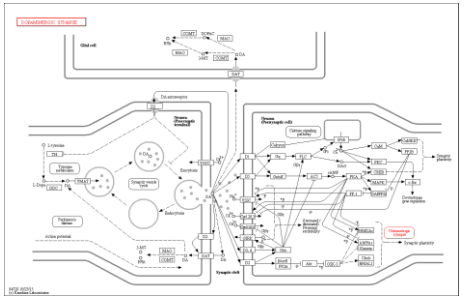
- Cholinergic neurones
- GABAergic neurons
- Glutamatergic neurons
- Dopaminergic neurons
- Serotonergic neurons

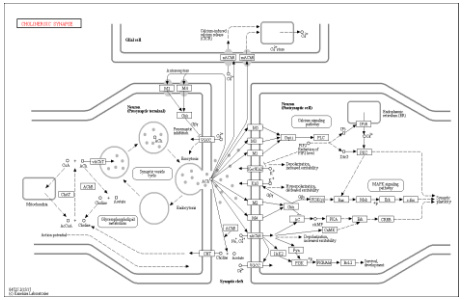
All types of synapse seem to be upregulated over the 28 days

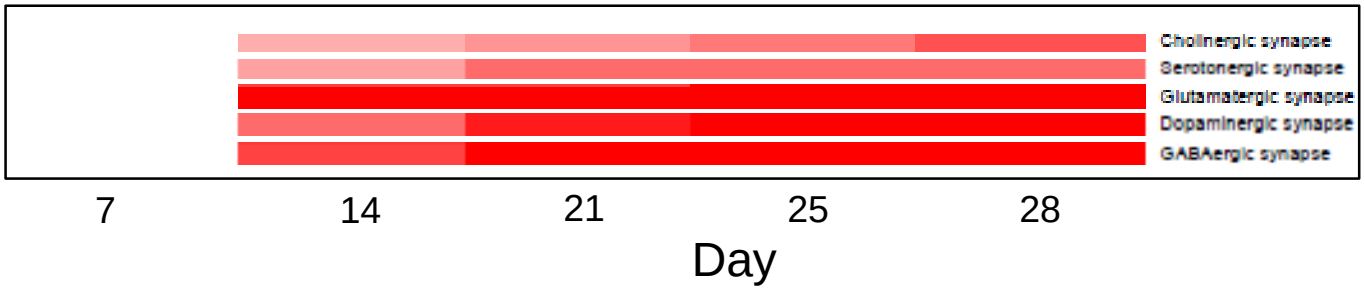








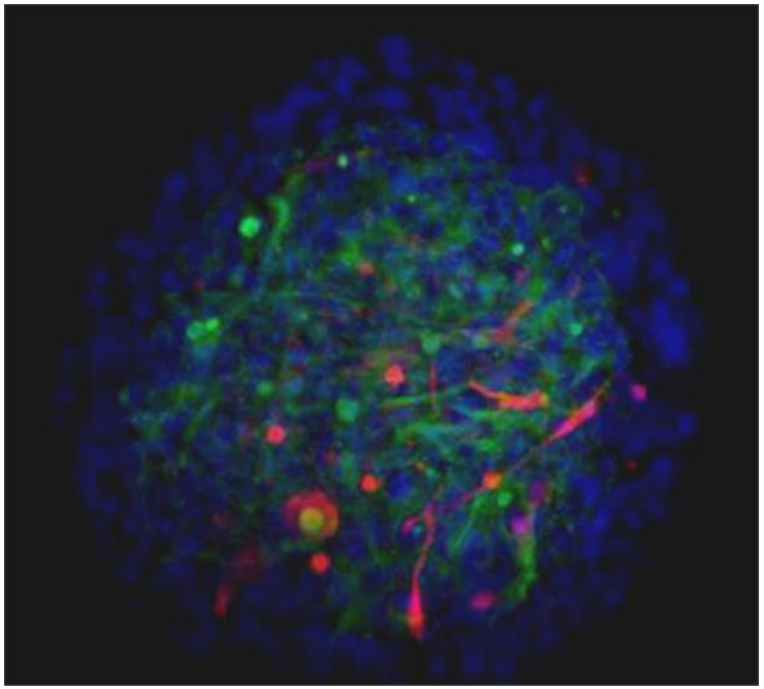




Confocal imaging of MT structure

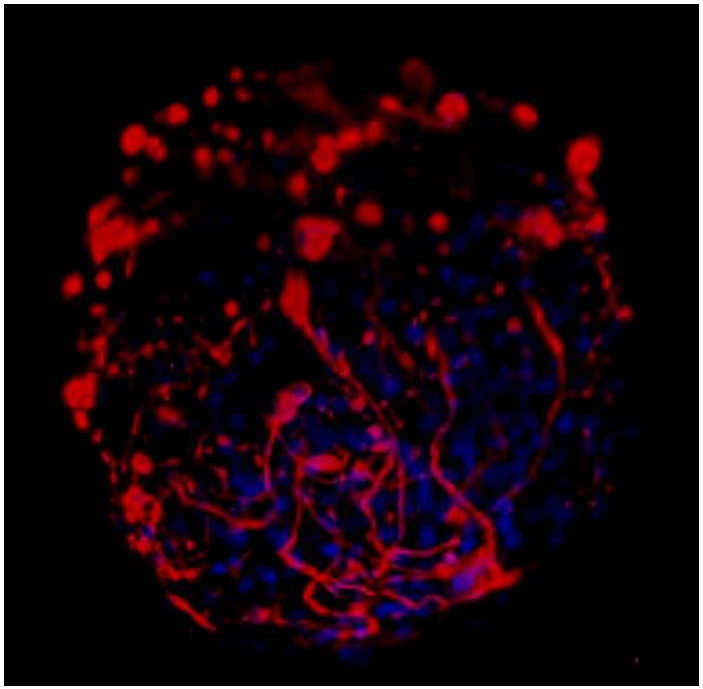
7000 cells/ MT

Day 0



βIII –Tubulin (Neurons)
GFAP (Astrocyes)
Hoechst (Nucleus)

