



CENTER FOR NEUROSCIENCE
AND CELL BIOLOGY
UNIVERSITY OF COIMBRA
PORTUGAL

Exploring high-content screening as a functional genomics tool in biomedicine

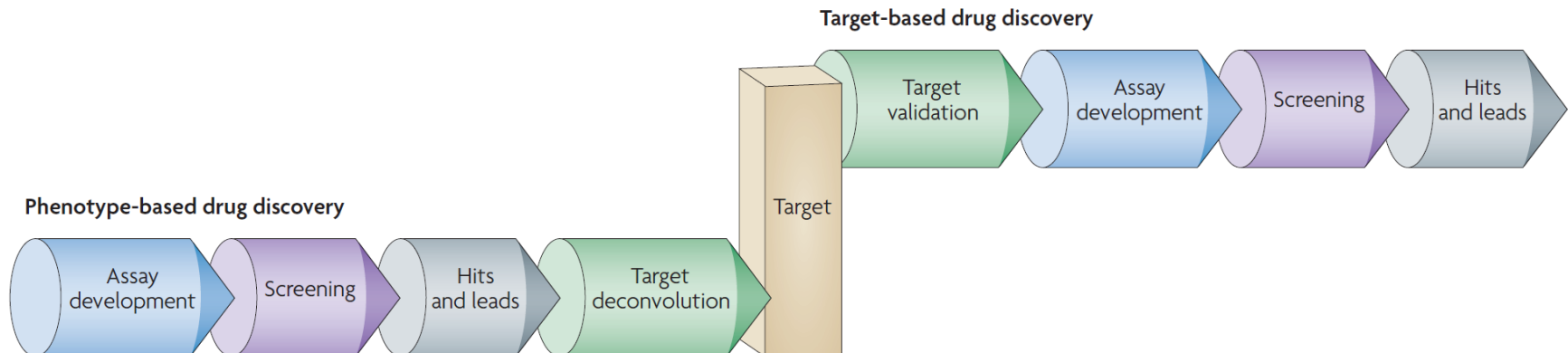
Miguel Mano

Functional Genomics and RNA-based Therapeutics Laboratory

Drug discovery and development



Roses, Nature Reviews Drug Discovery 2008



Terstappen et al., Nature Reviews Drug Discovery 2007

Functional genomics

Application of global (genome-wide or system-wide) experimental approaches to assess gene function

Genome Sequencing



***S. cerevisiae* 1997**

***C. elegans* 1998**

***D. melanogaster* 2000**

***A. thaliana* 2000**

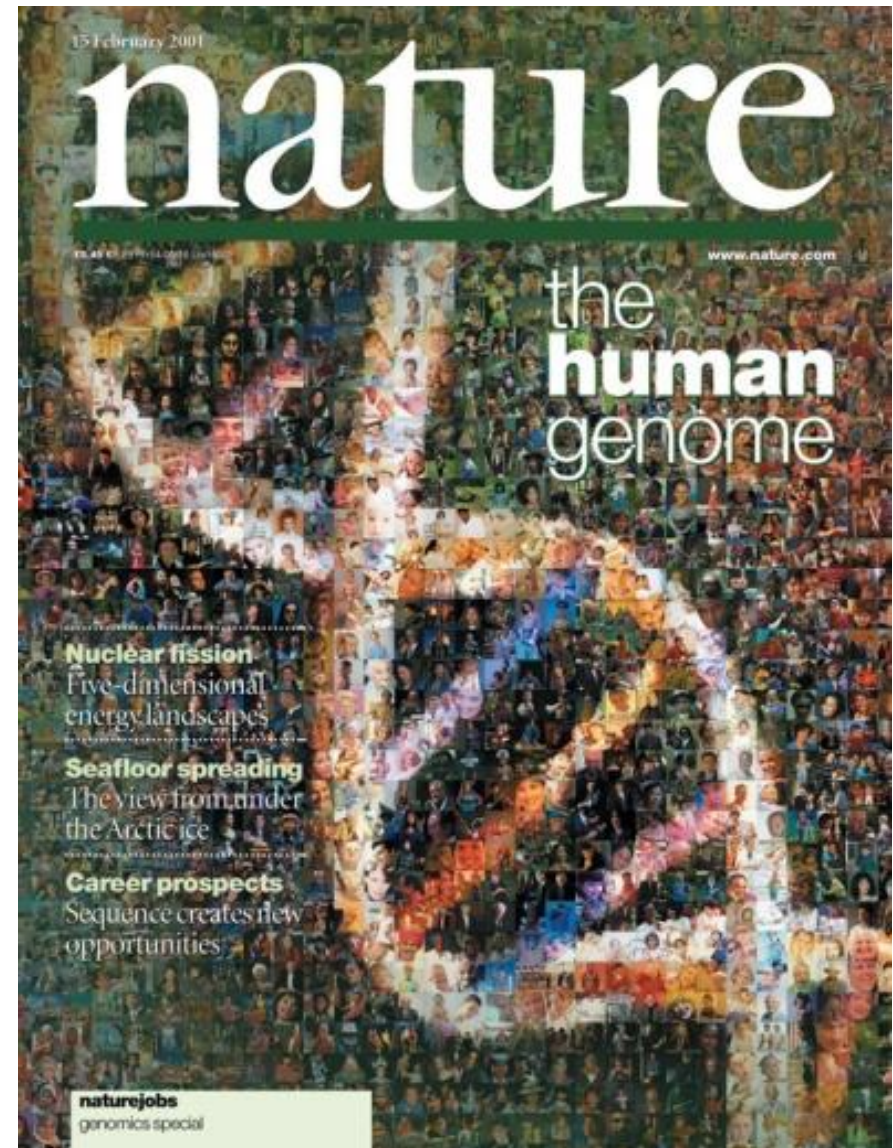
***H. sapiens* 2001**

***M. musculus* 2002**

***R. norvegicus* 2004**

(...)

