IRBM

passionate about science

IRBM

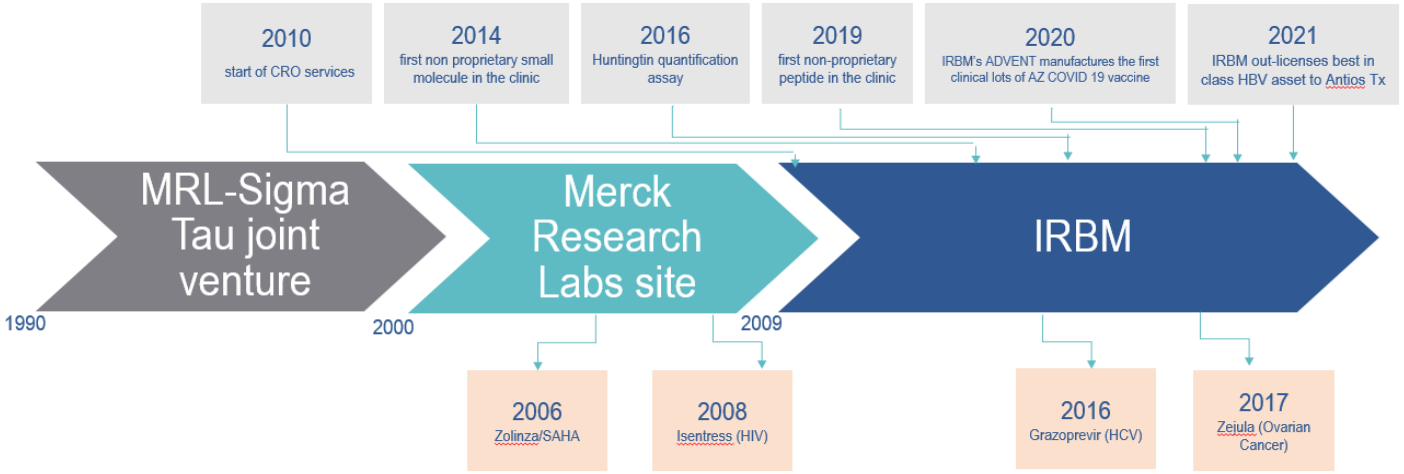
High Throughput Screening Overview

July 2023

Our history

- **70,527 m²** (759,146 sq.ft.) facility
- **Expertise in small molecule, peptide and antibody discovery**
- **State-of-the-art facilities all under one roof**
- **High science organisation with a large pharma heritage**

22 employees in 2010, ***200 plus employees today***



Our roots and focus

IRBM ROOTS

Roche
EMBL
NOVARTIS
HARVARD UNIVERSITY
gsk GlaxoSmithKline
AstraZeneca
SANOFI
AMGEN
Lilly
MERCK Be well
janssen Pharmaceutical Companies of Johnson & Johnson
THE UNIVERSITY OF TEXAS MD Anderson Cancer Center Making Cancer History
COLUMBIA UNIVERSITY
Karolinska Institutet

Drug hunters with a record of success

Scientific Leadership with a History of Success…

- Pivotal contributors to 4 currently marketed drugs
- Highly cited authors in hundreds of papers and patents
- 25 plus candidates in preclinical or clinical trials
- 20-25 average years of experience in the field

… and an Experienced Team of Researchers

- Over 180 researchers among the various units
- > 75% at a M.Sc. or Ph.D. level
- Significant experience in pharmaceutical and biotech R&D
- Extensive training in “big-pharma” problem-solving
- A cohesive team selected specifically for your project

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Fully integrated R&D capabilities: small molecules, peptides & mAbs

Molecular Biology	Biochemical Assay Development	Medicinal Chemistry	Peptide Chemistry	Target Engagement Assay
Biochemistry	Cell-based Assay Development	Computational Chemistry	mAb Discovery & Optimization	Biomarker Assay Development
Enzymology	320K Screening Library Collection	Virtual Screening	mAb Affinity Maturation	*In vivo* Pharmacology
Cell Biology	mAb Phage Library ($6x10^{10}$)	Compound Management	ADME-Tox	Preclinical Imaging
Protein Production and Purification	Peptide Phage Libraries	Analytical Chemistry	DMPK	Pathology, Histology & IHC
Cell Imaging	High-Throughput Screening	Biophysical Assays (SPR, BLI)	In vivo Pharmacology	Flow Cytometry
High-Content Screening	Phenotypic Screening	NMR Structural Biology	Immunoassays	

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External

Biochemical assays

Expertise with various target classes

- Enzymes e.g. kinases, phosphatases, metabolic enzymes, immuno-modulatory enzymes
- Protein receptors e.g. GPCRs, nuclear receptors
- Scaffolding proteins and bi- or multi-functional molecules

Assay development and compound characterization

- Determination of enzyme kinetics (Km, Vmax, etc.)
- Mode of action studies
- Evaluation of complex mechanisms using kinetic or end point assays (e.g. covalent inhibitors)
- Routine compound profiling
- HTS with IRBM compound collection and/or external libraries

Assay import

- Import client established assays, adapting the protocol to IRBM instrumentation and repeating key experiments to confirm assay conditions
  -e.g. Km of the substrate, enzyme linearity overtime, confirmation of protein concentrations, reagent addition and assay duration, test of selected molecules

Available readouts

- Luminescence, Absorbance, FI, FP, FRET, TR FRET, Alpha Technology

Cellular assays

Assay development and compound characterization

- Mechanistic studies with direct readout or via reporter systems (split-luciferase, HiBit)
- Functional studies: PTMs detection, pathway activation, transcriptional regulation, second messengers, proliferation/cytotoxicity, etc.
- Target engagement assays
- Routine compound profiling
- HTS with IRBM compound collection and external libraries

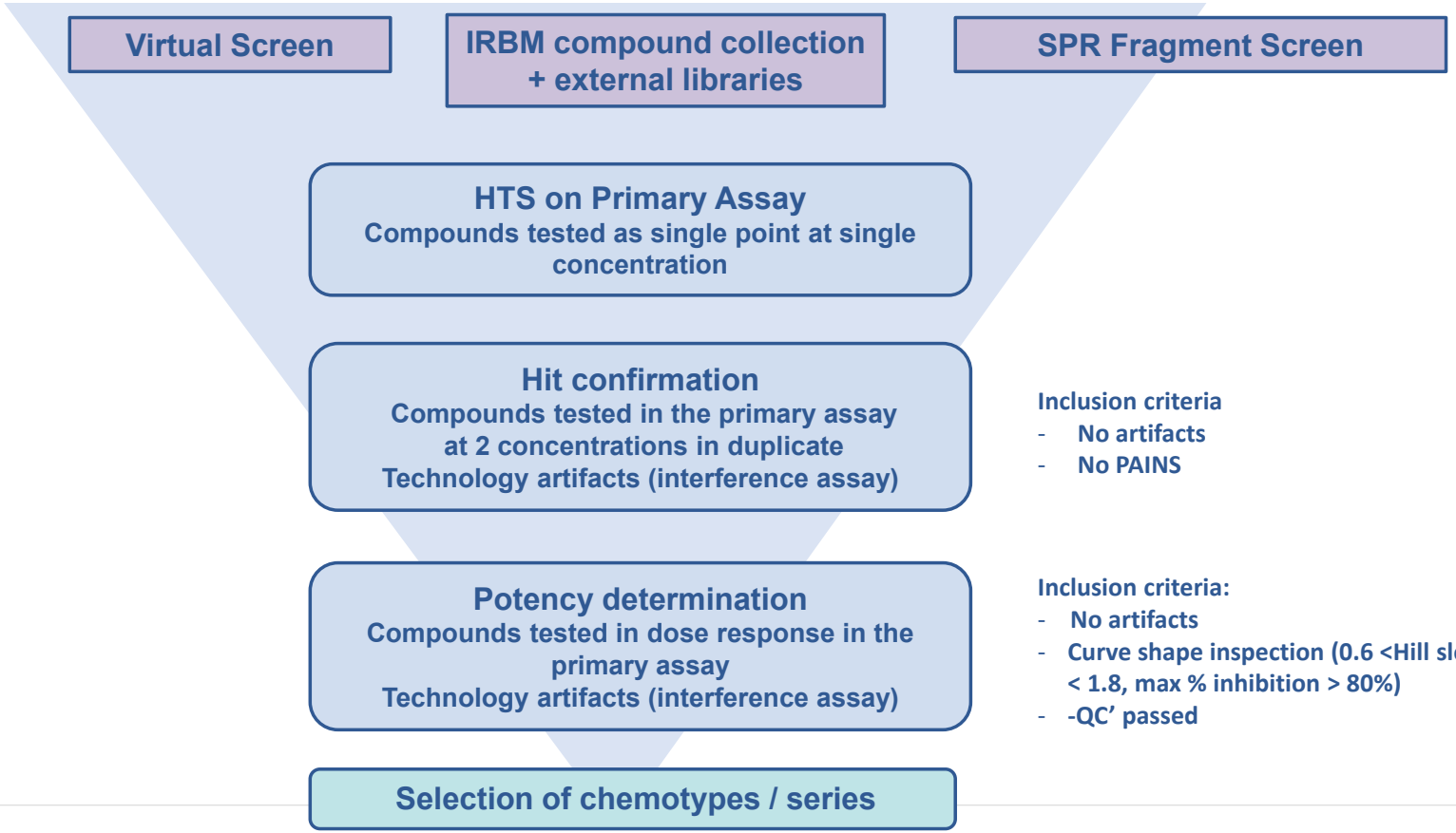
Assay import

- Readily import external assays as needed, adapting the protocol to IRBM instrumentation and repeating key experiments to confirm the assay conditions
  - e.g. cell density, reagent addition and assay duration, test of selected molecules