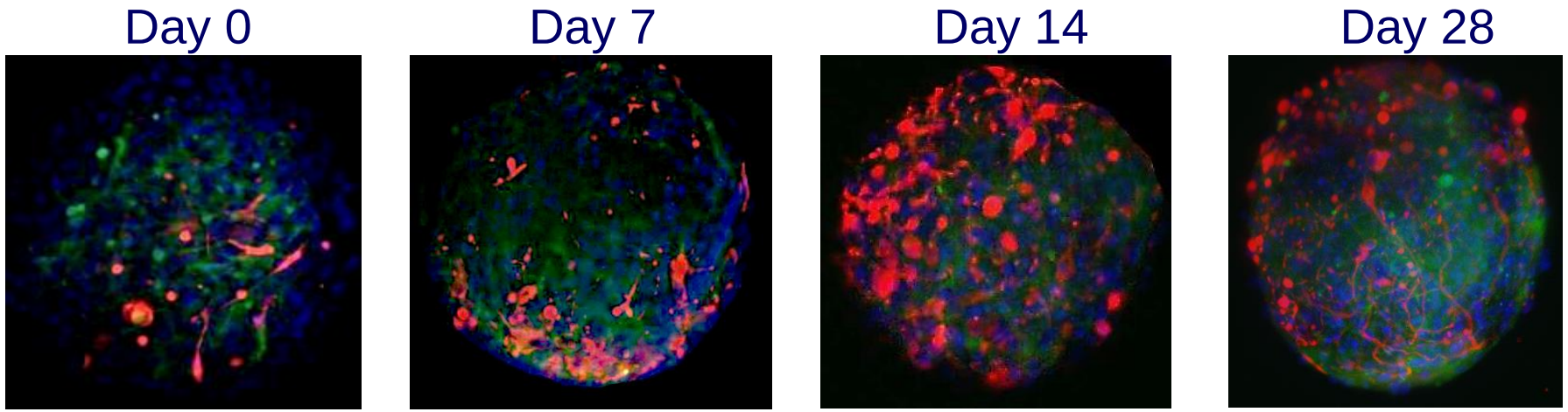
Day 28



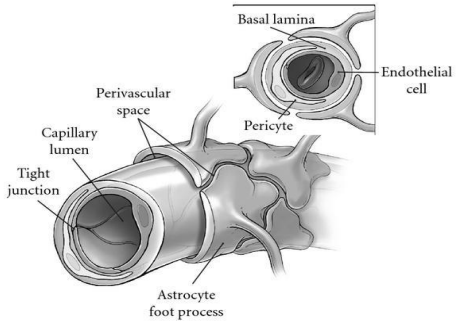
GFAP
Hoechst

Brain Microtissue Structure

βIII –Tubulin
GFAP
Hoechst

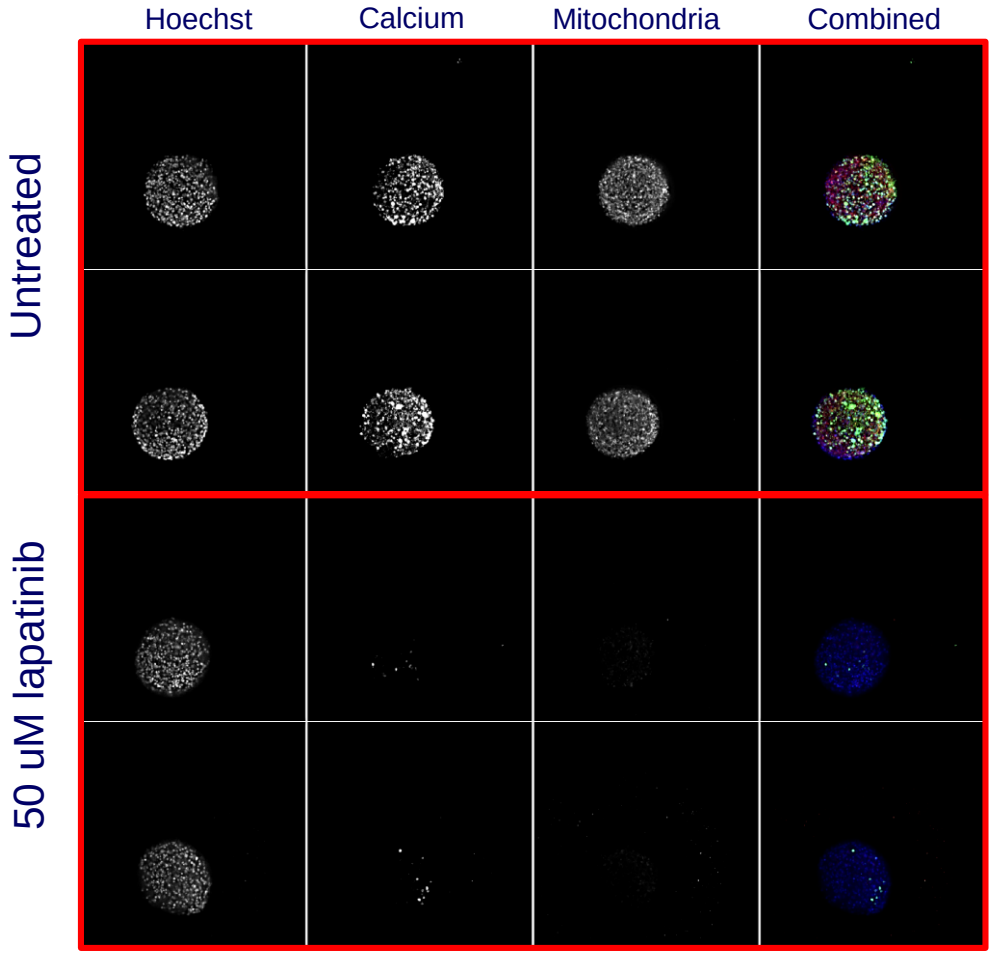


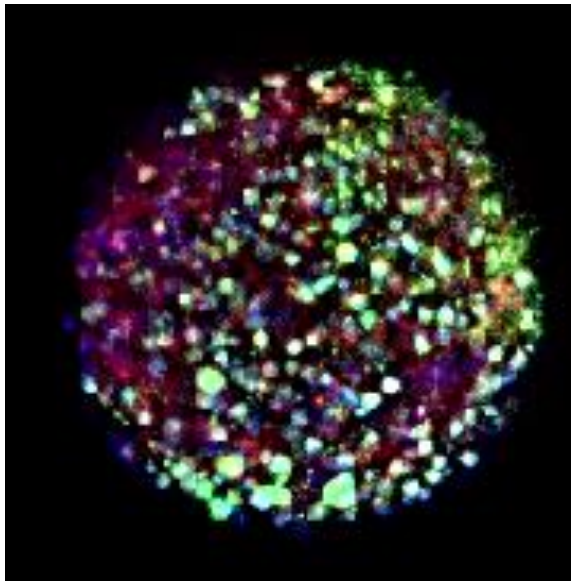
- Astrocyte cell number increases with microtissue age? and/or astrocytes migrate to the outer surface until day 14-28?
- At day 28 astrocytes show networking of processes throughout the microtissues
  - In the mature brain, there are 5 to 10 times as many astrocytes as there are neurons and they form spindle like processes of around 50 µm which extend throughout the CNS
  - Feet like processes at the media/vascular interface (in the absence of a vascular network)?