The Human Genome at 18 | Feb.2001-Feb.2019





haploid genome, font size 5

The Human Genome in numbers

23 chromosome pairs

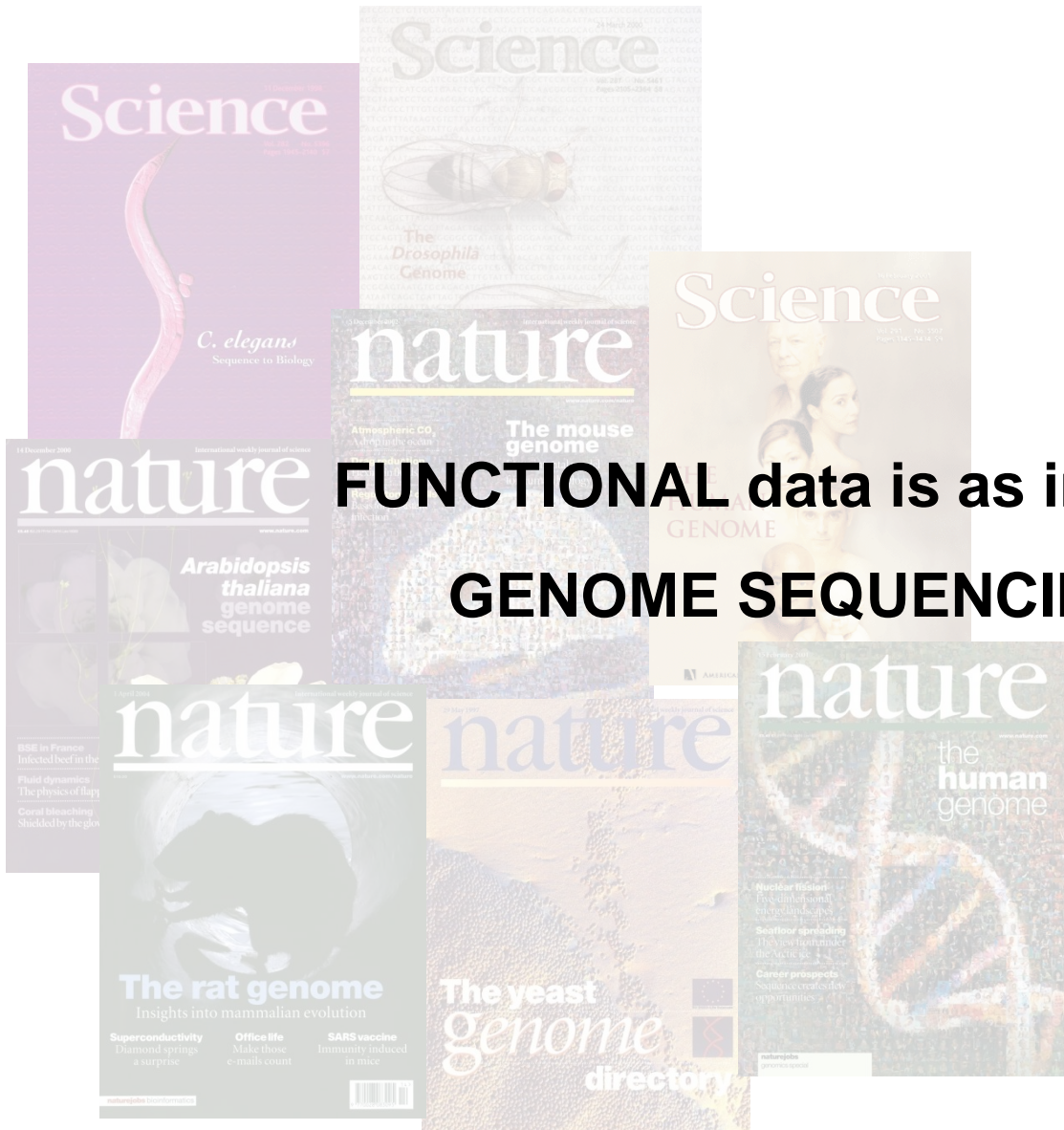
~ 6 500 000 000 base pairs

2-3 meters in length

~ 20 000 protein coding genes

< 2% genome encodes proteins

Genome Sequencing



**FUNCTIONAL data is as important as
GENOME SEQUENCING data!**

S. cerevisiae 1997

C. elegans 1998

D. melanogaster 2000

A. thaliana 2000

H. sapiens 2001

M. musculus 2002

R. norvegicus 2004

(...)

Functional genomics

Application of global (genome-wide or system-wide) experimental approaches to assess gene function

How can we study gene function?

Expression analysis vs functional screening

Expression analysis

- Analysis of mRNAs/microRNAs/lncRNAs present at different stages
- Characterization changes occurring after different stimuli

Functional screening

- Analysis of the functional impact of the perturbation of a single protein/microRNA/lncRNA
- Relevance of proteins/microRNAs/lncRNAs to a given function/
/cellular process/phenotype

Expression analysis vs. functional/phenotypic screening

Expression analysis

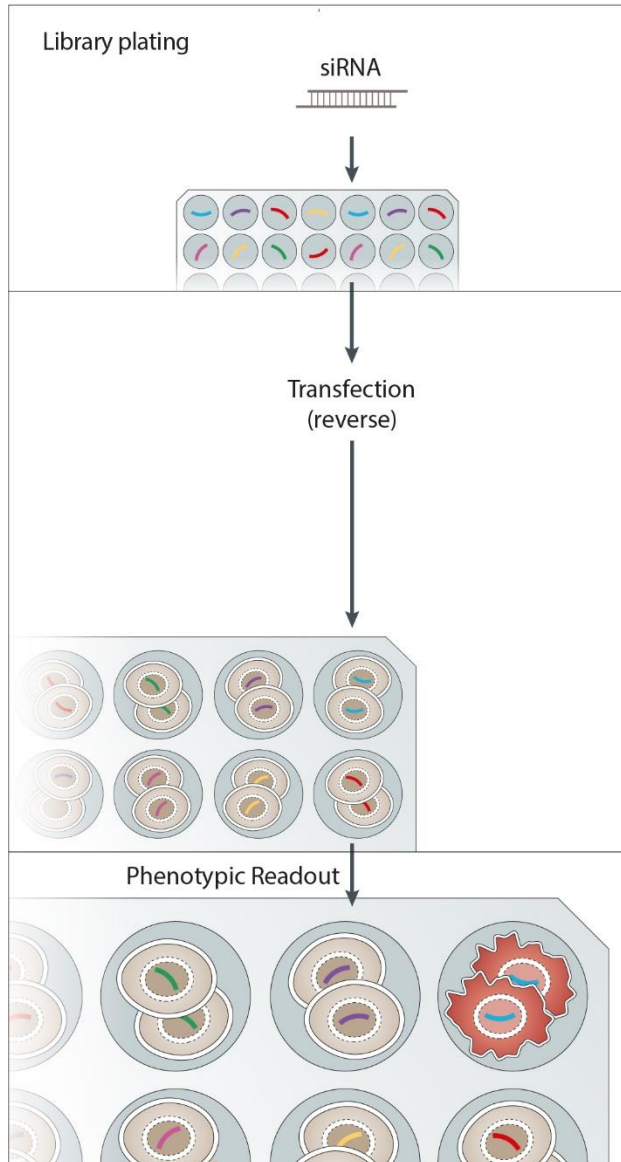
- **DNA/RNA sequencing**
(mRNA, ncRNAs)
- **Proteomics**
(proteins)
- **Lipidomics**
(lipids)
- **...omics**

Functional screening

- **Gene silencing**
(RNAi)
- **Gene overexpression**
(expression libraries)
- **Gene knockout**
(CRISPR)
- **miRNA modulation**

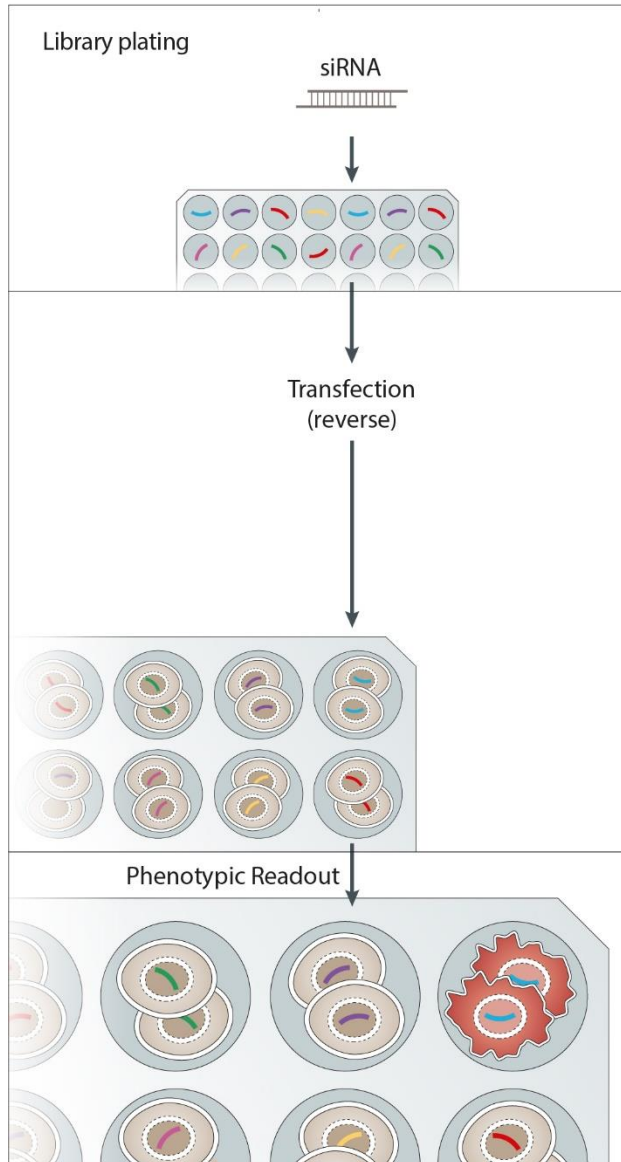
Phenotypic Screening in Functional Genomics