Available readouts

- Luminescence, Absorbance, FI, FP, FRET, TR FRET, Alpha Technology

Screening funnel for hit identification

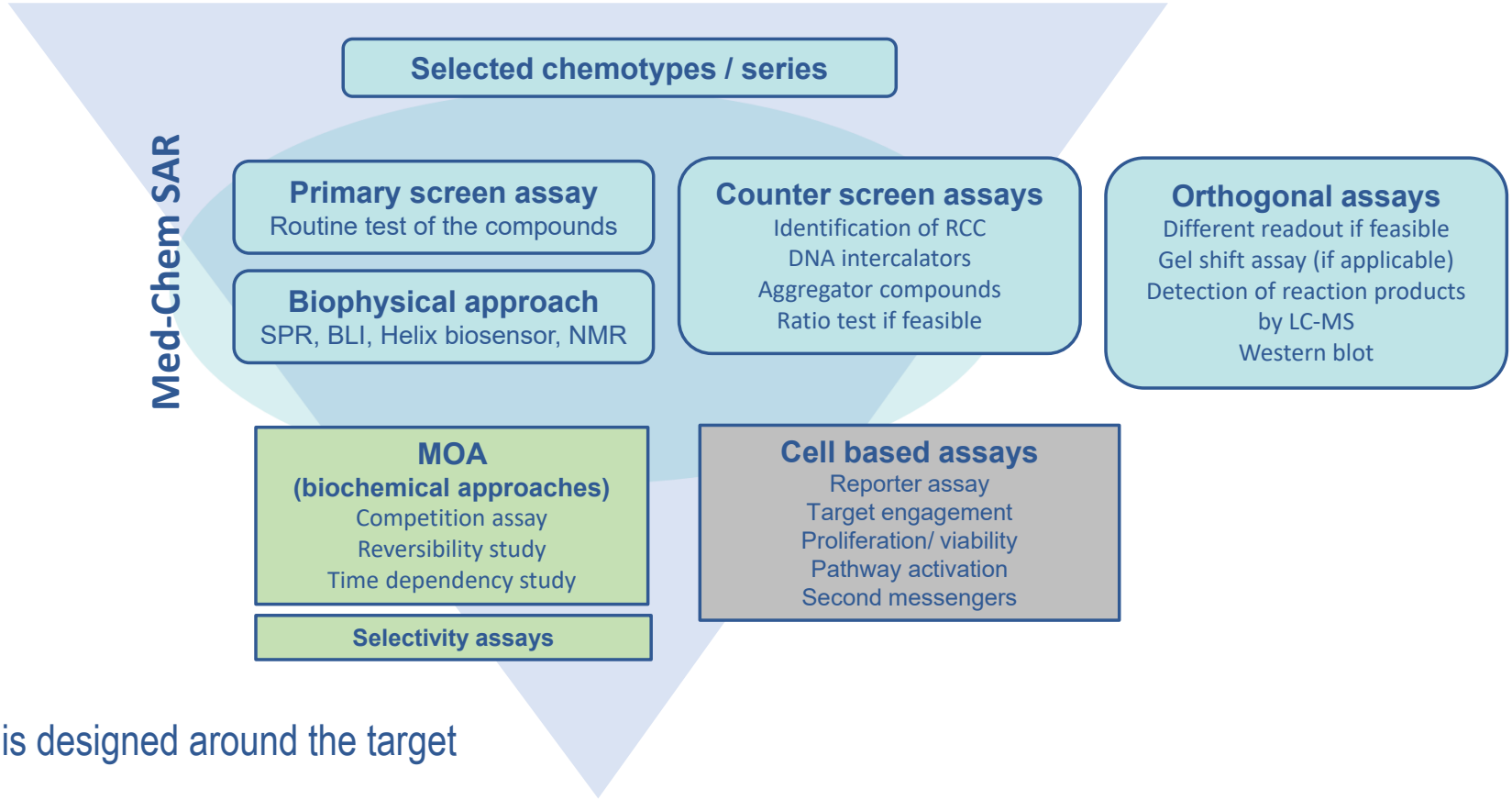


Inclusion criteria
- No artifacts
- No PAINS

Inclusion criteria:
- No artifacts
- Curve shape inspection (0.6 <Hill slope < 1.8, max % inhibition > 80%)
- -QC' passed

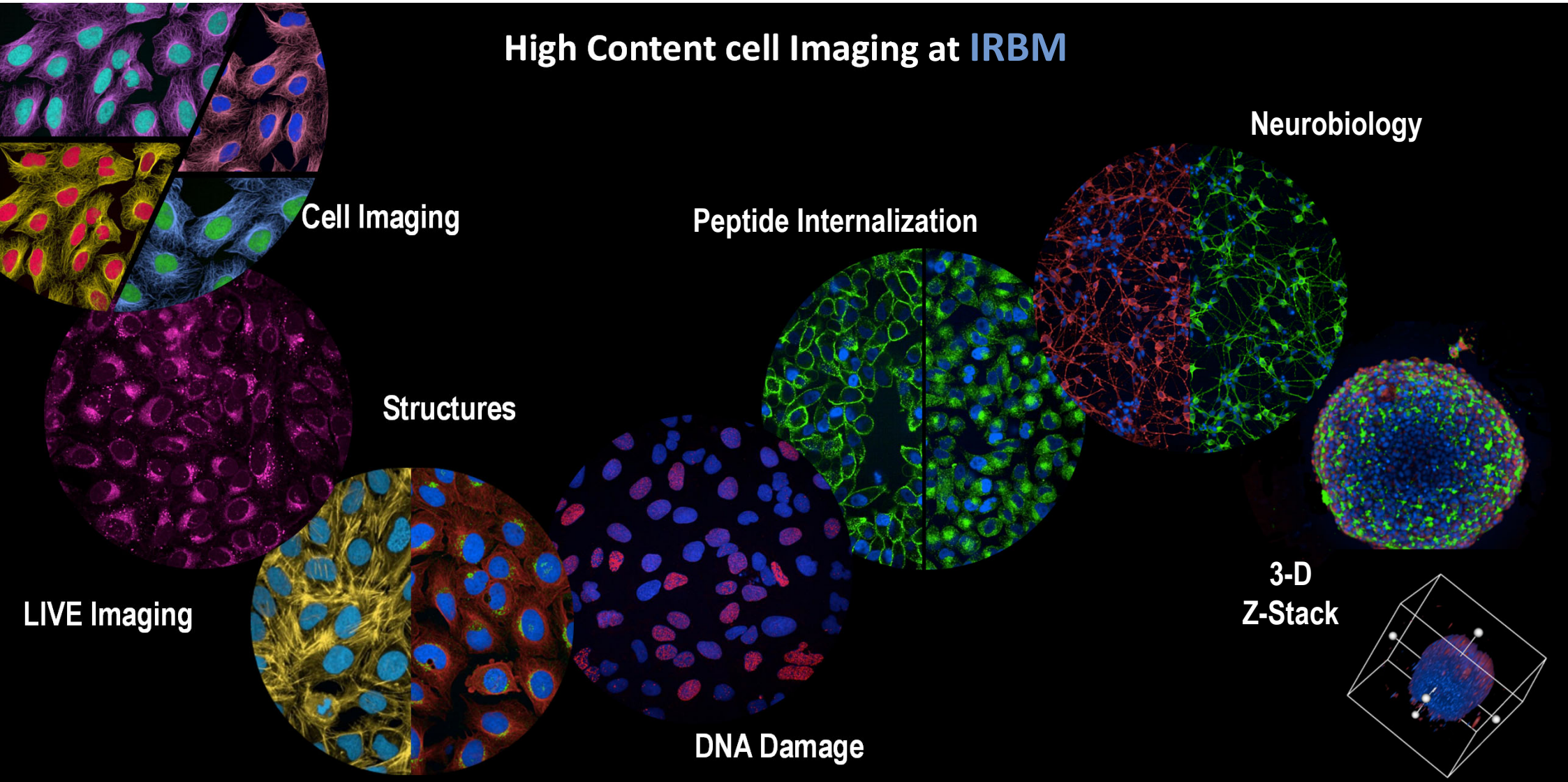
Screening funnel

Inclusion criteria
- No artifacts
- Evidence of the interaction between molecules and target



Tailored screening funnel is designed around the target

High Content cell Imaging at IRBM



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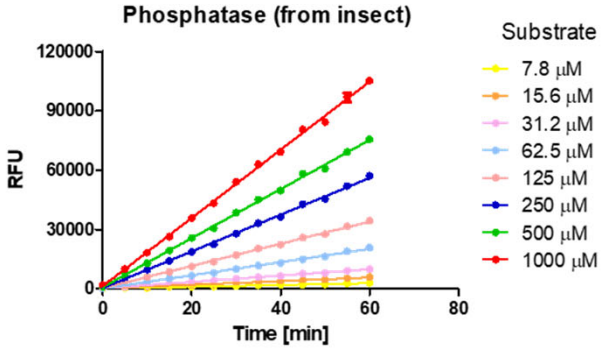
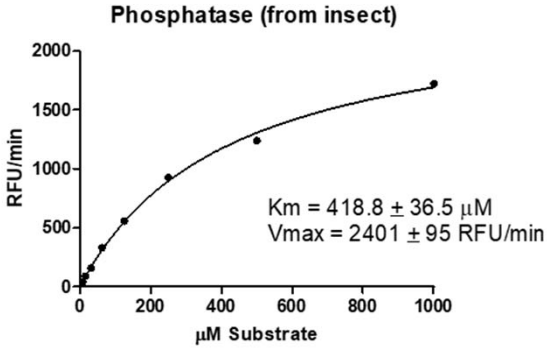
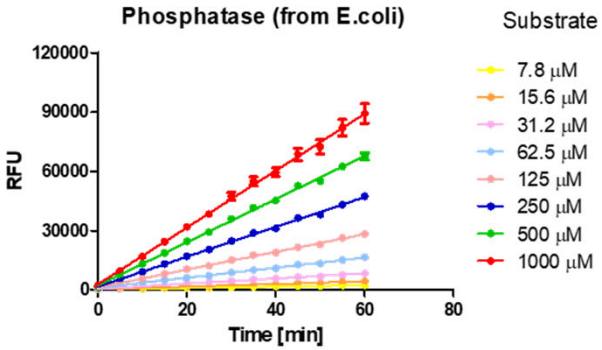
Examples