High content imaging assay development

Nuclear features, Calcium Content, and Mitochondria





Hoechst
Calcium
Mitochondria

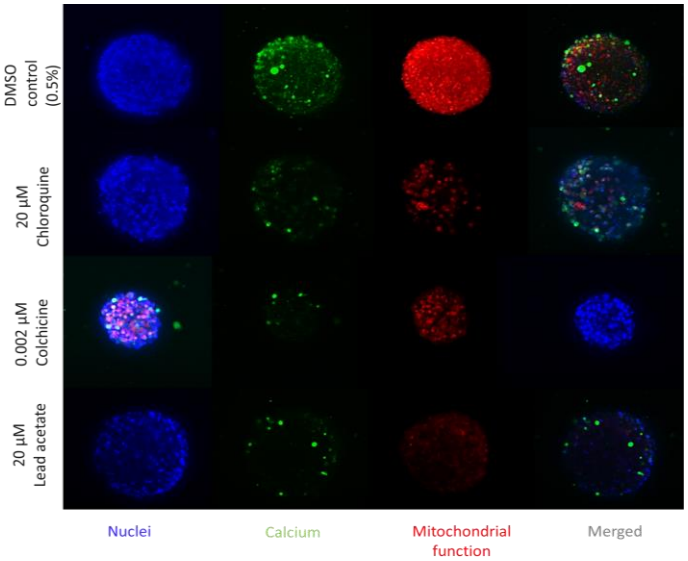
- 3 hour treatment with 50 µM lapatinib shows loss of calcium homeostasis and mitochondrial function
- 7000 size MT's handled well during HCS assay washes, positioned well in the ULD plate

Brain microtissue safety testing

14 day MTs vs 2 day MTs (maturity) & 72h vs 336h dosing

Compound	Expected outcome	C_{max} (µM)	100 x Cmax (µM)	2 day brain MT		14 day brain MT		Wilson *et al.*, 2014 (6 day)		
				72 hr	336 hr	72 hr	336 hr	SH-SY5Y	PC12	hN2
				MSM HCS and ATP				MSM neurite outgrowth HCS		
Amoxicillin	Non-toxic	0.87	87	NR	NR	NR	NR	NR	500uM	NR
Acetaminophen	Toxic, Non-neurotoxic	130	13000	8790	6450	13800	3230	1mM	NR	1mM
Chloroquine	Neurotoxic	1.62	162	52.5	13.1	28.2	13.1	100uM	100uM	100uM
Colchicine	Neurotoxic	0.015	1.5	0.0138	0.00748	0.00954	0.00488	100 nM	1uM	100 nM
Lead acetate	Neurotoxic	1.3	130	NR	>200	>200	23.1	NR	500uM	NR
Lidocaine	Neurotoxic	25.6	2560	>3000	1650	>3000	2120	500uM	500uM	1000uM
Paclitaxel	Neurotoxic	2	200	45.4	NR	16.7	<0.08	10uM	10uM	10uM
Tamoxifen	Neurotoxic	0.083	8.3	NR	6.37	9.45	5.45	1uM	1uM	NR
Vinblastine	Neurotoxic	0.24	24	0.0429	<0.008	<0.008	<0.008	1uM	1uM	100nM

Negative (>100 x Cmax)
Positive (<100 x Cmax)

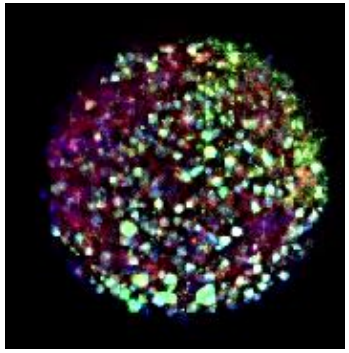


- Brain microtissues will be maintained for either 2 days or 2 weeks before test compound exposure
- Microtissues were treated to reference compounds for either 72 hours or 14 days
- Microtissue size, DNA structure, calcium homeostasis, mitochondrial function and ATP determined.
- Responses compared to HCI neurite out growth assay (Wilson *et al., 2014)*
- Positive response based on 100x C_{max}
- Small evaluation set however brain microtissues improve compound prediction (14 day with 336 hour exposure).