ARRAYED SCREENINGS



Phenotypic Screening in Functional Genomics

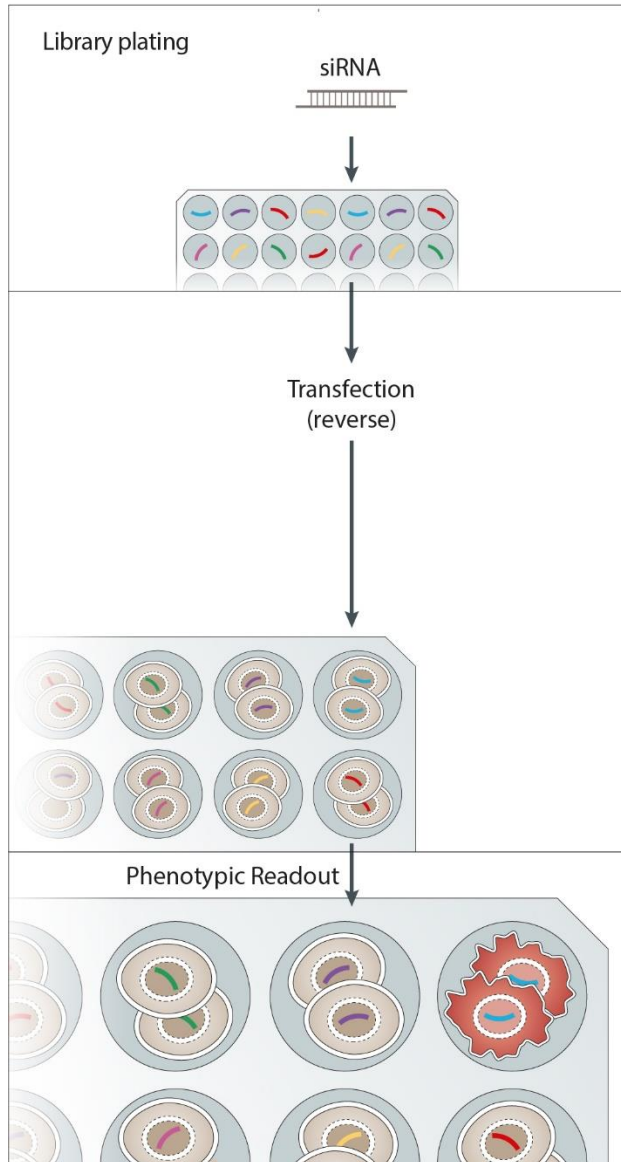
ARRAYED SCREENINGS



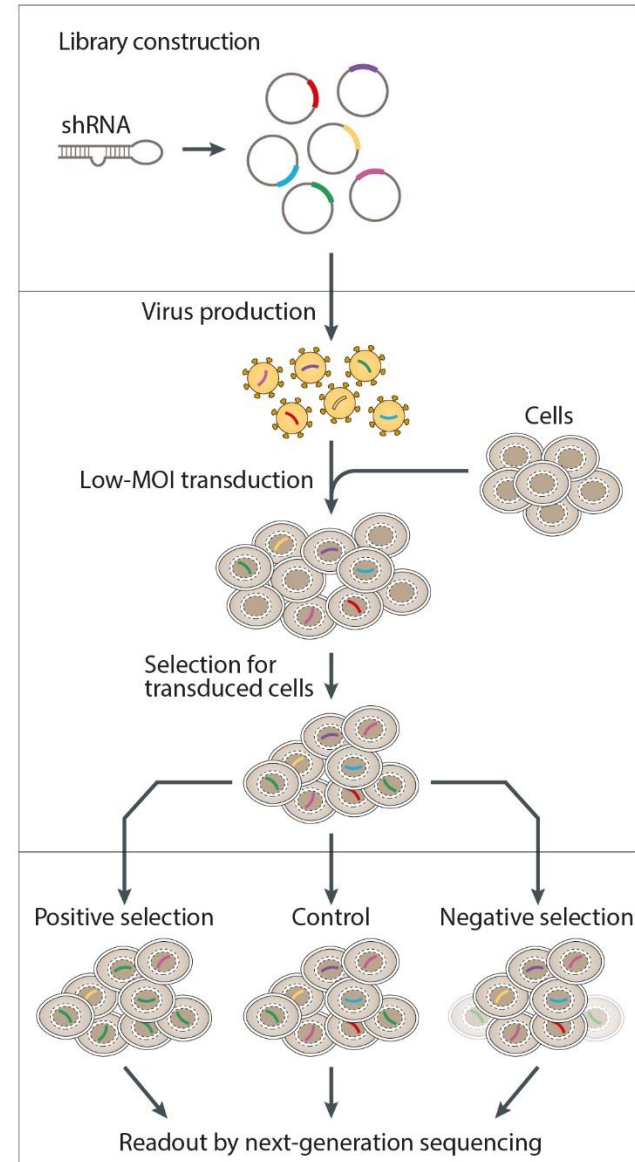
siRNAs
microRNA mimics
microRNA inhibitors