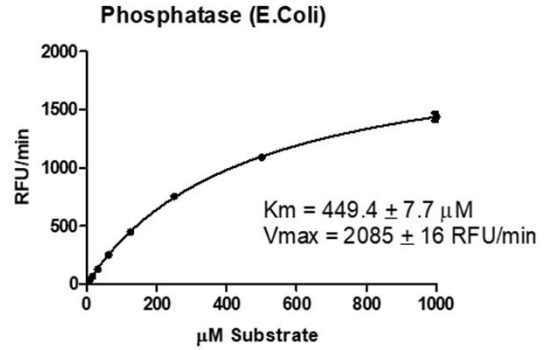
Enzyme characterization

- Enzyme: Phosphatase
  - Substrate: generic phospho-substrate
- Comparison of enzyme activity; enzyme produced in different systems (E.coli vs insect cells)
- Linearity of the reaction was measured at different substrate concentrations: slope values were used to calculate substrate K_m
- In the final assay condition, substrate concentration is used at K_m value



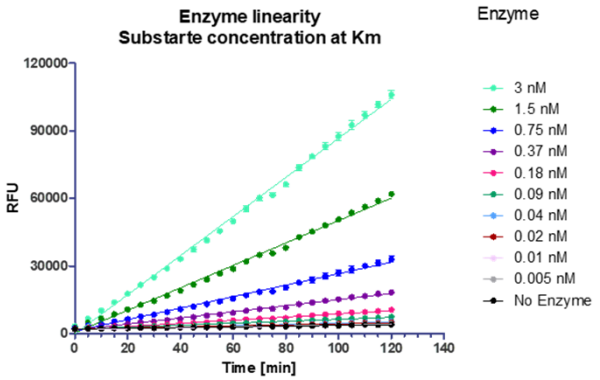


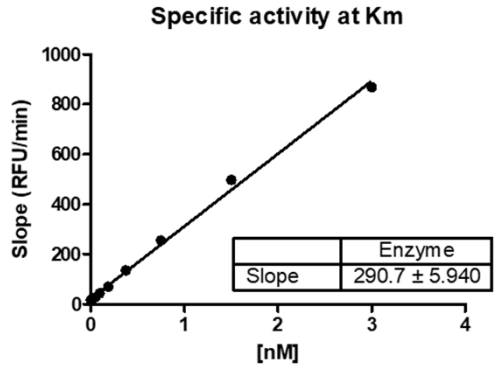




Specific activity at Km and identification of final assay conditions

- Enzyme: Phosphatase
  - Substrate: generic phospho-substrate
- Enzyme linearity using substrate concentration at Km
- Definition of specific activity
- In the final assay condition, substrate concentration is used at Km value; concentration of enzyme with S/B >3 and substrate conversion below 10%
- Assay was used to support the SAR
- S/B, Z' > 0.5, IC_{50} of reference standard compound were monitored

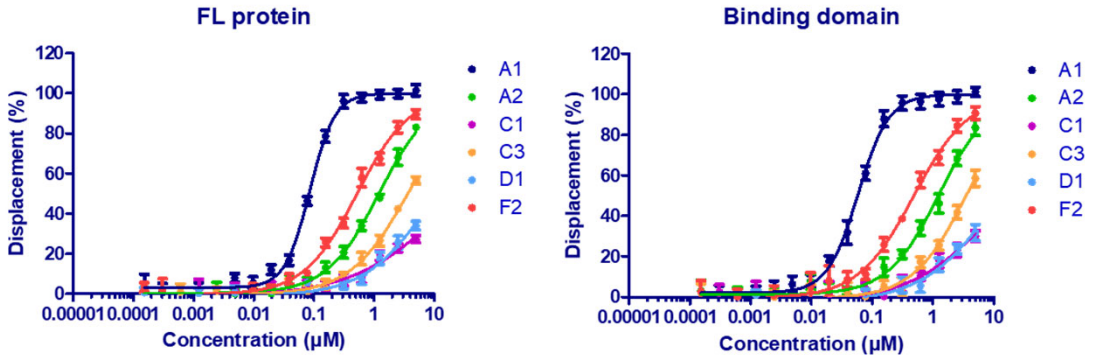




Enzyme characterization

Florescence Polarization (FP) technology

- Full length (FL) protein vs binding domain
- Effect of known inhibitors evaluated in both proteins
- FL protein was used to run HTS



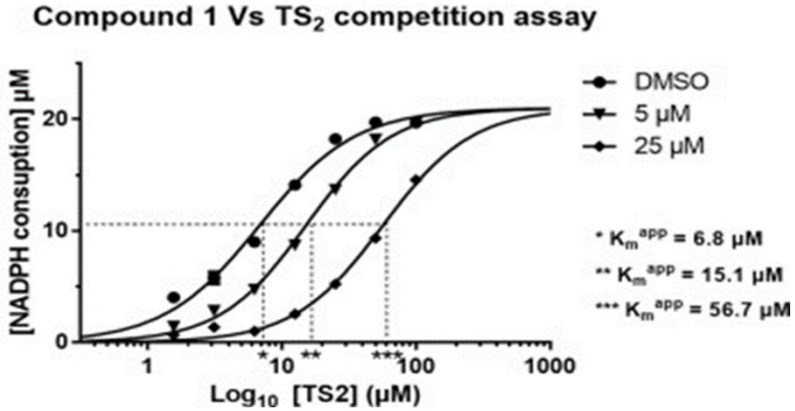
	FL protein		Binding domanin	
Compound	Hill slope	DC50 (µM)	Hill slope	DC50 (µM)
A1	2.10	0.086	1.72	0.059
A2	1.00	1.21	1.04	1.25
C1	25% D at top con		30% D at top con	
C3	0.90	3.62	0.99	3.46
D1	34 % D at top con		31 % D at top con	
F2	0.99	0.520	0.96	0.480

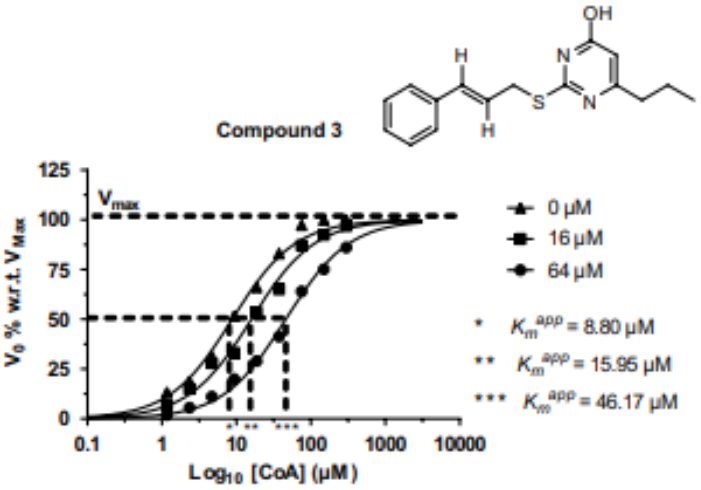
Compound Mechanism of Action (MOA)

Define type of inhibition

- Competitive
- Noncompetitive
- Uncompetitive

Example of competitor compounds were reported in the two graphs (as part of publications)





-Spiro-containing derivatives show antiparasitic activity against *Trypanosoma brucei* through inhibition of the trypanothione reductase enzyme, Turcano et al, 2020
**Identification by High-Throughput Screening of Pseudomonas Acyl–Coenzyme A Synthetase Inhibitors, Turcano et al. 2017

Reversibility study

Define type of inhibitor

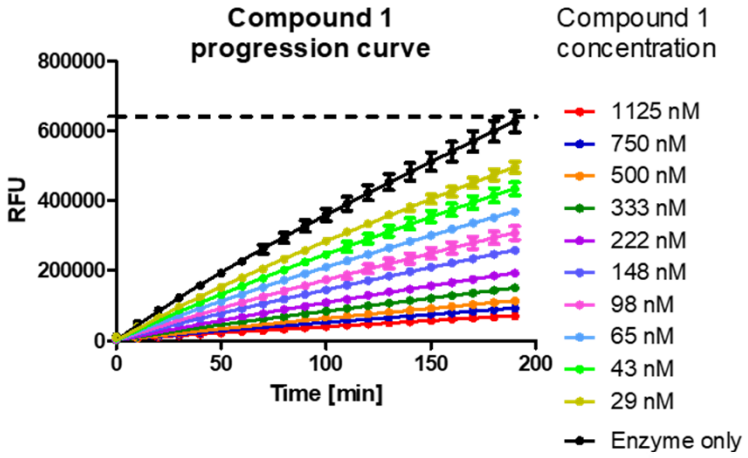
- Reversible
- Slow binding
- Covalent

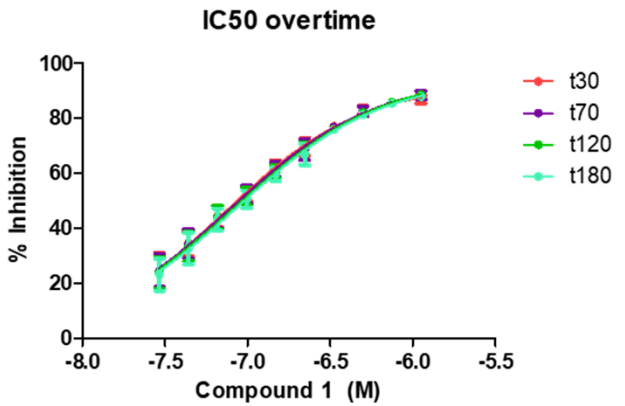
Progression curve and jump dilution using kinetic assay under specific conditions as needed

