European ScreeningPort

Cellular Screening in 2D and 3D

–

Identification of cancer specific modulators

ELRIG.de, March 2013

Introduction

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- International FP7 program
- Aim is the identification of novel compounds with anti-cancer properties from marine fungi
- Work packages include: isolation, fermentation and screening of marine fungi extracts and compounds

Marine natural products

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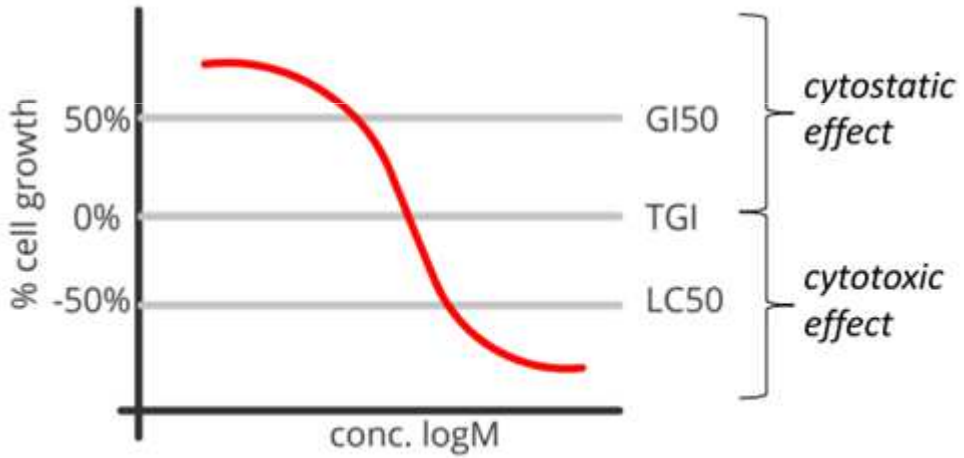
Source	Extracts	Organisms	No. hits	Hit Rate (%)
Mediterranean sponge fungi	754	206	78	10.3
Chilean macro-algal fungi	125	125	48	38.4
Indonesian coral fungi	331	105	47	16.5
Totals	1210	436	173	14.3

- Hypha Discoveries Stimulation Fermentation technology gave rise to 1210 crude extracts from 600 fungal strains
- Bioactivity guided fractionation was used to identify individual active fractions.
- 78 pure (> 80% by weight) active natural product compounds were then obtained following strain re-fermentation, induction, isolation and purification.
- Compound structures were elucidated using NMR

Assay principle

- Recording of cytotoxicity profiles of the fungal extracts and compounds
- Obtain GI50, TGI and LC50 in the whole NCI 60 panel



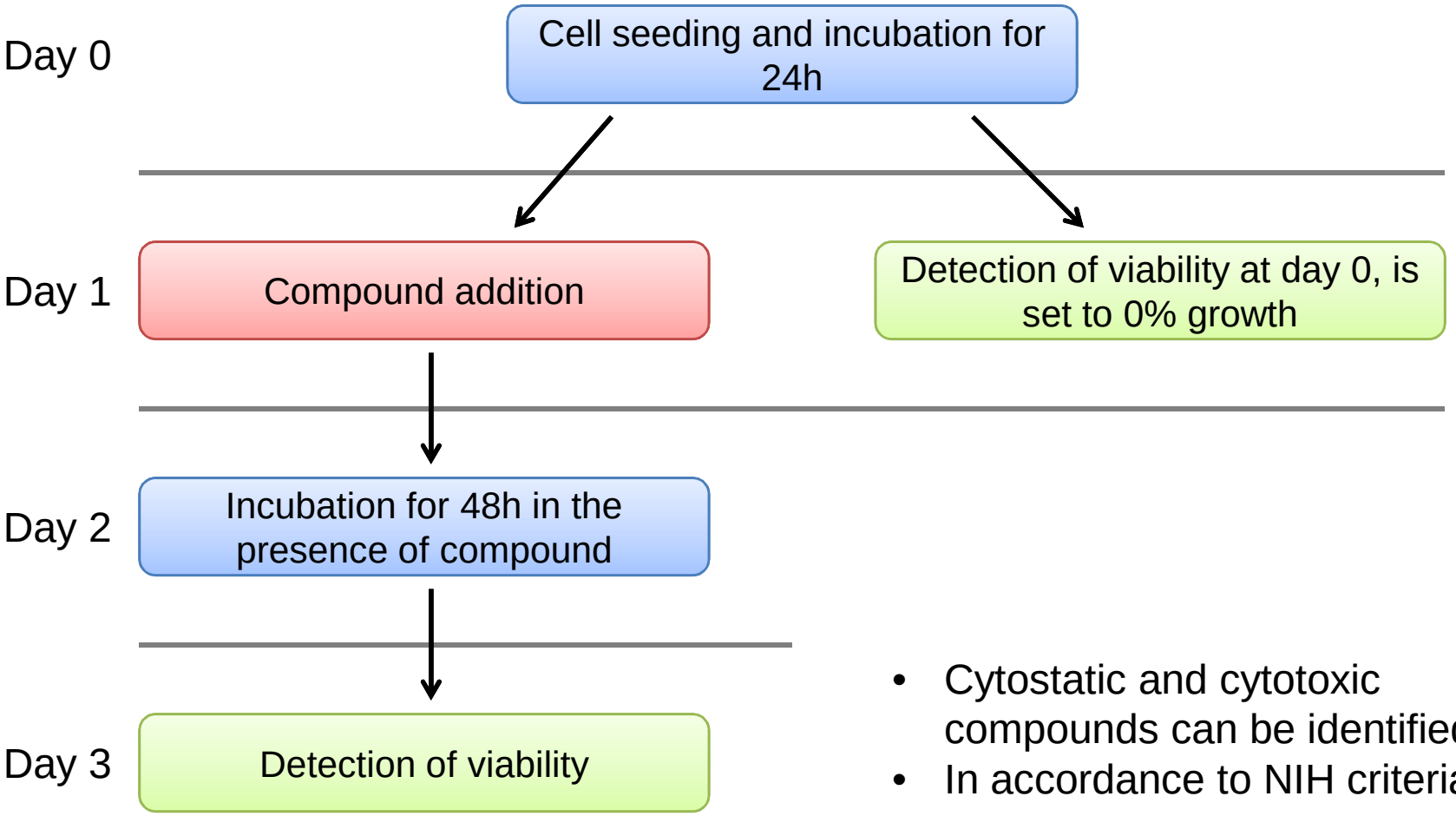
Concentration at:

- 50% cell growth = GI50
- 0% cell growth = TGI
- -50% cell growth = LC50

Tissue origin	No. of cell lines
Leukemia	6
Non-Small Cell Lung	9
Colon	7
CNS	6
Melanoma	9
Ovarian	7
Renal	8
Prostate	2
Breast	6

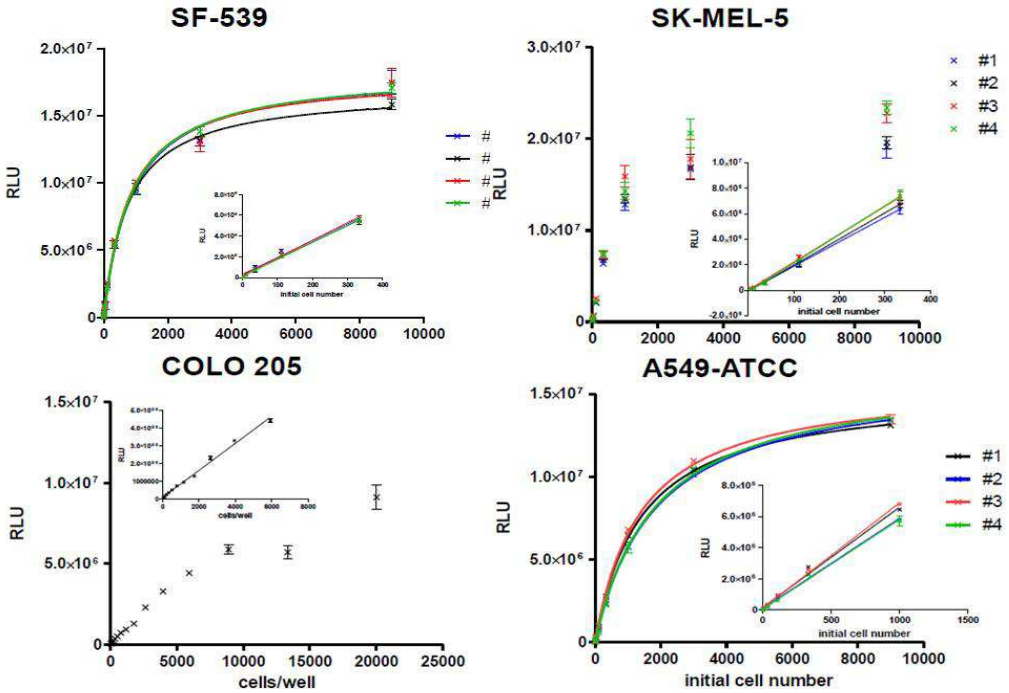
NCI 60 panel

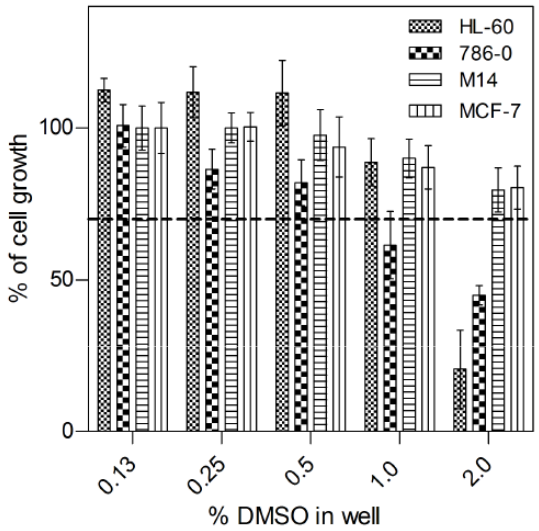
Measurement procedure



- Cytostatic and cytotoxic compounds can be identified
- In accordance to NIH criteria