Phenotypic Screening in Functional Genomics

ARRAYED SCREENINGS

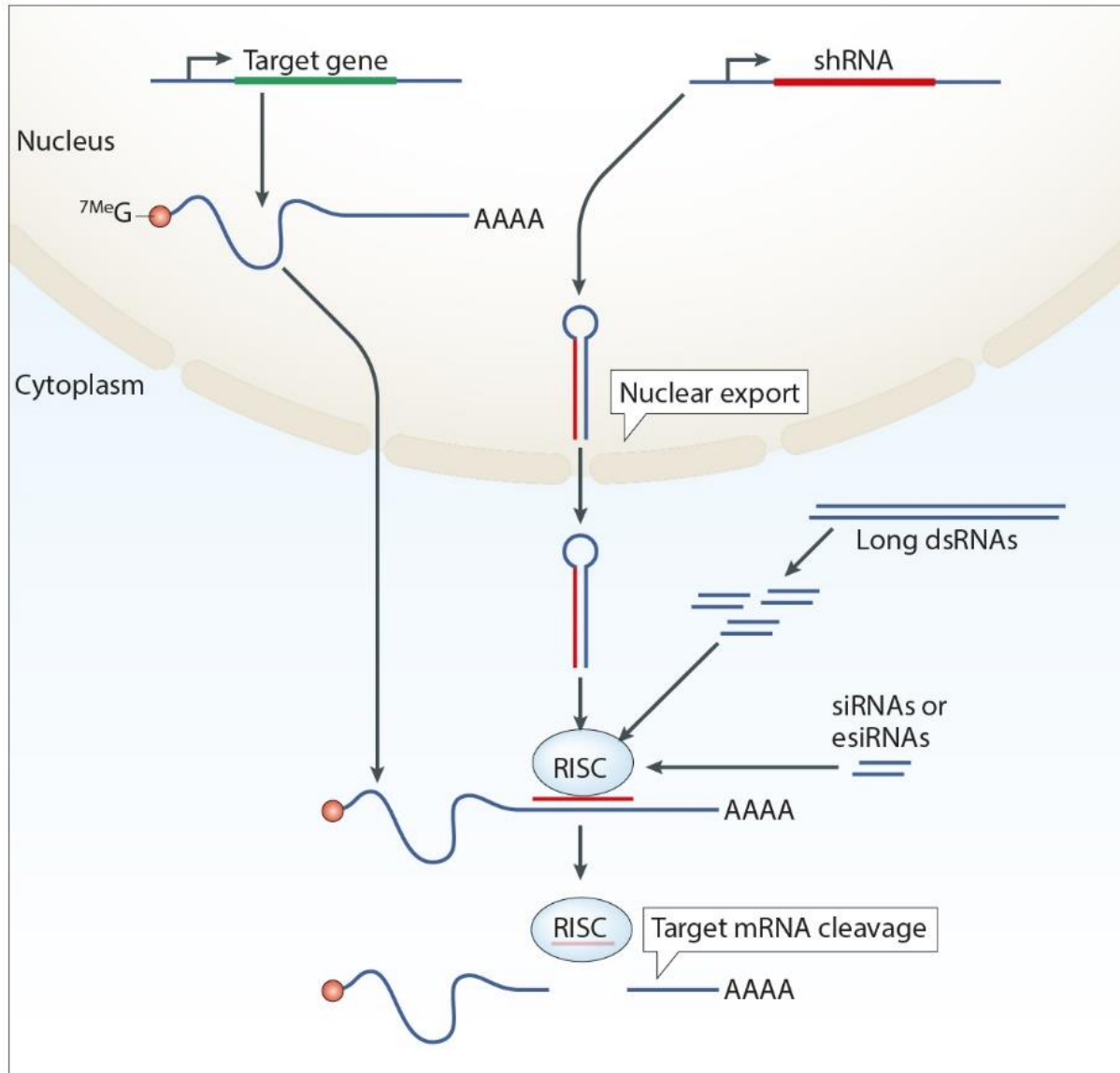


POOLED SCREENINGS



adapted from Shalem et al., Nature Reviews Genetics 2015

RNA interference

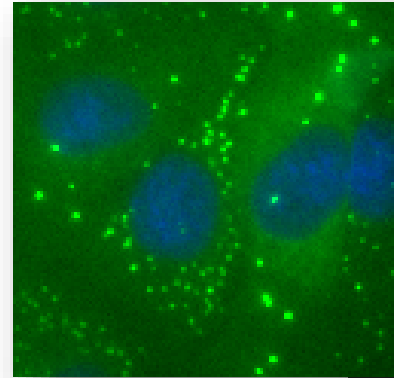


Homogeneous assays vs. Microscopy-based readouts

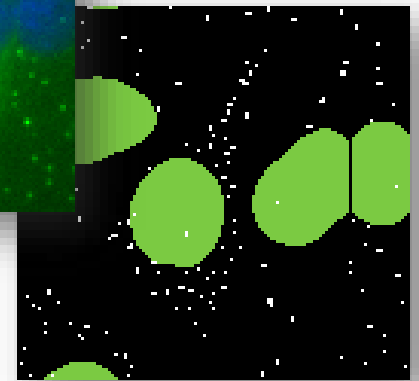


Plate-reader Readouts
High-Throughput Screening

original image



analysis



Microscopy Readouts
High-Content Screening

Simple optimization
Fast
Quantitative

PROS

Quantitative
Multiparametric
Rich biological information
Subcellular resolution

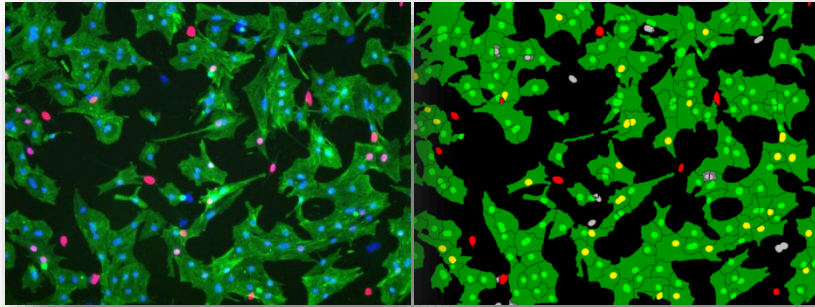
Binary results
Poor biological information

CONS

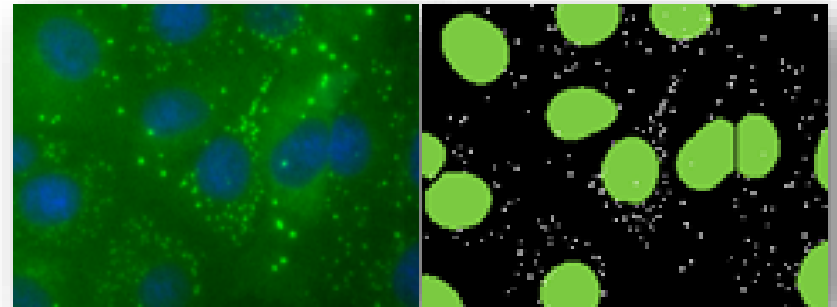
Slow (acquisition and analysis)
Complex optimization
Complex analysis