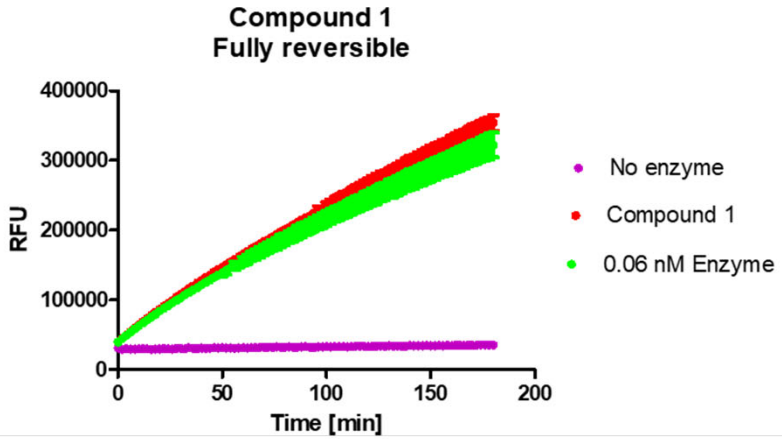
Intact mass spectrometry can be used to confirm binding for covalent compounds

Example of reversible compound

- Progression curve reported in the first graph
- IC_{50} values did not change over time
- Jump dilution confirmed reversibility





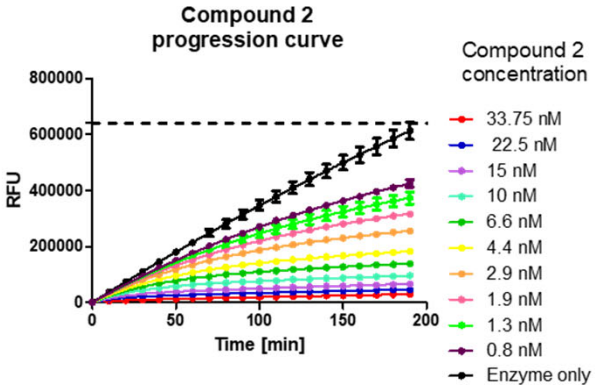


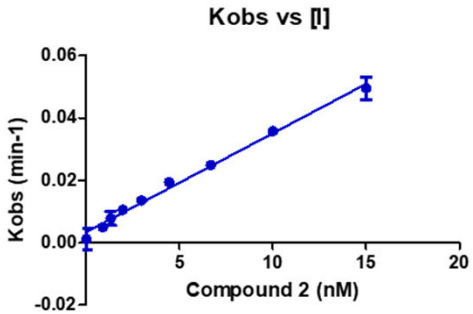
	IC50 nM	Slope
T30	74.67	1.04
T70	80.17	0.99
T120	85.09	0.97
T180	86.33	0.99

Quantifying the Interactions between Biomolecules: Guidelines for Assay Design and Data Analysis, Tonge, 2019
Enzymes: a practical introduction to structure, mechanism, and data analysis, Copeland, 2000
Evaluation of enzyme inhibitors in drug discovery: a guide for medicinal chemists and pharmacologists, Copeland, 2013

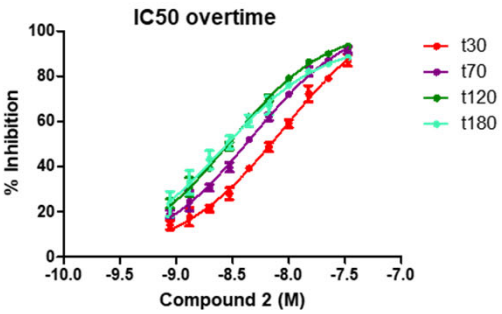
Example of slow binding compound

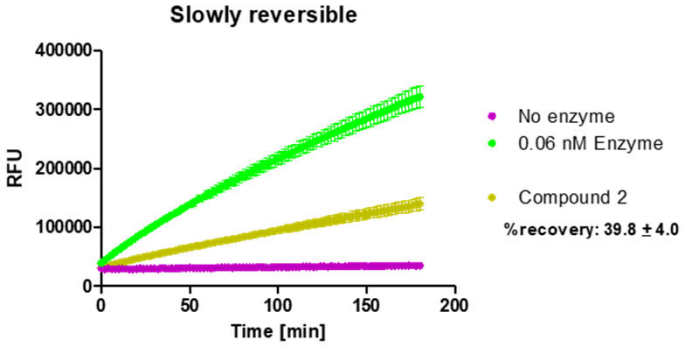
- Progression curve reported in the first graph. Compound 2 classified as slow binding with a one step binding mechanism
- IC_{50} values decreased over time
- Jump dilution confirms slow binding





	IC50 nM	Slope
T30	9.1	0.92
T70	4.7	0.97
T120	3.0	1.02
T180	2.6	0.98





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Cell based assays

Depending on the target, we can include different assays in the screening funnel

- Reporter assay
- Target engagement
- Proliferation/viability
- Pathway activation
- Detection of second messengers

Assays are usually performed in 384-well format as they are used to support the SAR in parallel with the biochemical assay

Multiple approaches for deeper analysis of MOA in cells

- High content imaging (Opera Phenix)
- Live cell analysis (Incucyte)
- Multiplexed flow cytometry analysis
- Gene target expression levels (WB, qRT-PCR, and ddPCR)
- Genome editing technologies (CRISPR/Cas9)

Target engagement with NanoBRET technology from Promega

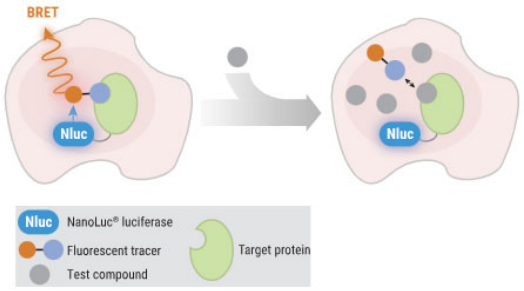
Measured effect of selected compounds on a panel of cell lines in

- Cell proliferation assays
- pERK assays

The selected compounds were also tested in the NanoBRET assays to obtain a deeper understanding of their MOA

6 Target Engagement assays were imported from Promega and validated

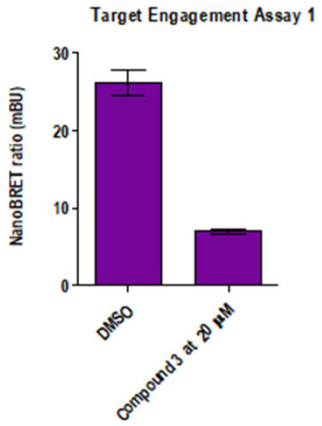
- RAS Pathway Drug Discovery (https://ita.promega.com/applications/small-molecule-drug-discovery/ras-oncogene/)

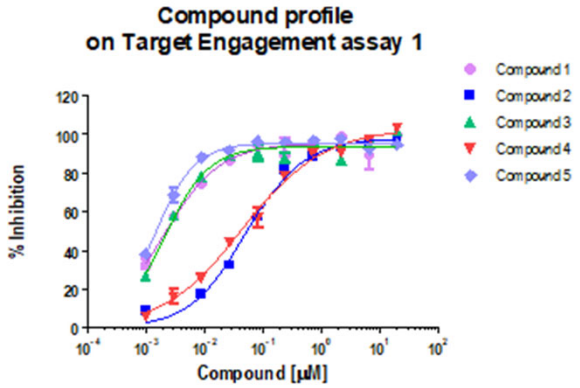


Principle of the NanoBRET™ Target Engagement Assay.

Example of target engagement study results

- At optimized concentration of tracer (1 µM), results were in agreement with Promega's data
  - S/B = 3.6; Z' = 0.7
- A small set of inhibitors were used to validate the assay; data obtained were again in agreement with Promega data
- Same approach was used for the other five assays
  - S/B, Z'>0.5 and EC_{50} value of standard compound in agreement with Promega data
- Assays were used to profile a set of inhibitors





	EC50 nM IRBM	EC50 nM Promega
Compound 1	2#	6.2**
Compound 2	53	55.4* / 55.0**
Compound 3	2.1#	5.9* / 4.7**
Compound 4	47	12.0**
Compound 5	1.4#	5.2* / 5.2**