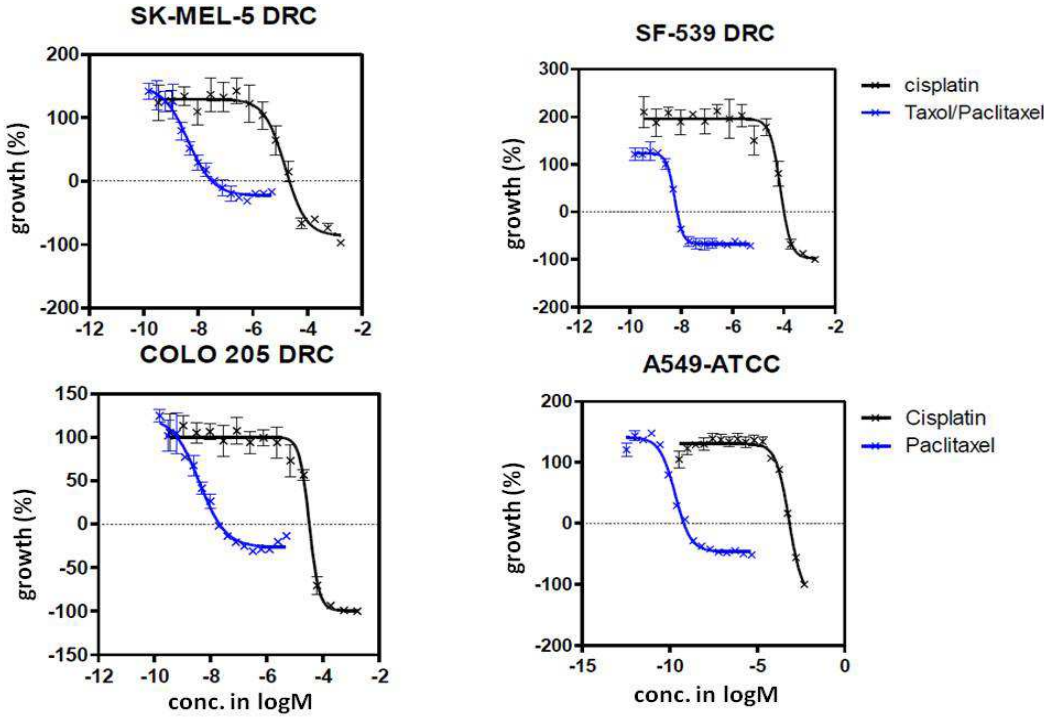
Assay development

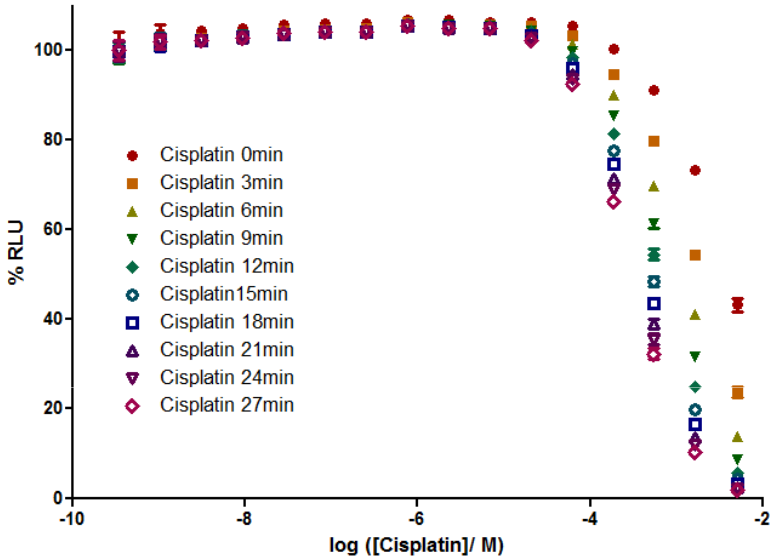




- RPMI1640 supplemented with 10% FCS and 2mM L-Glutamine
- doubling time between 18 and 80h
- NCI assay protocol includes 96 well plates, 200µl assay volume and a staining protocol
- current protocol includes 384 well plates, 25µl assay volume and ATP determination using CellTiter-Glo (Promega)

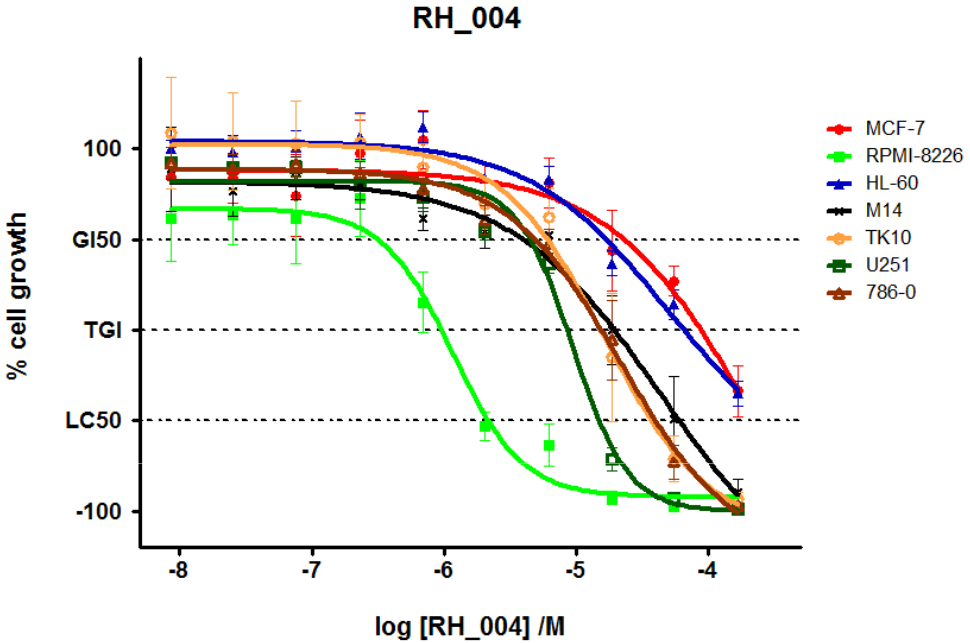
Assay development

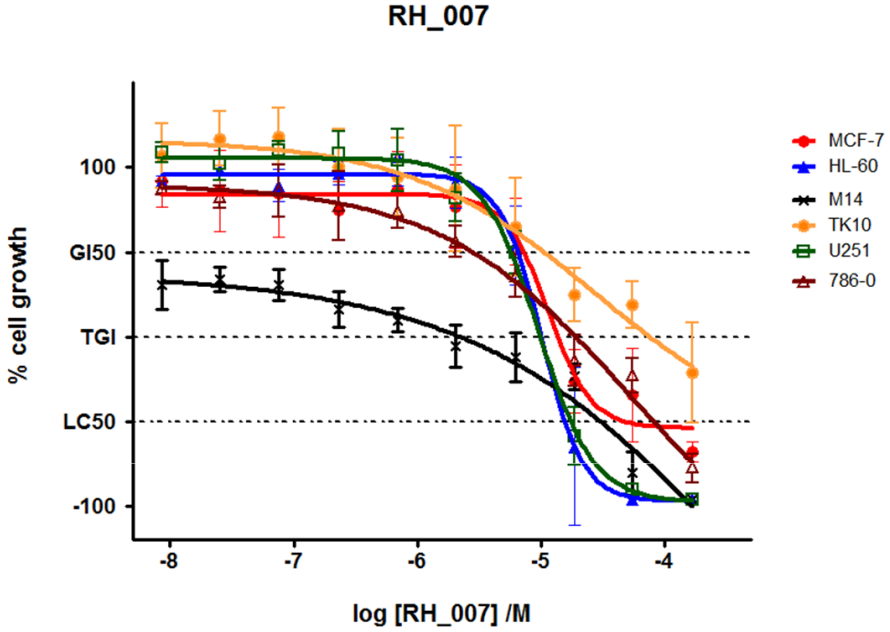




- Assay development includes: cell titration, DMSO tolerance and standard compounds (Cisplatin, Paclitaxel, 6-Mercaptopurine, Staurosporine)
- Luciferase counter assay has to be performed for all tested compounds
- Cisplatin inhibits luciferase at a concentration of 100µM

Physiological relevance of NCI60 panel – kinase inhibitors





- RH004 selective kinase inhibitor against VEGFR2
- RPMI-8226 cells do express VEGFR2

- RH007 selective kinase inhibitor against V600E BRAF-mutation
- only the M14 cell lines has this mutation