High-Content Screening: getting the whole picture

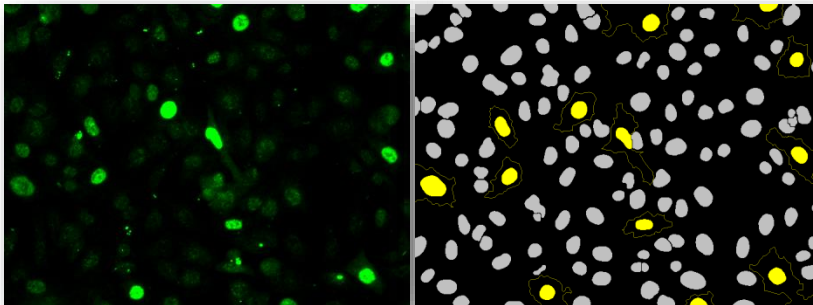
Cell proliferation



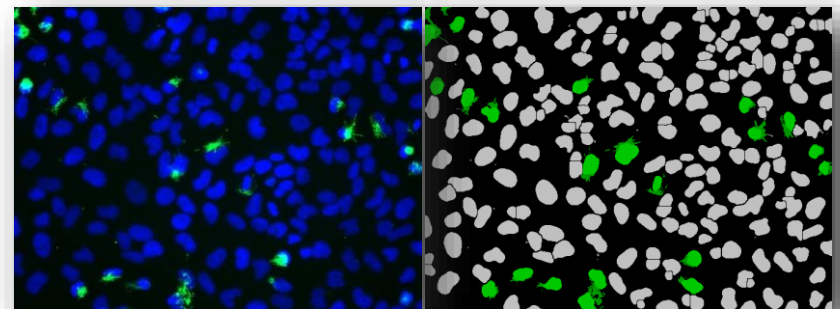
Viral infection



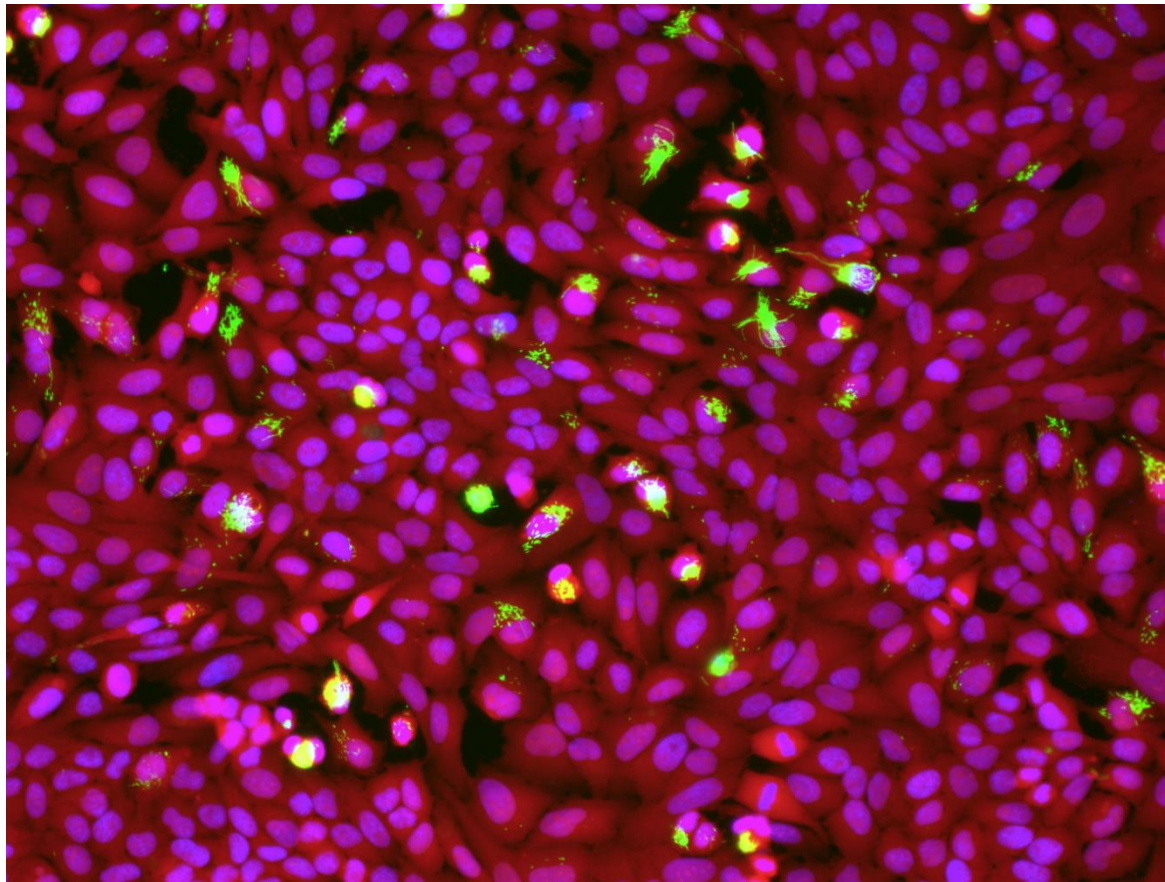
Nucleo-cytoplasmic translocation



Bacterial infection

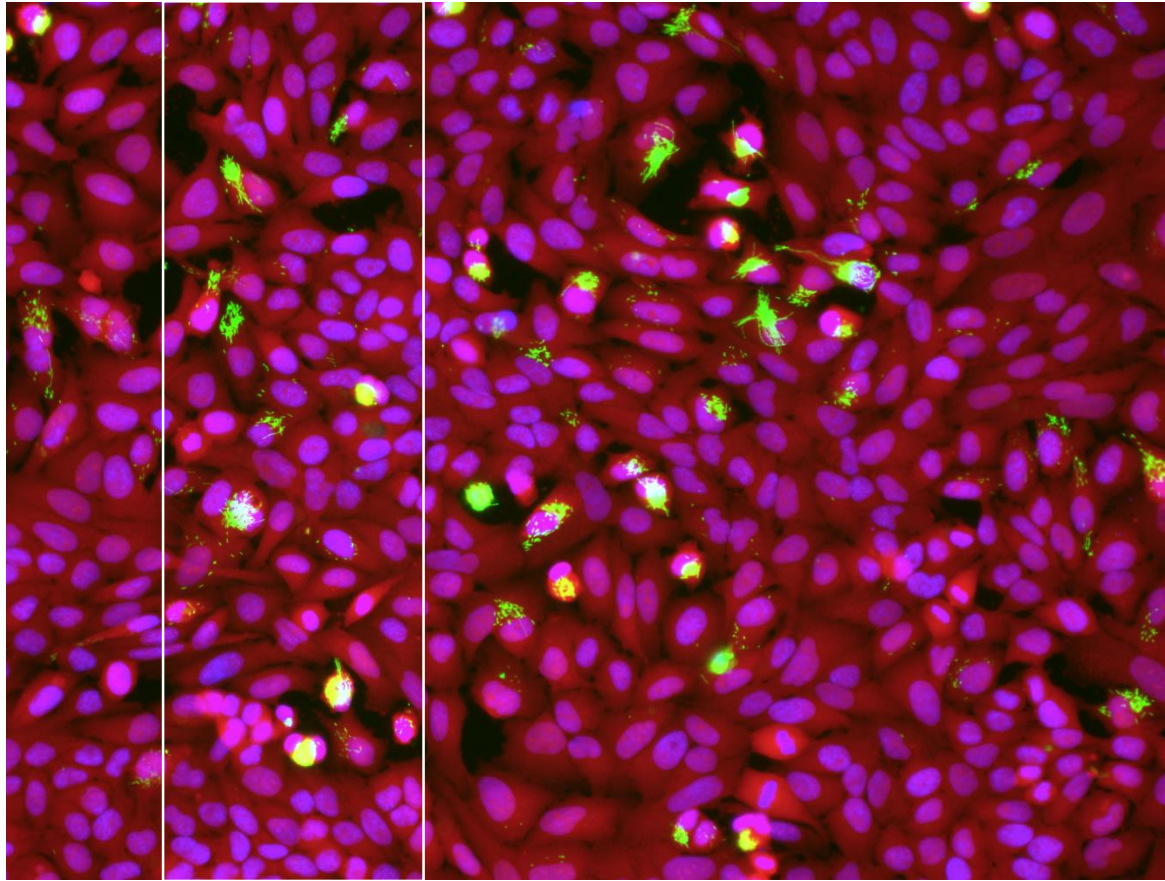


Automated image analysis

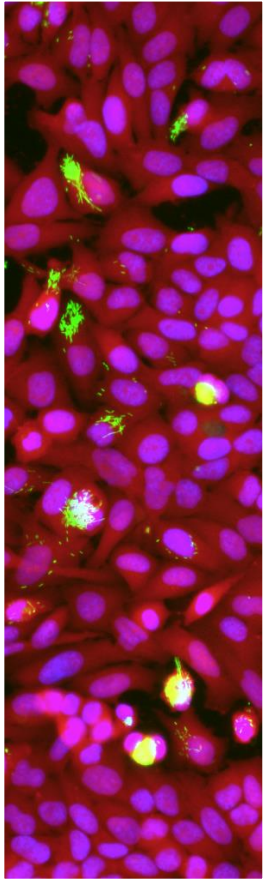


Hoechst *Salmonella Typhimurium* CellMask