Extrapolated values

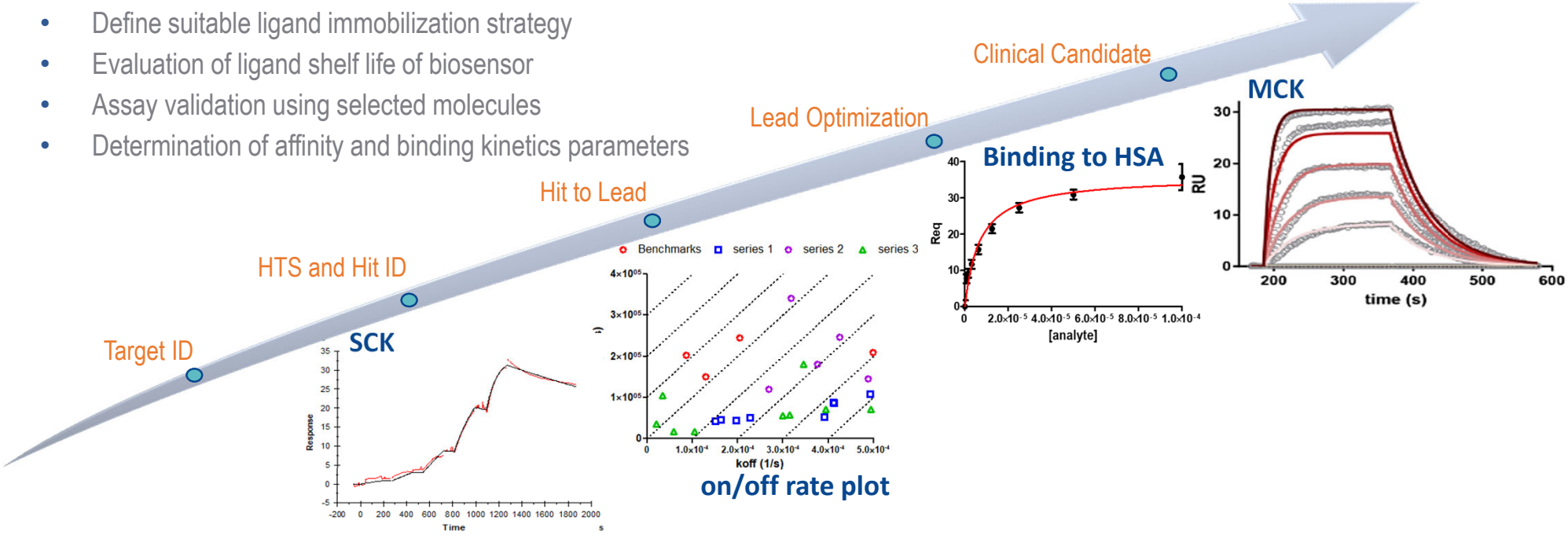
* 2h-cmpd incubation
** 24h-cmpd incubation

Biophysical approaches

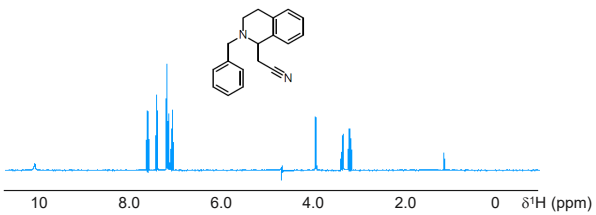
Biophysical assays are used to determine accurate binding kinetics (on/off rates, affinities) and binding specificity of small molecules or peptides to their target protein in any phase of drug discovery

Assay design based on the target

- Define suitable ligand immobilization strategy
- Evaluation of ligand shelf life of biosensor
- Assay validation using selected molecules
- Determination of affinity and binding kinetics parameters

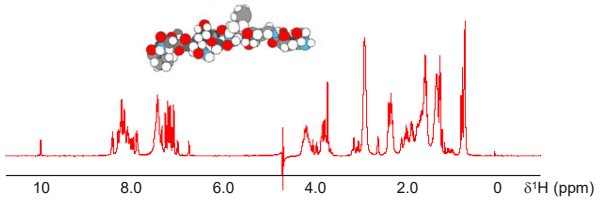


Overview of NMR capabilities and applications



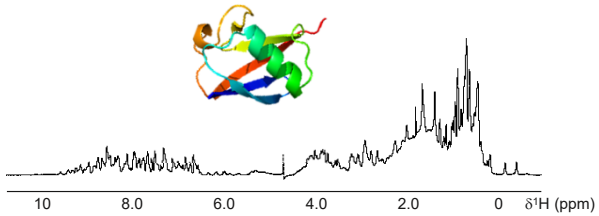
Small molecules

- Purity
- Solubility
- Stability
- Chemical shift assignment
- Structure elucidation or confirmation
- Conformational analysis



Peptides

- Purity
- Solubility
- Stability
- Chemical shift assignment
- Secondary structure analysis
- Oligomerization state



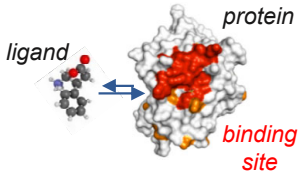
Proteins

- Solubility and stability
- Overall folding state
- Chemical shift assignment
- Secondary structure analysis
- 3D structure determination
- Backbone dynamics
- Intra- and intermolecular interactions

Fragment-based drug discovery

Ligand-observed NMR

- Ligand/protein quality control
- Fragment screening
- Binding affinity determination
- Hit to lead optimization

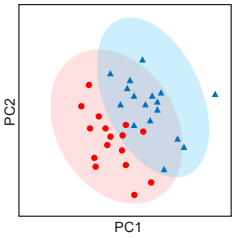


Protein-observed NMR

- Hit validation
- Binding site identification
- Binding affinity determination
- Structure-activity relationship (SAR)

Metabolomics

- Multi-metabolite fingerprints
- Search for biomarkers associated with diseases
- Targeted/untargeted analysis
- Clinical studies



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Bio-NMR: ligand-protein binding

Ligand-observed NMR

No isotopically-labeled protein required

Protein-observed NMR

Isotopically-labeled (^{13}C, ^{15}N) protein required, MW < 40 kDa

Hit identification/ validation

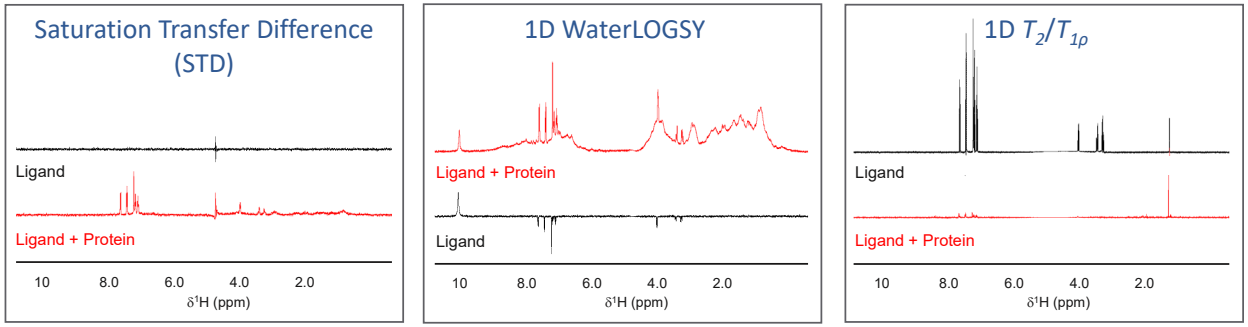
- **Fragment screening**
- **Hit validation (from other screenings)**
- **SAR by NMR, identification of allosteric binding sites**
- **Hit-to-Lead optimization**

Lead optimization

Ligand-observed NMR spectroscopy can be used as primary screening tool for Fragment-Based Drug Discovery (FBDD) or to confirm hits from other screening methods. This method is particularly suited for studying weak-medium binders (mM<k_D<μM)

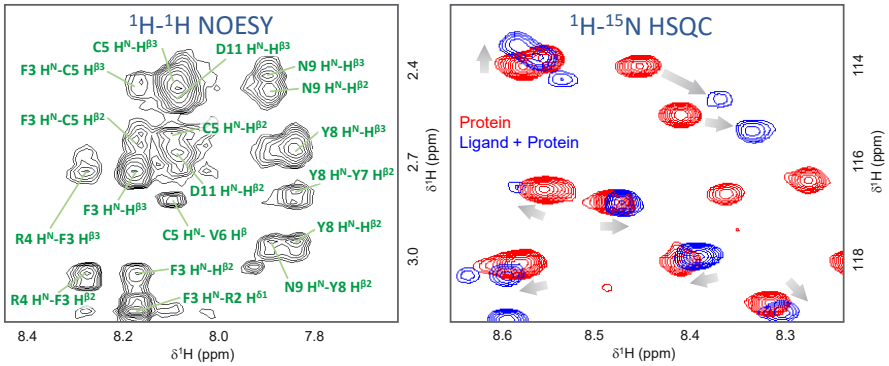
Protein-observed NMR spectroscopy can be used to identify the ligand's interaction site on the protein, its binding orientation and affinity, thus enabling SAR by NMR which is used to guide further optimization of the ligand. This method allows to study medium-strong binders (μM<k_D<nM)

Ligand-observed NMR - Fragment screening / hit validation



- Ligand/protein integrity and solubility
- Possibility to screen fragment libraries or validate hits from other screenings (STD, WaterLOGSY, $T_2/T_{1\rho}$)
- Possibility to combine fragments (5-10) in cocktails to increase the throughput

Protein-observed NMR - Chemical shift perturbation, SAR by NMR



- Target protein characterization (3D structure, backbone dynamics)
- Chemical shift perturbation and chemical shift mapping
- SAR by NMR

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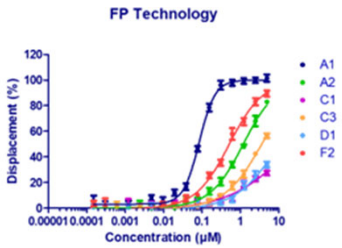
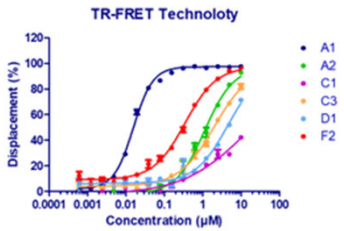
Orthogonal assays

Orthogonal assays for hit validation

- Assay with different readout (if feasible)
- Gel shift assay (if applicable)
- Detection of reaction product by LC-MS
- Western blot