Physiological relevance of NCI60 panel – COMPARE algorithm

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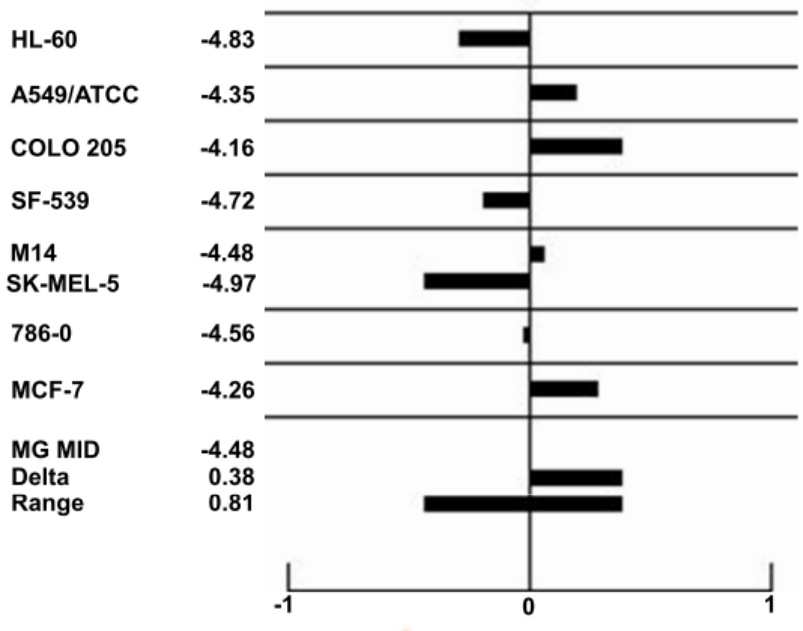
- Mean values and deviation from the means has to be calculated
- All data have to be transformed to be uploaded to the NCI COMPARE database
- Output is a likelihood analysis of similar compounds and involved pathways
- Data is shared within the consortia by an encrypted web portal



NCI mean graph analysis

Data

GI50, TGI, LC50

→

COMPARE online tool

NCI Mean graph

→

Output

Correlation of test compounds to database

→

MoA studies

Target directed studies

Physiological relevance of NCI60 panel – COMPARE algorithm

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Rank	Compound	Common cell lines	Description of mode of action
1	glycoxalic acid	30	natural exfoliant
2	asaley	25	alkylates and crosslinks DNA
3	methyl-CCNU	29	alkylates and crosslinks DNA
4	AZQ	28	alkylates and crosslinks DNA
5	cisplatin	30	crosslinks DNA

- Cisplatin data from 30 cell lines were used to perform a COMPARE analysis
- Cisplatin was found within the top 5
- 18 of the top25 alkylate or crosslink DNA

Automatisation of the cell culture processes

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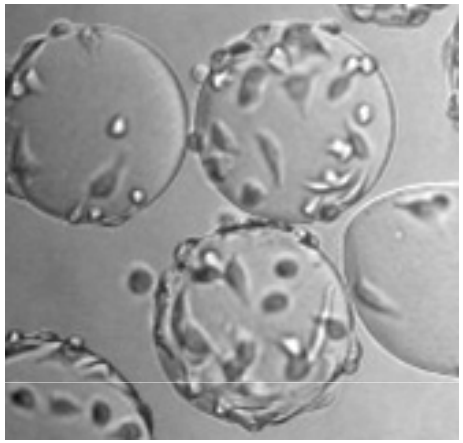
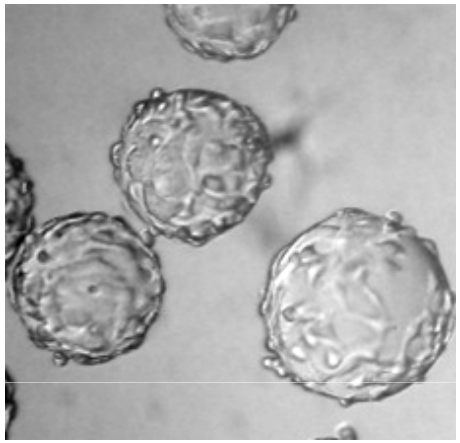