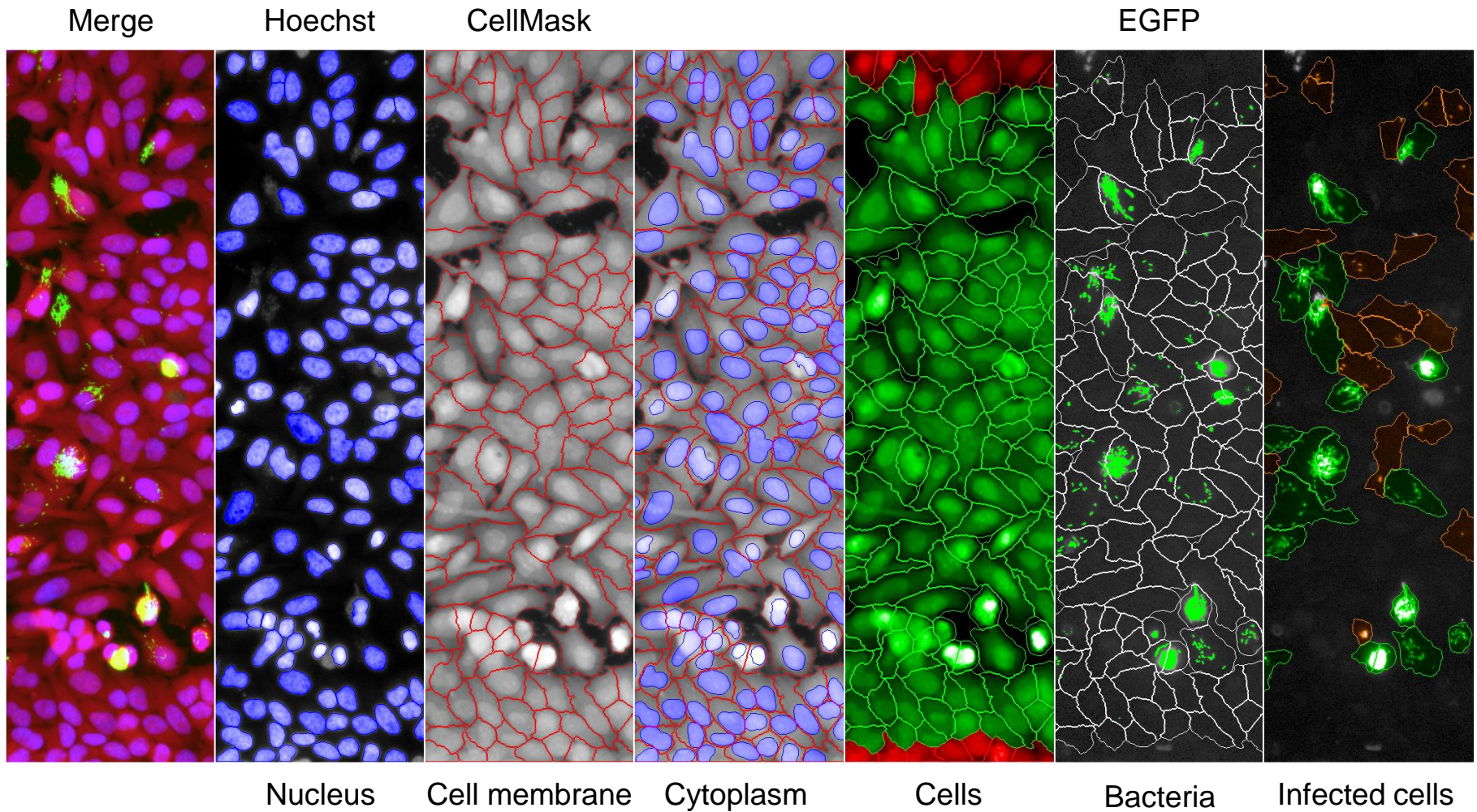
Automated image analysis



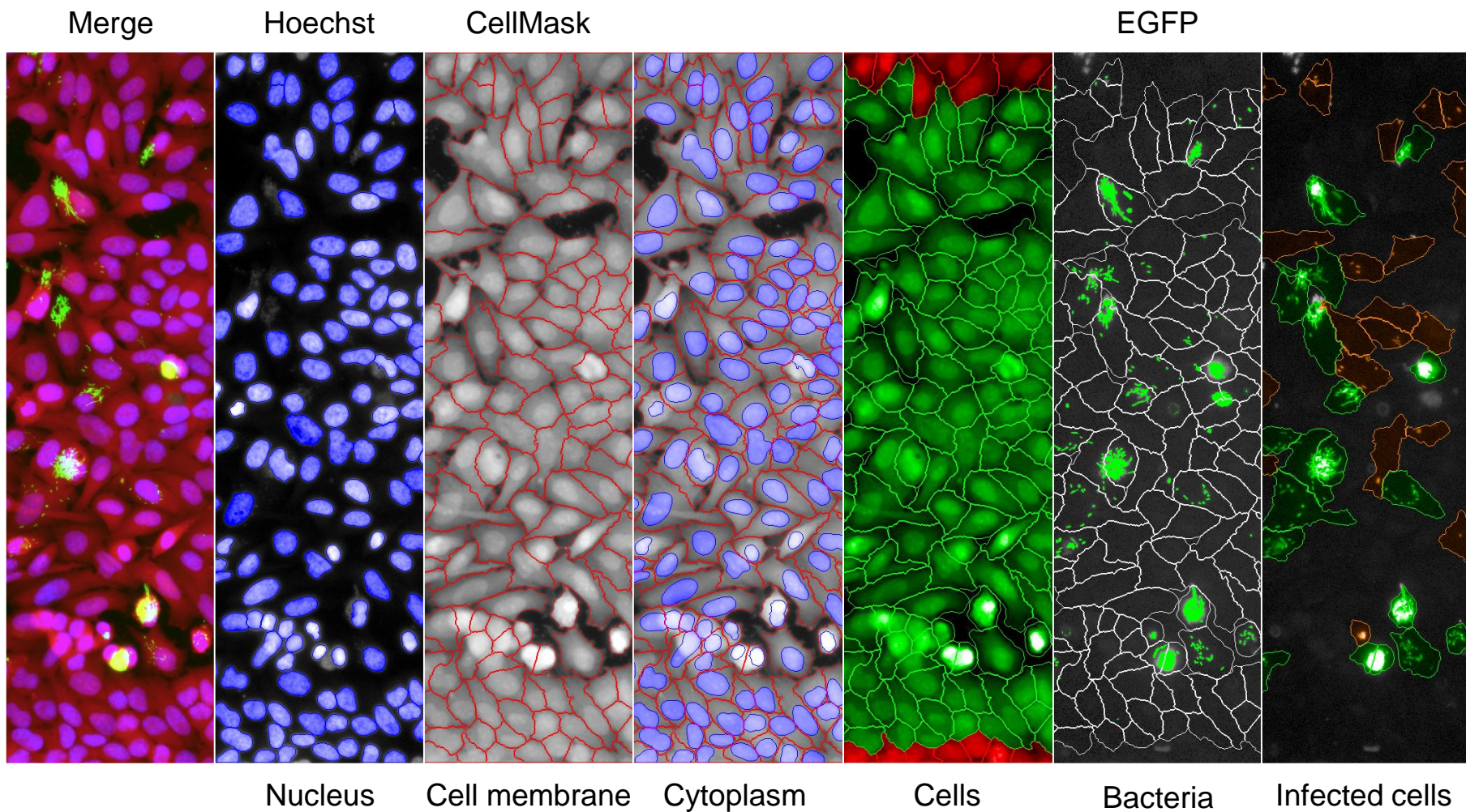
Automated image analysis



Automated image analysis



Automated image analysis



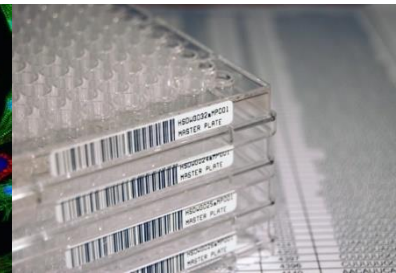
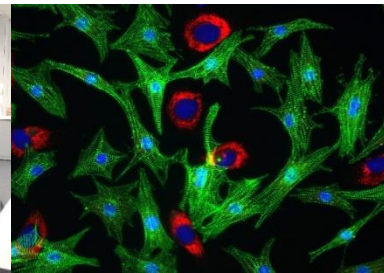
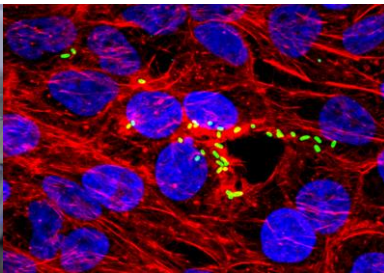
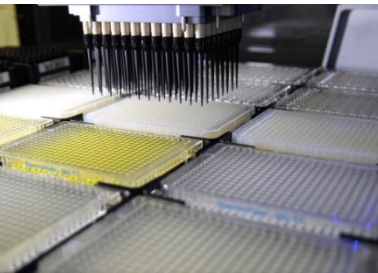
Unbiased extraction of multiparametric numerical data from images