Potency of compounds measured in two readouts

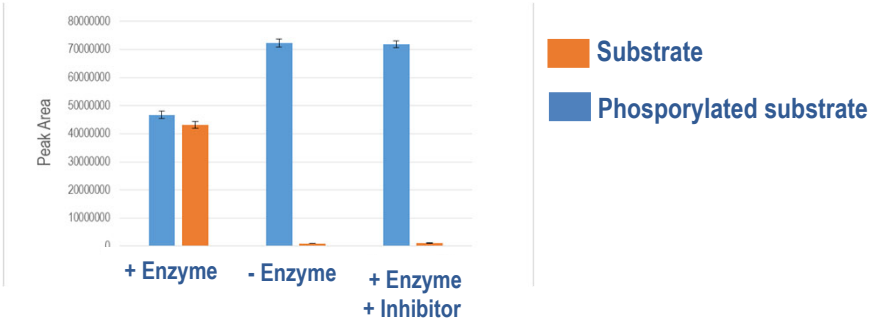




	FP Technology		TR-FRET Technology	
	Hill slope	EC50 (µM)	Hill slope	EC50 (µM)
A1	2.10	0.086	1.71	0.016
A2	1.00	1.21	1.15	1.14
C1	25% D at top con		42% D at top con	
C3	0.90	3.62	0.94	2.04
D1	34% D at top con		1.01	4.62
F2	0.99	0.520	1.06	0.349

Similar potency observed; discrepancy in the most active compound (A1) is due to the protein concentration used in the assay (125 nM in FP vs 15 nM in TR-FRET)

Dephosphorylation assay by LC-MS



Blocking of enzymatic activity confirmed by LC-MS

Key aspects of assay automation and screening

Assay metrics

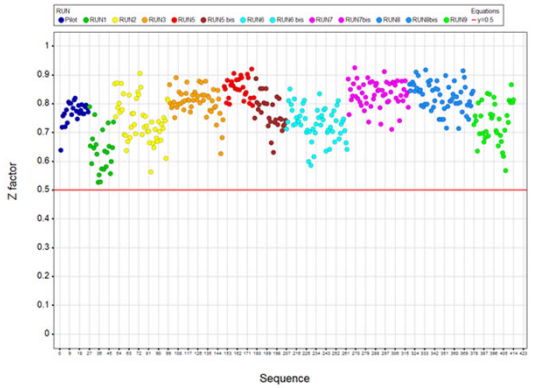
- Z'/robust Z'
- S/B or ΔmP for FP technology
- Stability/ uniformity
- Performance of reference standard

Test of selected molecules to validate the assay

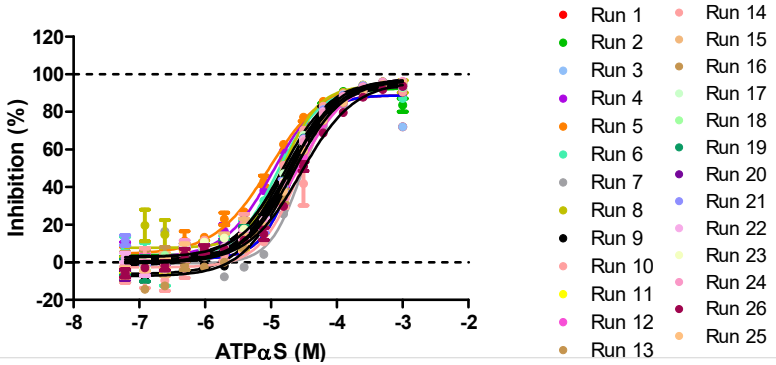
Validated assay can be used for

- Routine work to support the SAR
- Pilot screen: approximately 8K compounds are tested in HTS mode
- HTS of the IRBM compound collection or external libraries

Data obtained from cell-based assay HTS with Nanoluc as reporter



Performance of standard in a biochemical HTS



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Key aspects of HTS follow-up

Hit identification

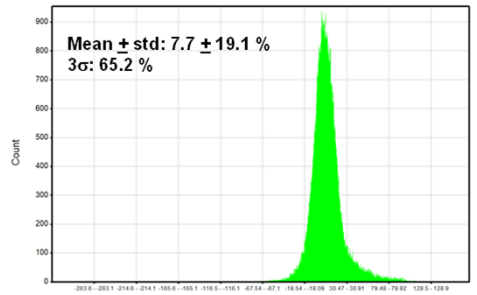
- Standard approach is based on the activity distribution around non-actives
- Hits are identified based on sigma distribution (≥3s)

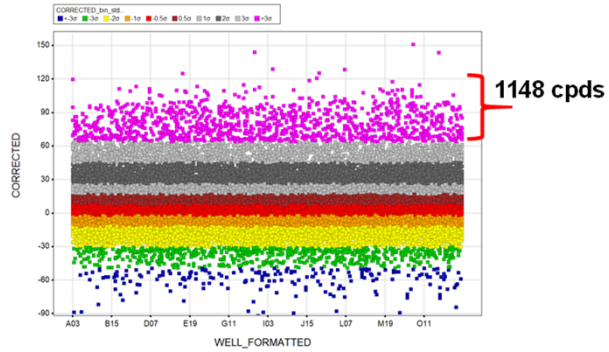
Potency determination and preliminary selectivity

Compound clustering and QC

Counter screening

- Identification of RCC compounds
- DNA intercalators
- Aggregator compounds
- Ratio test (if feasible)
- Interference assay (if feasible)





Data obtained from cell-based assay HTS with Nanoluc as reporter

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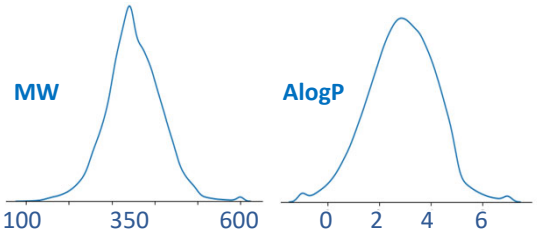
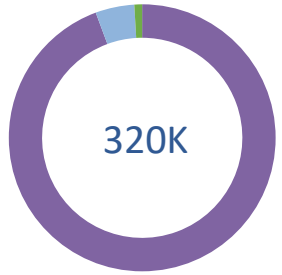
IRBM small molecule screening library

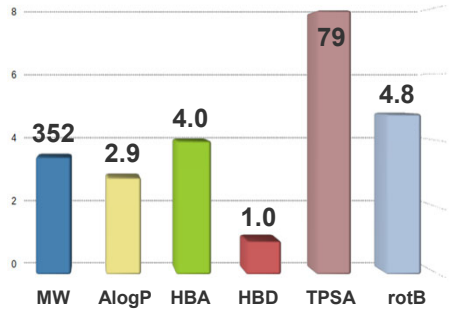
320,000 compounds

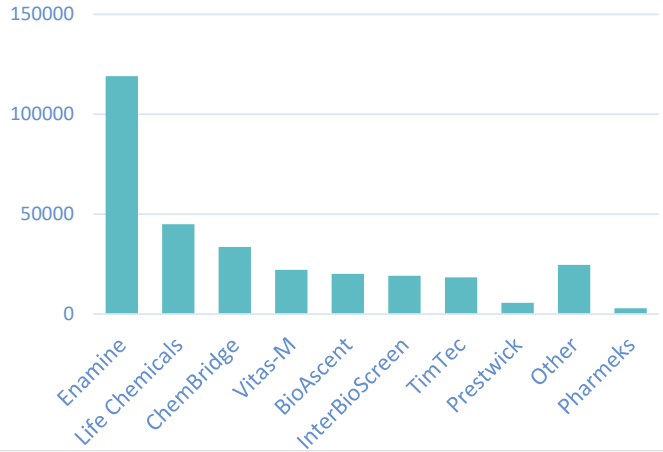
Built and curated by IRBM chemists

- No unwanted groups and focused on desirable calculated properties
- Med. Chem. focused
- Diversified sources









Full collection ready to be screened

- 100% in DMSO solution at -20°C under nitrogen

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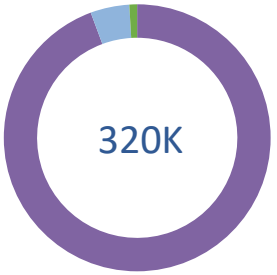
IRBM small molecule screening library

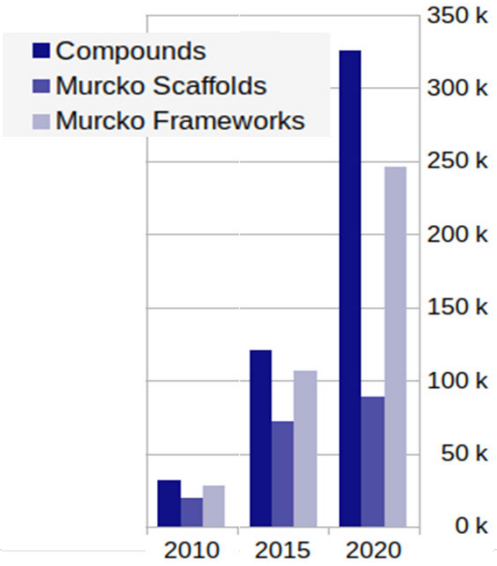
Constantly evolving to adapt to industry needs