SF-593

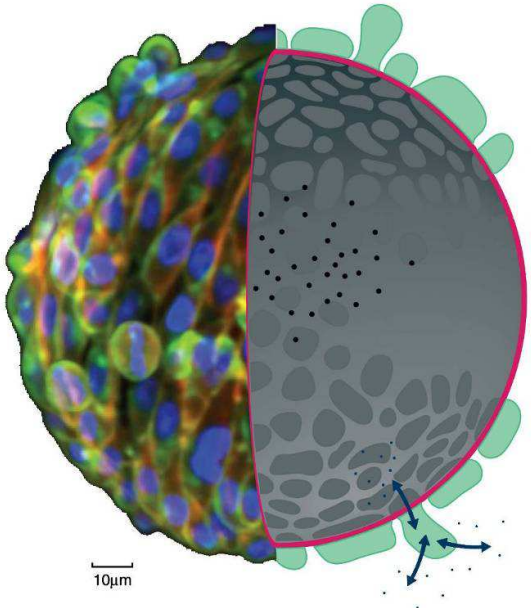


A549-ATCC

M14

786-0

The bead –
the core of the technology

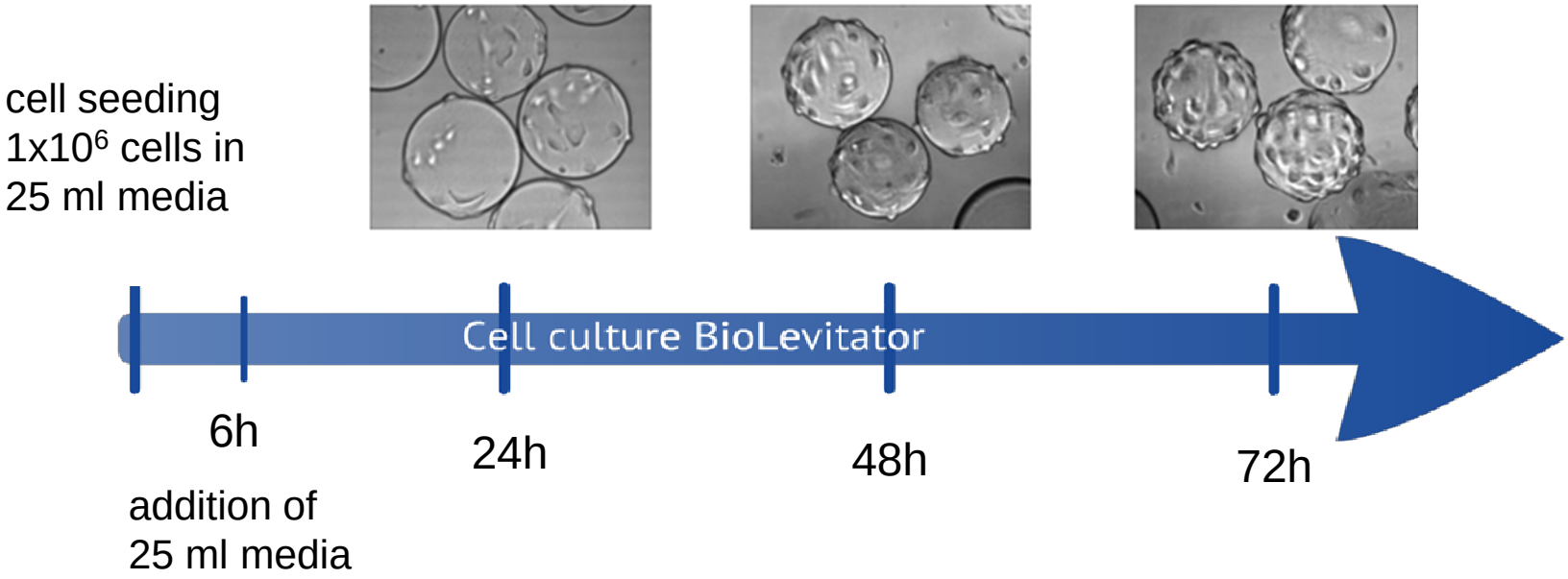


scheme of a bead

Brand name	Specification
GEM	Dextran beads with iron particles for magnetic separation
Cytodex 1	Dextran beads with positive-charged DEAE groups throughout matrix
Cytodex 3	Dextran beads coated with denatured porcine-skin collagen bound to surface.

Cell line	Tissue type	Bead type
M14	Melanoma	Cytodex 3
786-0	Renal	GEM or Cytodex 1
A549/ATCC	Non-Small Cell Lung	Cytodex 1
SF-539	CNS	Cytodex 1

Optimisation – cell number



- Doubling times between 18 to 80 hours
- Bead coating is also critical for growth

Optimisation – counting

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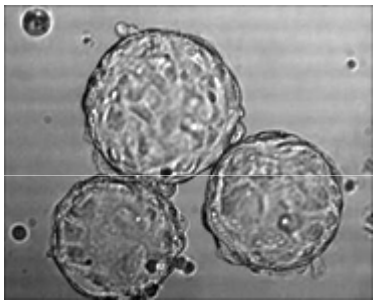
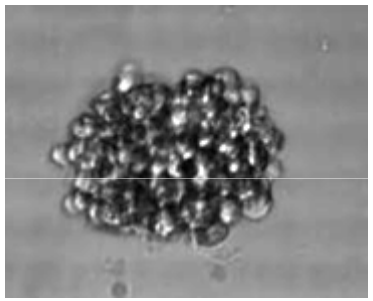
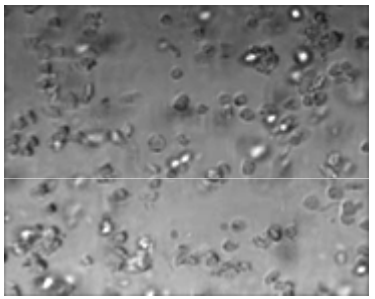
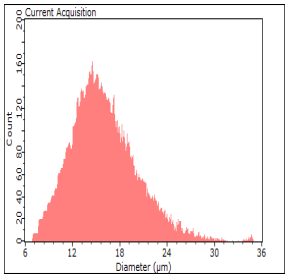
1 vol. 10% dextranase → 10 min → wash; add trypsin → 10 min → resuspend









Measurement by Millipore Scepter

- Direct addition of trypsin to the beads not possible
- Dextranase has to be removed before trypsin addition

Optimisation – known drugs

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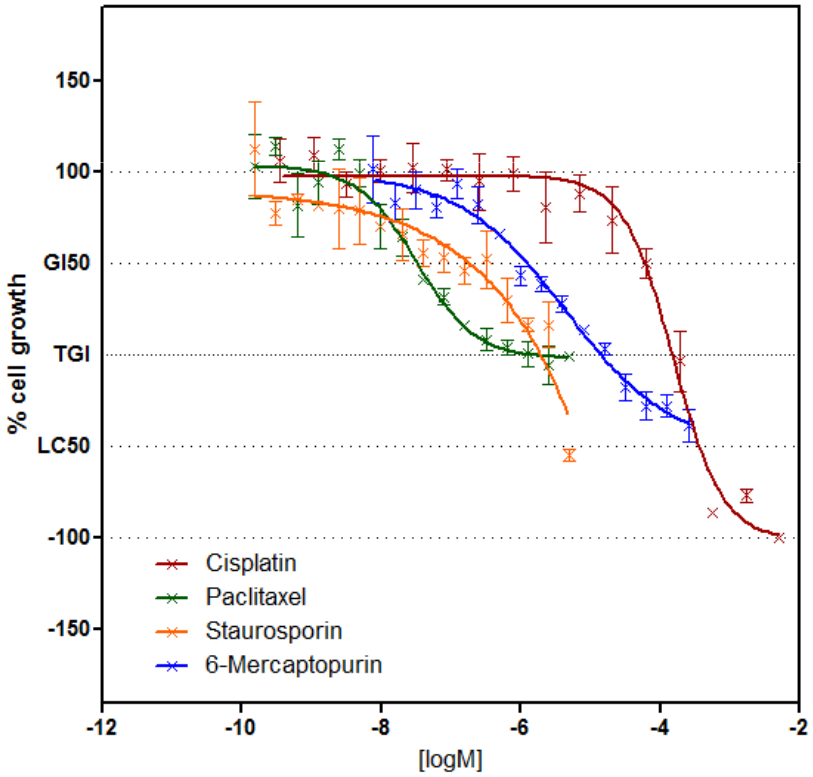
- 4 known compounds used for comparison of 2D and 3D
- Reference compounds for HTS screening



Data from 3D experiment using A549-ATCC

			GI50	TGI	LC50
Cisplatin	M14	2D	10	15.00	20
		3D	22	30.81	42
	A549-ATCC	2D	31	42.85	60
		3D	13	20.45	34
Paclitacel	M14	2D	0.2	0.4	0.8
		3D	NA	0.7	2
	A549-ATCC	2D	0.06	0.1	NA
		3D	NA	0.3	0.9