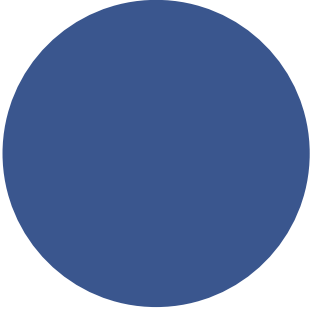



Functional Genomics and RNA-based Therapeutics laboratory

CNC/UC-Biotech | University of Coimbra, Portugal

- **Fully automated state-of-the-art high-throughput/high-content screening platform for functional screenings in human and mouse cellular models (BSL2 environment)**
- **Libraries:**
 - human genome-wide siRNA (18,175 gene targets; 4 siRNAs + pools)
 - mouse genome-wide siRNA (16,872 gene targets; 4 siRNAs + pools)
 - human microRNA mimics (miRBase v21; 2,588 mature sequences)
 - human microRNA inhibitors (miRBase v21; 2,588 mature sequences)
 - FDA approved drugs (640 chemical compounds)
- **Technologies:**
 - High-throughput screening
 - High-content microscopy screening
 - Live-cell microscopy screening



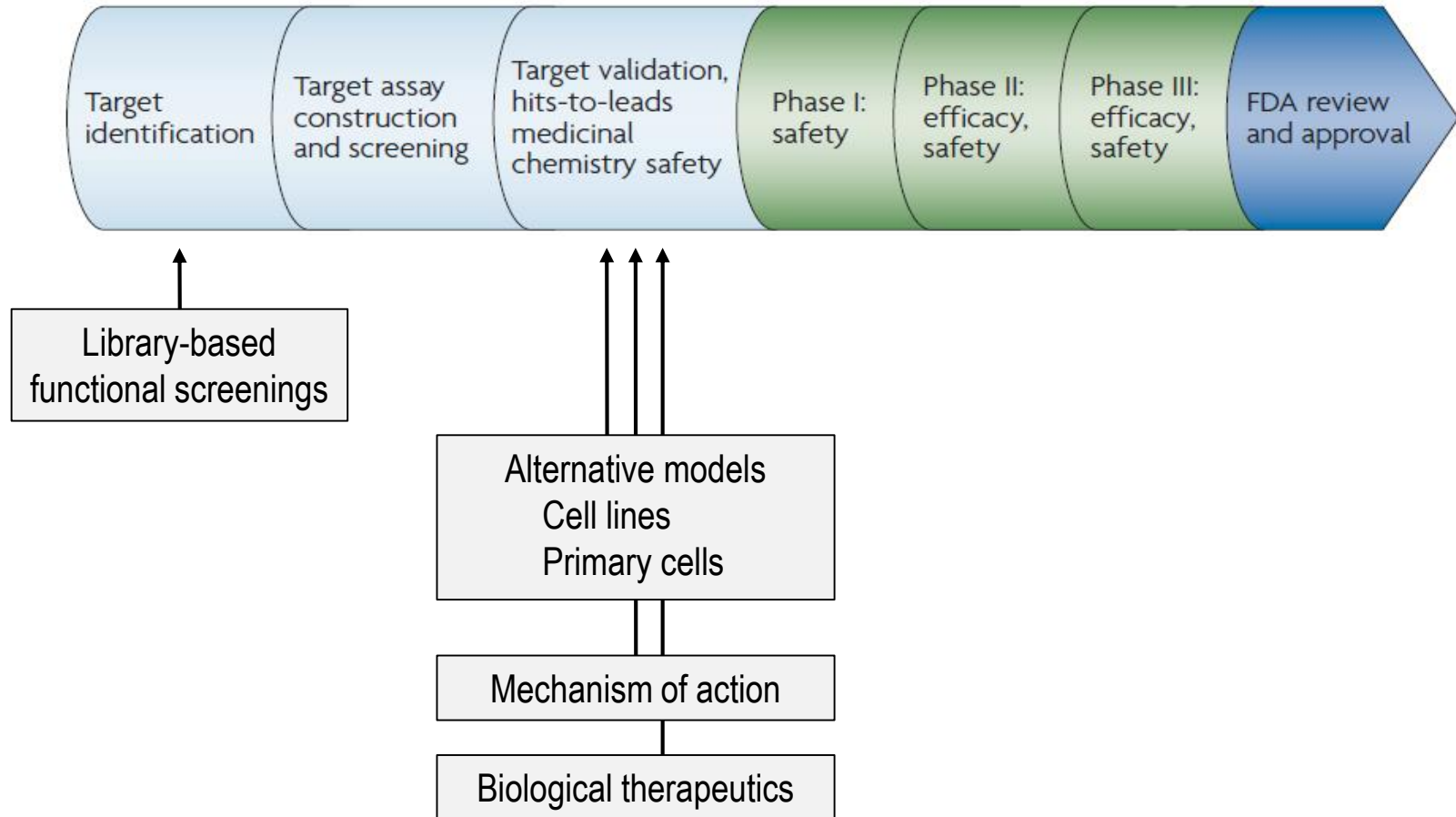
Miniaturization: costs and benefits

	6-well	96-well	384-well	1536-well
				
Well area (cm ²)	9.5	0.3	0.06	0.02
Number cells	1×10^6	3×10^4	8×10^3	2×10^3
Working volume	2-3ml	100-200 μ l	25-50 μ l	5-10 μ l

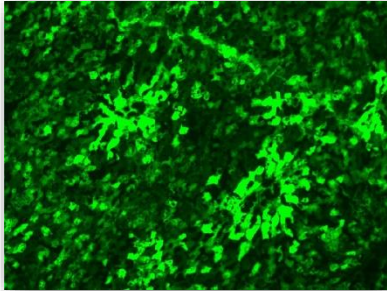
Assay complexity

Throughput

Functional genomics screening as a tool for the development of novel therapeutics



Functional screening: examples of applications

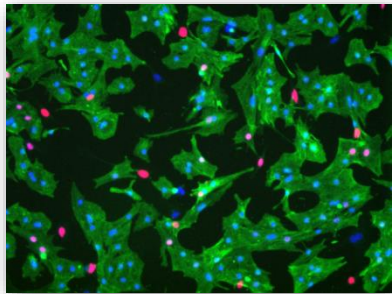


Functional high-throughput screening identifies the miR-15 microRNA family as cellular restriction factors for Salmonella infection. Nature Communications (2014) 5:4718

Genome-wide RNAi screening identifies host restriction factors critical for in vivo AAV transduction. PNAS (2015) 112:11276

Analysis of host microRNA function in Shigella infection reveals a role for miR-29b-2-5p in bacterial capture by filopodia. PLOS Pathogens (2017) 13:e1006327

Cellular TRIM33 restrains HIV-1 infection by targeting viral integrase for proteasomal degradation. Nature Communications (2019) 10:926



Functional screening identifies miRNAs inducing cardiac regeneration. Nature (2012) 492:376

Id genes are essential for early heart formation. Genes and Development (2017) 31:1325

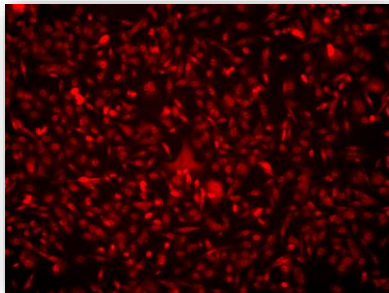
Metabolic control of YAP and TAZ by the mevalonate pathway. Nature Cell Biology (2014) 16:357

Glucocorticoid receptor signalling activates YAP in breast cancer. Nature Communications (2017) 8:14073

High-throughput screening uncovers miRNAs enhancing glioblastoma cell susceptibility to tyrosine kinase inhibitors. Human Molecular Genetics (2017) 26:4375-4387

Mechanical cues control mutant p53 stability through a Mevalonate/RhoA axis. Nature Cell Biology (2018) 20:28-35

Sterol Regulatory Element Binding Protein 1 couples mechanical cues and lipid metabolism. Nature Communications (2019) 10:1326