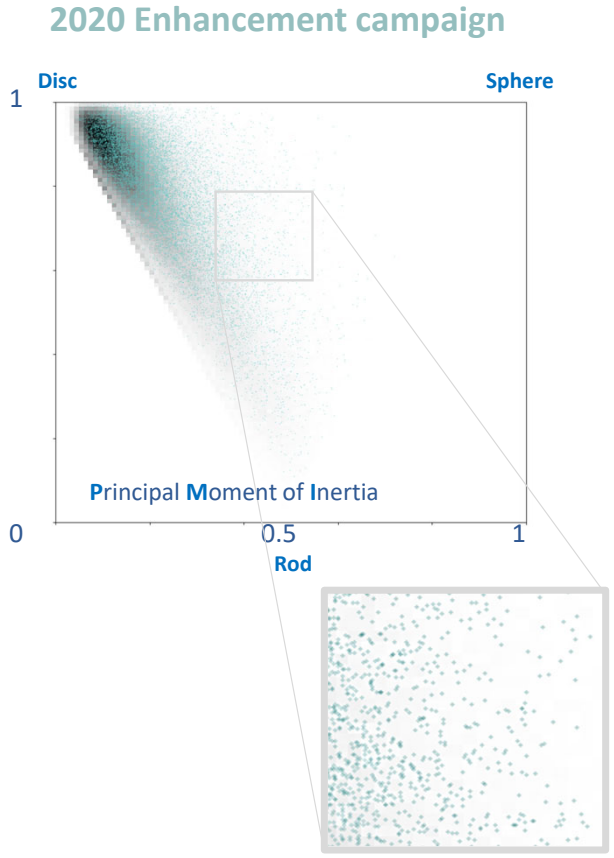
- Yearly Improvement programs
  - Focused Sets (e.g. RNA set)
  - General Diversity and Property Enhancement (e.g. 3D'ness)
- Pre HTS enrichment
- Quality management: Sample QC before addition
- Fully automated handling

Focused subsets

- Natural products
- CNS
- Kinase Inhibitors
- RNA interacting
- Safe in man
- Anti-cancer compounds
- Fragments



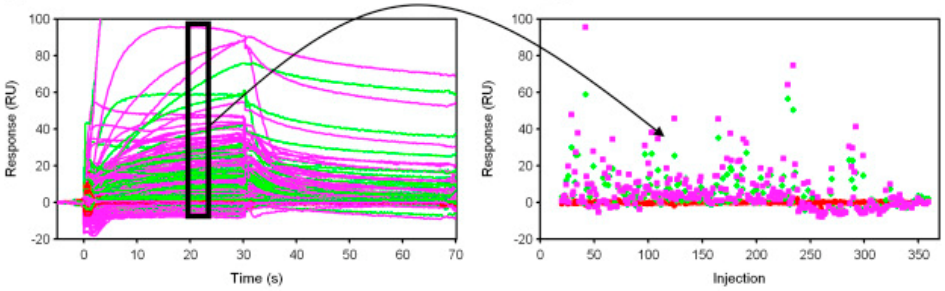




Fragment-based drug discovery by SPR

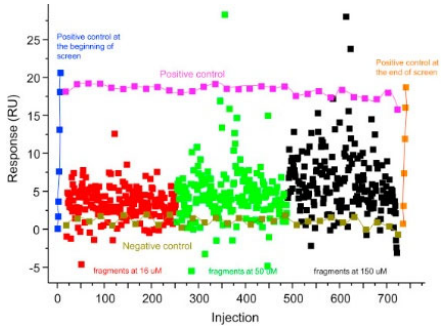
SPR based biosensors are attractive tools for fragment screening

- Low target consumption
- High quality interaction-data
- Higher throughput than X-Ray crystallography and NMR



Main goals achieved by FBDD

- Remove compounds forming aggregates
- Identify promiscuous binders with solubility issues
- Eliminate false positives from HTS
- Assess specificity by parallel testing on multiple targets



Progress in Biophysics and Molecular Biology, 116, 2014, 113

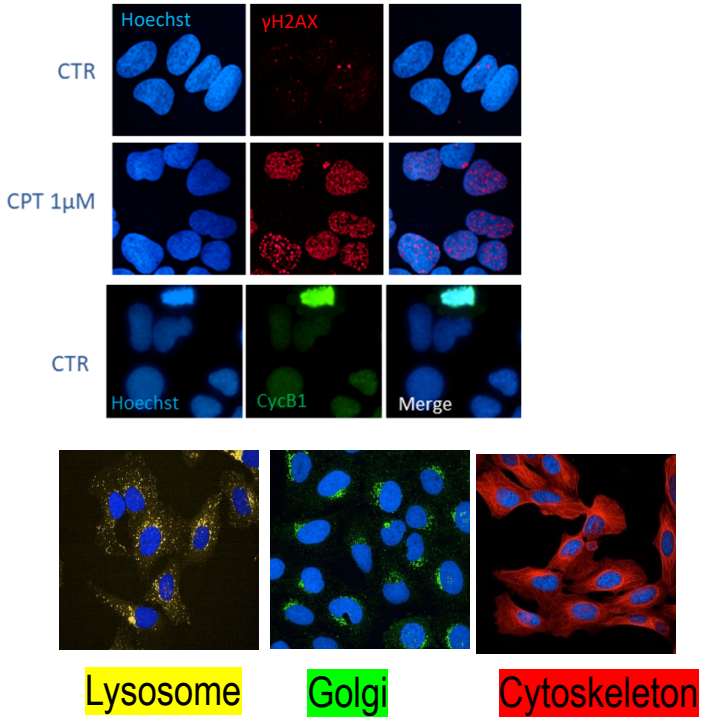
HCS assays

Cell proliferation and Viability

- DNA Damage
- Cell Cycle
- Cell Death
- Cytotoxicity

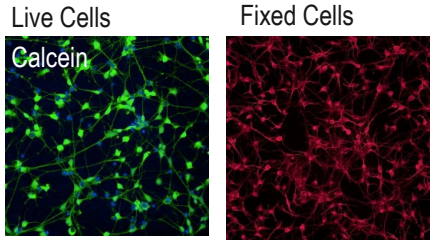
Cell proliferation and Viability

- Lysosome
- ER
- Golgi
- Mitochondria
- Cytoskeleton

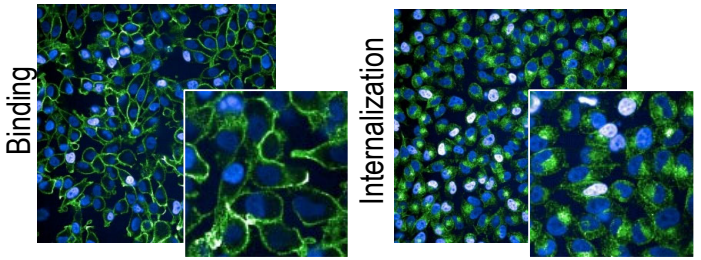


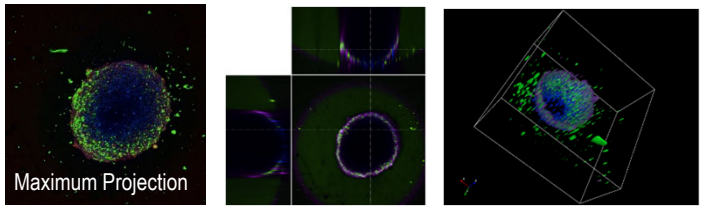
HCS assays

- Binding and Internalization assay for biotinylated peptides
- Neuronal Branching
- 3D analysis



Neurite
Number
Length
Roots
Morphology

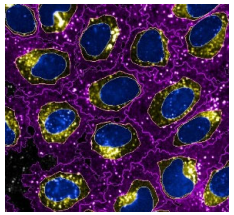




Object No	Volume	Area [µm²]	Roundness	Perimeter [µm]	Width [µm]	Length [µm]	Ratio Width to Length

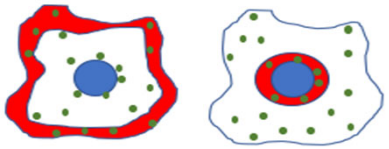
Image Analysis

Cell Segmentation



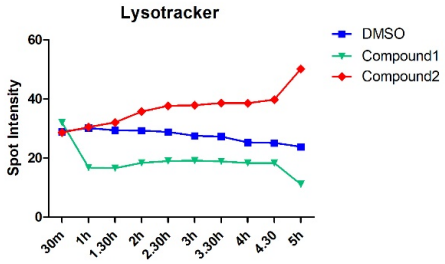
Cell	Parameter
Nuclei	Intensity
Cytoplasm	Number
Membrane	Classification