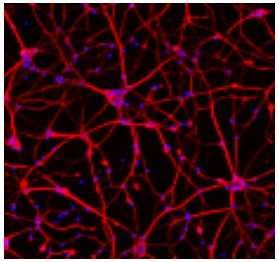
MEC = minimum effective concentration

Evaluating a combined approach

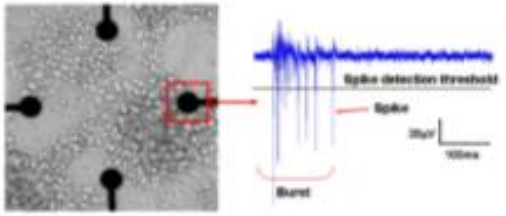
Structural/Cytotoxicity in Brain MTs



eCiphr®Neuro



- Primary rat cortical neurons
- Validation of iPS cell derived neurones ongoing

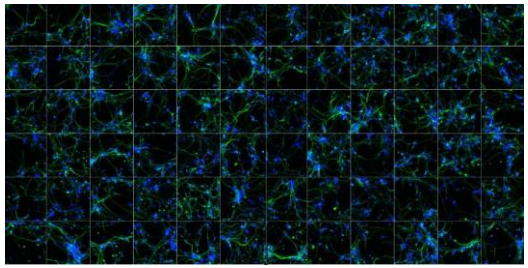


Axion Biosystem's Array

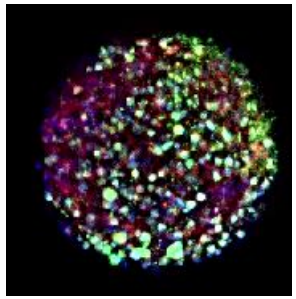
Real time cell activity

- Firing Rate
- Spike Burst Rate
- Interburst interval
- Burst Duration
- Spike Synchrony

Neurite outgrowth Assay (HCS)



Summary and next steps



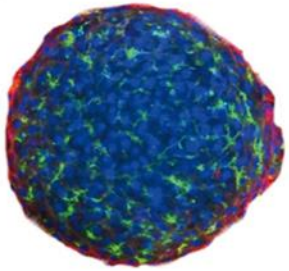
- Brain **MTs developed** 7000 cells/ well (ratio 1:1)
- Brain microtissues are **resistant to 0.5% DMSO** up to 28 days
- GFAP (astrocyte marker) and βIII-tubulin (neurone marker) immunofluorescence show an **increase in astrocyte number on the microtissue periphery** suggesting astrocyte proliferation and/or migration.
- Microtissues amenable to **High Content Imaging** Techniques
- Brain microtissues slight improvement compared with neurite outgrowth assay (Wilson *et al* 2014), limited data set.
- 336hr (**chronic exposure**) more sensitive
- Complements **other approaches** such as function ePhys assay (eCiphr®Neuro) and neurite outgrowth assay, further validation on going.
- Inclusion of **enodothelial cells**?
- Increased **validation/reference compound** data sets.

Any Questions?

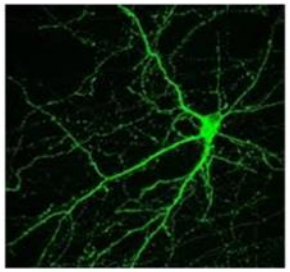
- Collaborative approach
- Custom assay development
- Ranking compounds based on individual and combined endpoints
- Compound databases and modelling
- Integrating exposure of dose (e.g. C_{max})
- Specific Client Report Formatting and Data Formats
- 3D cellular assays

High content screening

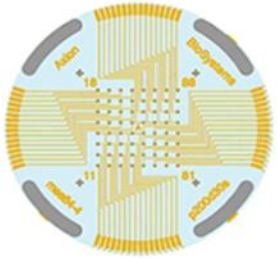
3D cellular models



Stem cell models



Microelectrode array



iQue screener

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