Fold difference

- 1 – 1.5
- 1.5 – 2.5
- > 2.5

Screening in 2D and 3D

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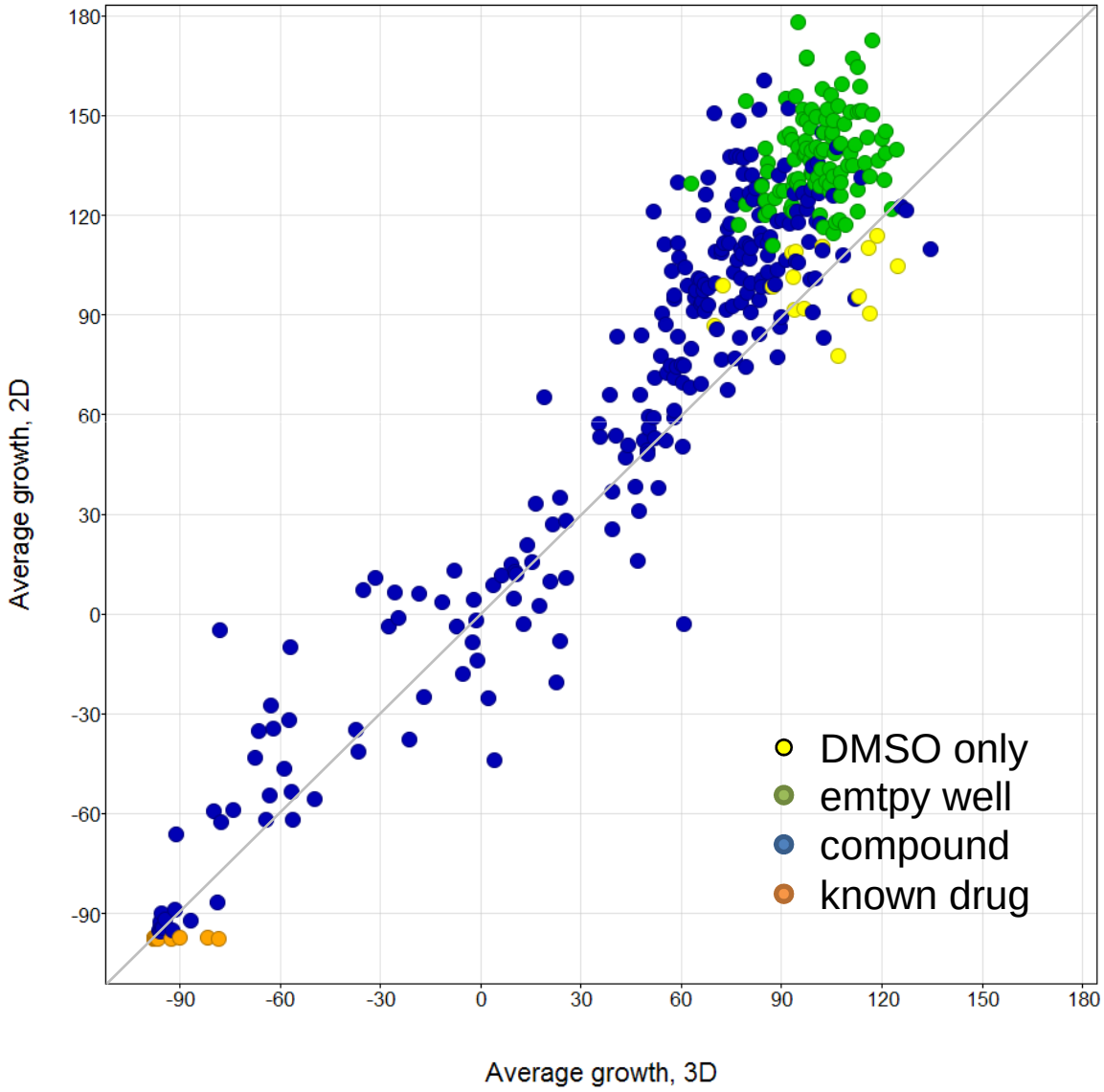
- 241 compounds of marine origin were screened in 2D and 3D
- $R^2 = 0.95$
- 50 µM compound concentration, single point
- Known drug: 5 mM cisplatin



Dose-Response curves

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- 56 dose response curves where recorded
- No significant differences are observed
- 3D data confirm 2D experiments

	3D – GI50	2D – GI50		3D – TGI	2D – TGI		3D – LC50	2D – LC50
compound 1	62.4	69.1		80.6	86.6		93.8	100.6
compound 2	74.5	97.4		87.2	98.7		96.8	99.7
compound 3	85.8	87.5		92.8	93.3		97.3	97.3
compound 4	28.4	22		59.5	52.9		107.5	99.6
compound 5	86.7	88.3		98	98.6		111.3	105.9
compound 6	84.9	69		92.8	86.8		97.7	101.5
compound 7	88.2	71.2		96.3	89.7		100.9	114.9
compound 8	64.2	56.7		84.8	80.6		100.6	99.8
compound 9	83.1	84.8		89.8	91.2		95.1	95.9
compound 10	89	85		97.9	95		103.4	101.4
compound 11	100.5	93		109.5	107.1		115.1	115.1
compound 12	97.2	94.4		101.9	105.8		105.1	113.2