Positional analysis



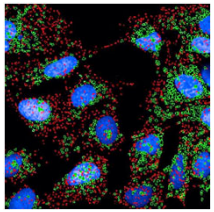
Position
Peripheral
Nuclear

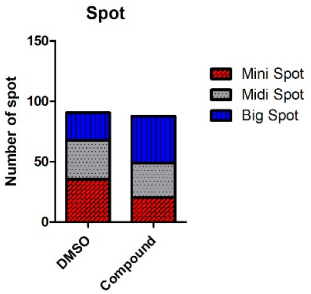
Time course/Tracking Object



Morphological analysis

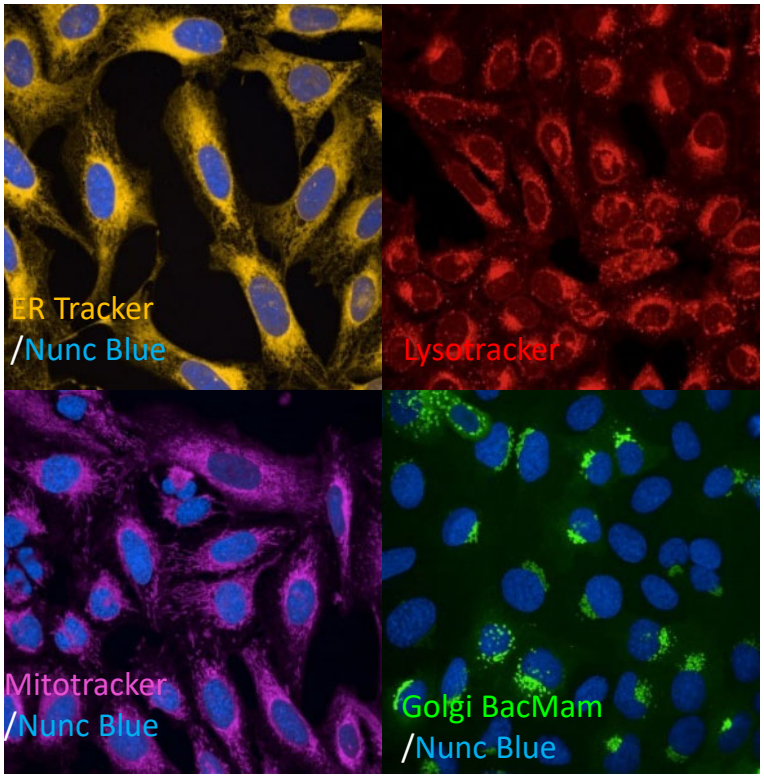
Big Spot/Mini Spot



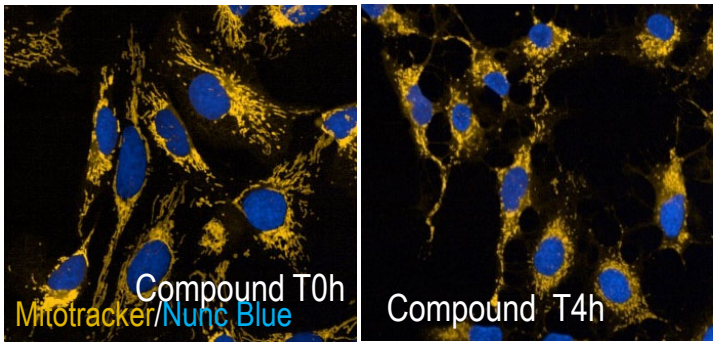


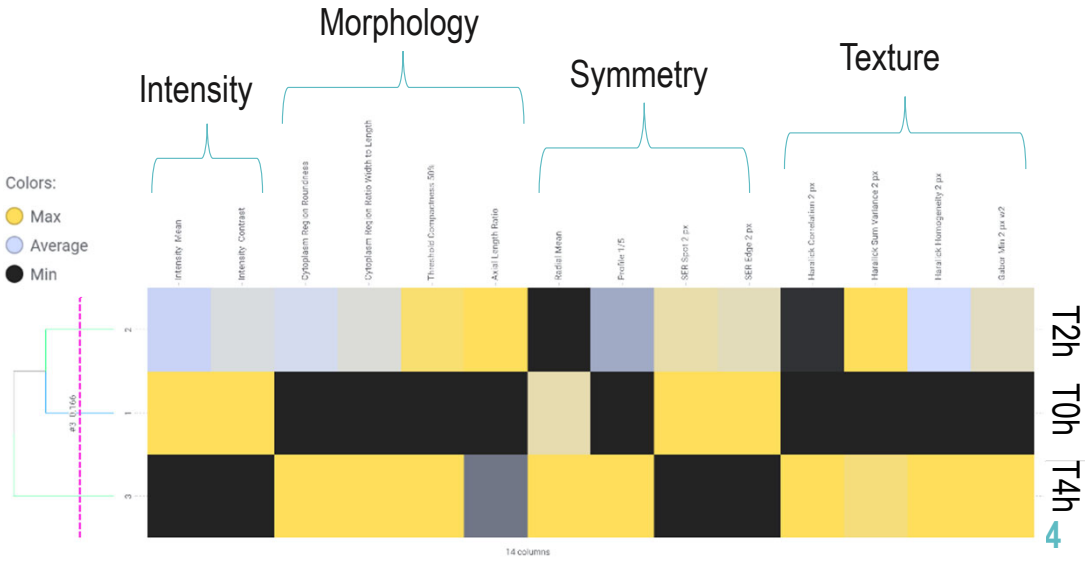
Other example HCS

Live Trackers



Structure-activity profiling





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Fully automated compound management platform

- Liquid handling
- Decapper
- Centrifuge
- Acoustic droplet ejection
- Plate handling
- Sealer and peeler

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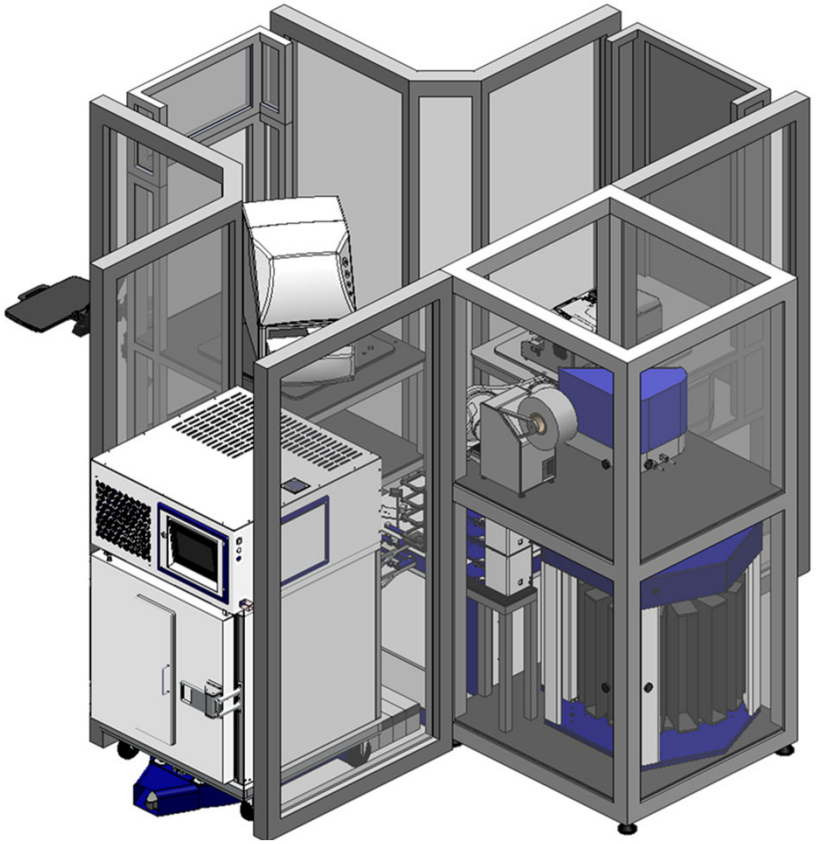
Fully automated, modular screening robot



Standalone instrumentation

Standalone instruments used to support assay development and routine work

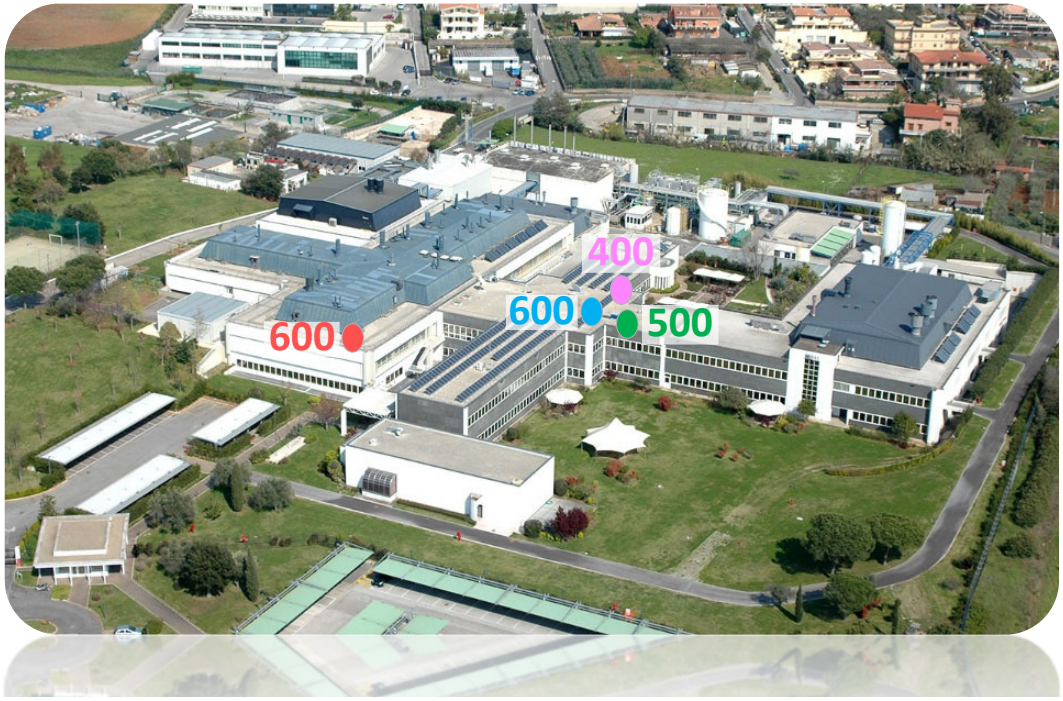
- Echo 650 acoustic droplet ejection
- Tempest Liquid Handler
- Envision plate reader
- Meso Scale Discovery Platform

Other instruments

- Opera Phenix
- Incucyte S3

NMR instrumentation

IRBM Science Park S.p.A.



SampleJet
96 individual tubes
480 tubes in thermostatic racks

- **Bruker Avance 600 MHz**
  equipped with a 5 mm BBI probe/1.7 mm TXI probe | SampleJet
- **Bruker Avance III HD 600 MHz**
  equipped with a 5 mm TXI cryo-probe | SampleJet
- **Bruker Avance Neo 500 MHz**
  equipped with a 5 mm BBI probe | SampleCase
- **Bruker Avance Neo 400 MHz (coming soon!)**
  equipped with a 5 mm BBO probe | SampleCase

Contact Details

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