Difference in percent

- 0 – 5
- 5 - 10
- > 10

All data in µM

Automatisation of the measurement processes

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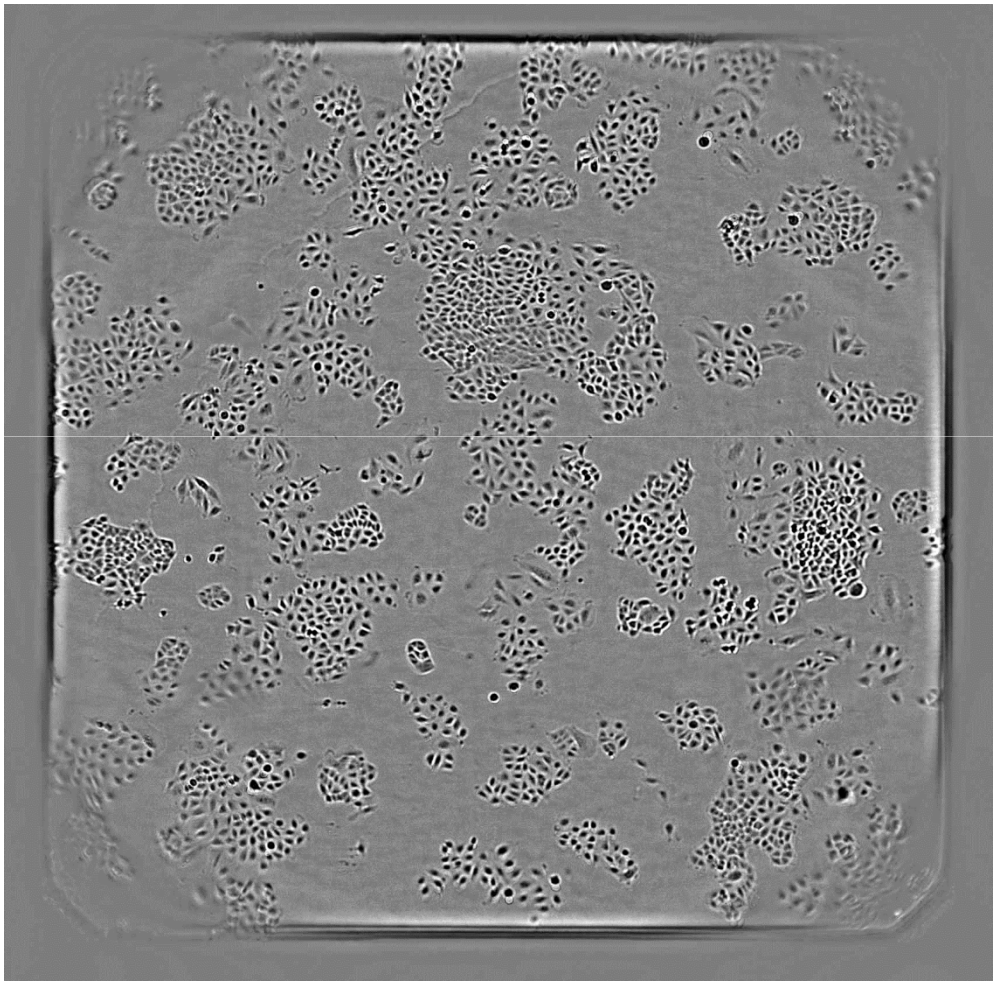
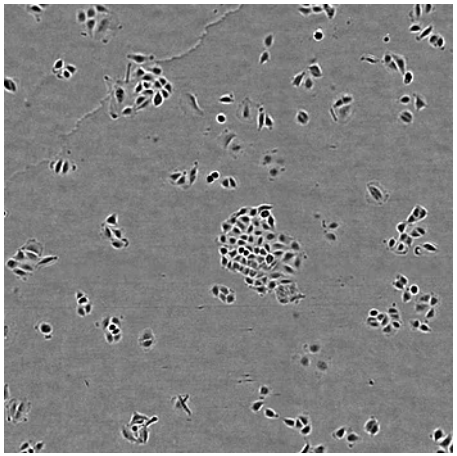
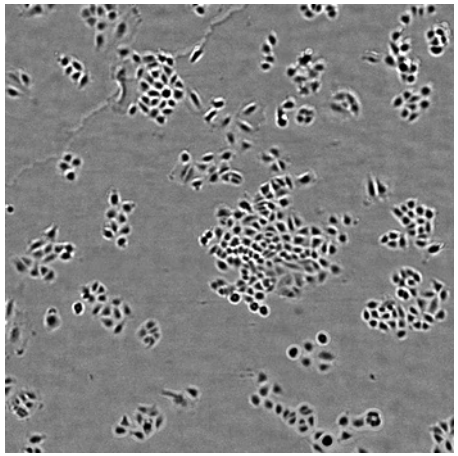


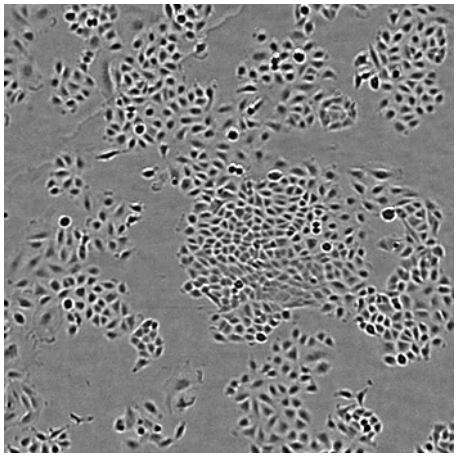
Confluence analysis using Cell Metric



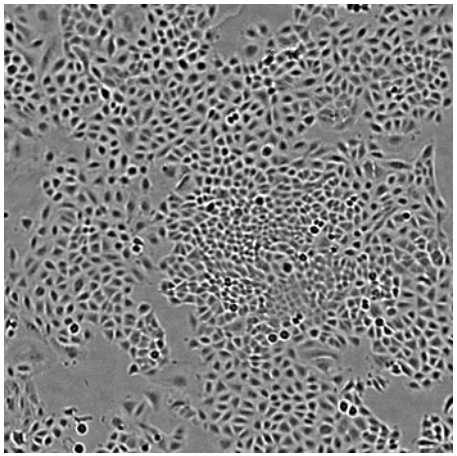
3h



24h

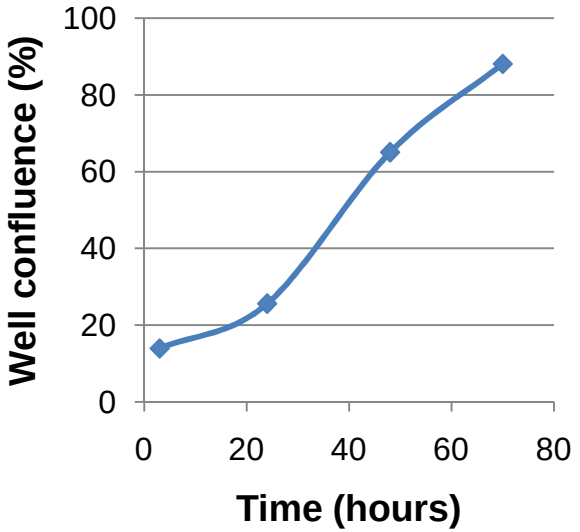


48h



70h

- A549-ATCC cell line shown
- analysis done by Cell Metric from Solentim
- non-invasive measurement enables the recurrent analysis of a single plate
- no additional day 0 plate necessary



Confluence and viability analysis

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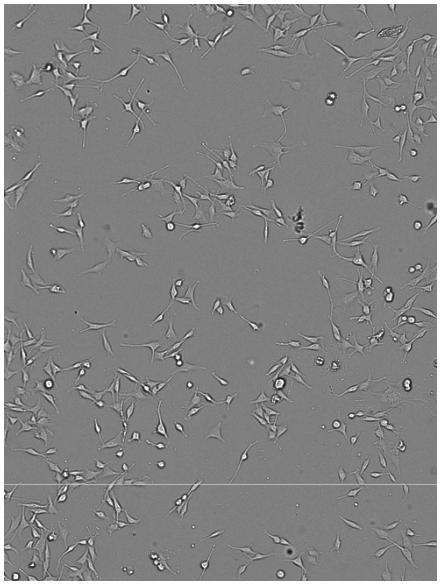
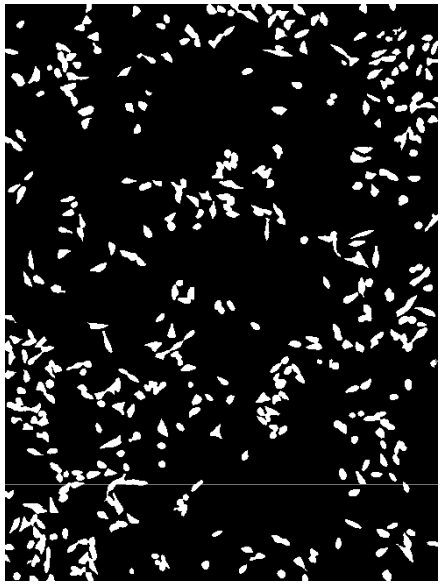
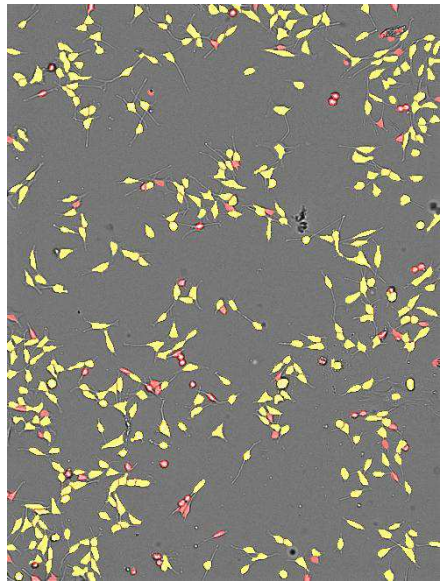


brightfield



whole cell analysis

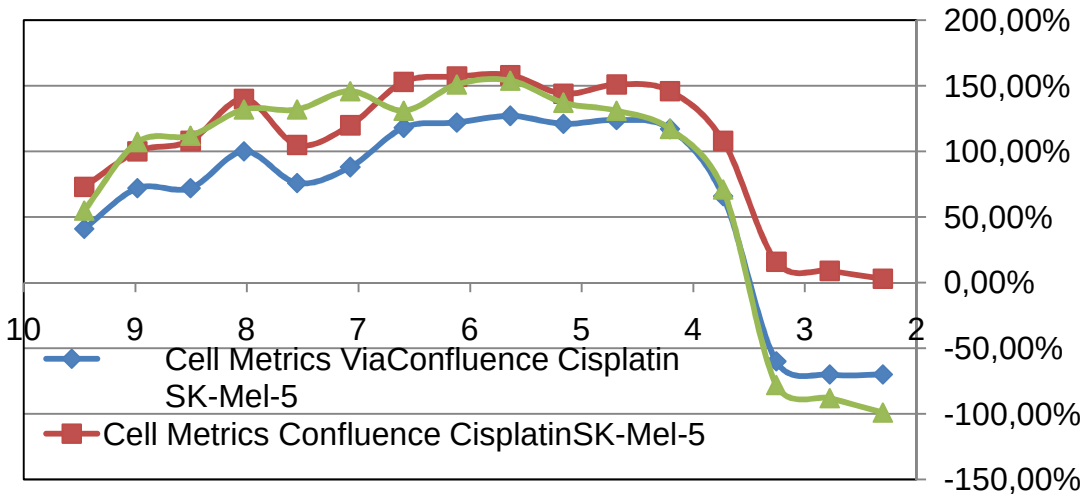
live/dead analysis



composite image

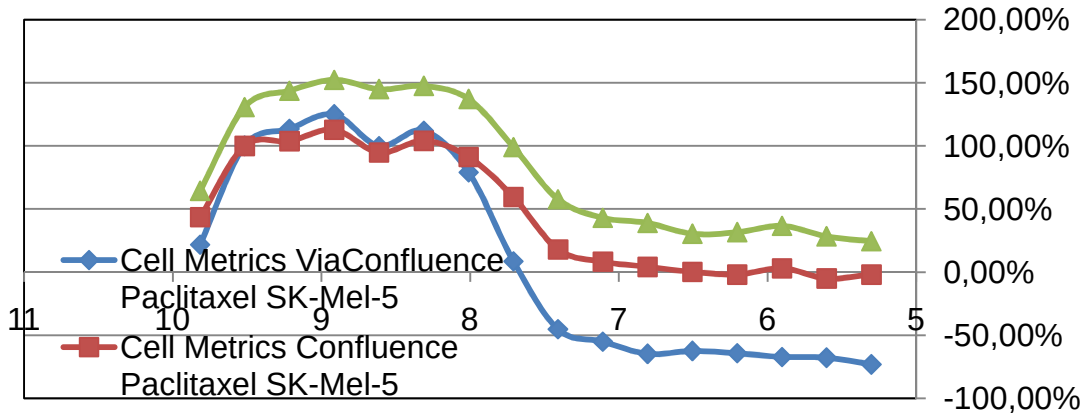
- Two measurements necessary for live/dead analysis
- Fast confluence analysis, image stacks to identify dead cells (diffraction index and roundness)
- 5 min per plate

Analysis of standard compounds



Cisplatin analysis

- SK-MEL5 cell line shown
- Viabibilty analysis performs superion over confluence analysis
- good comparison in the case Cisplatin
- discrepansies in the case of Paclitaxel/Taxol



Paclitaxel analysis

Partners

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This work was supported by the EU within the FP7 framework

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