High-Content Screening

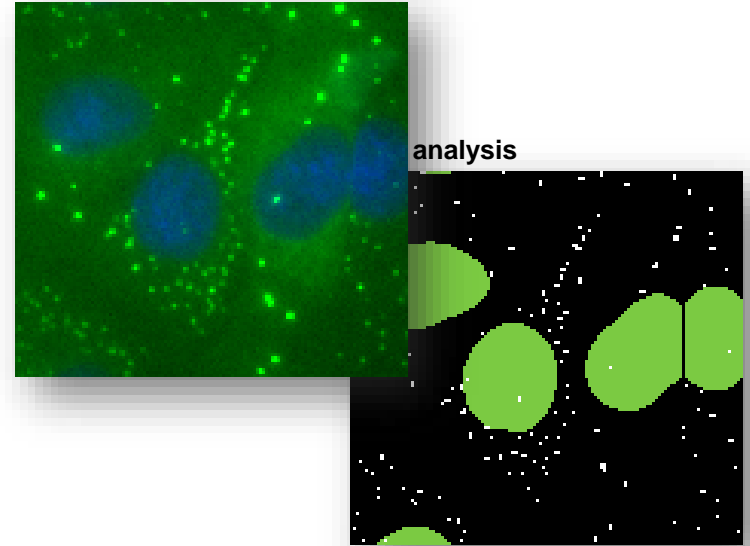
How high is high?

Homogeneous assays vs. Microscopy-based readouts



Plate-reader Readouts
High-Throughput Screening

original image



Microscopy Readouts
High-Content Screening

Simple optimization
Fast
Quantitative

PROS

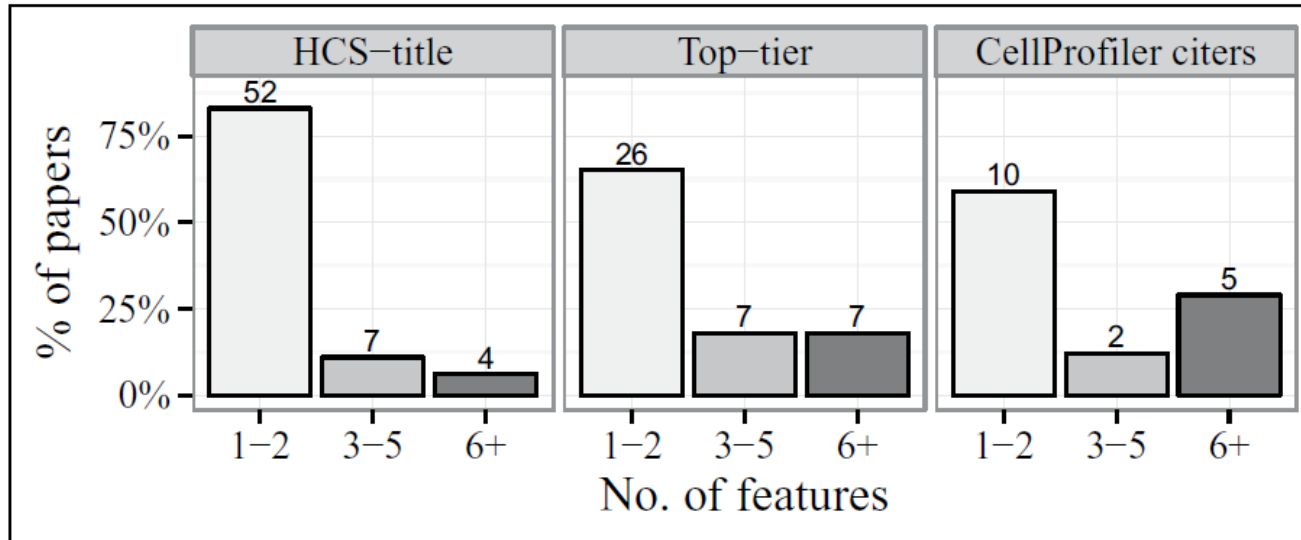
Quantitative
Multiparametric
Rich biological information
Subcellular resolution

Binary results
Poor biological information

CONS

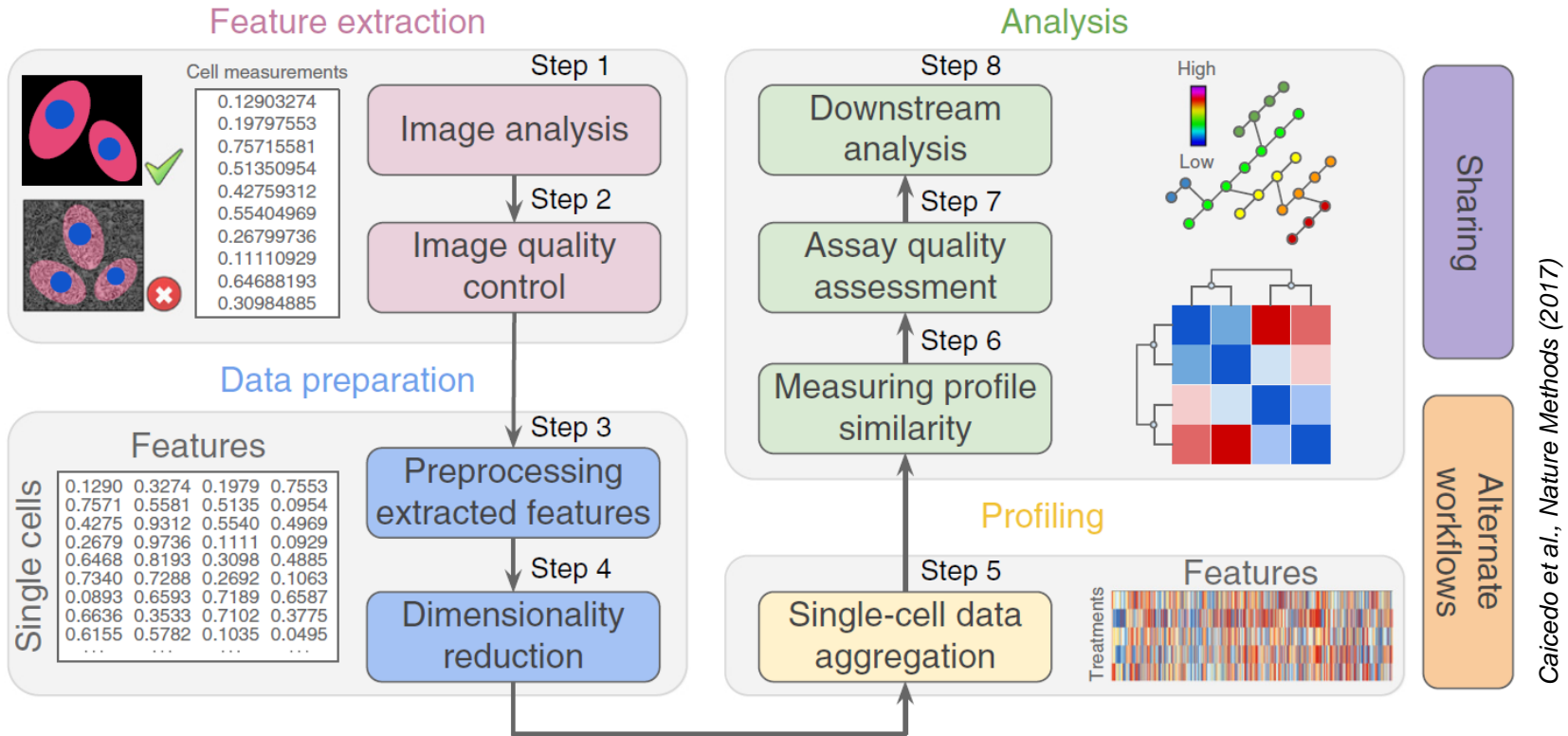
Slow (acquisition and analysis)
Complex optimization
Complex analysis

High-content screening... really?



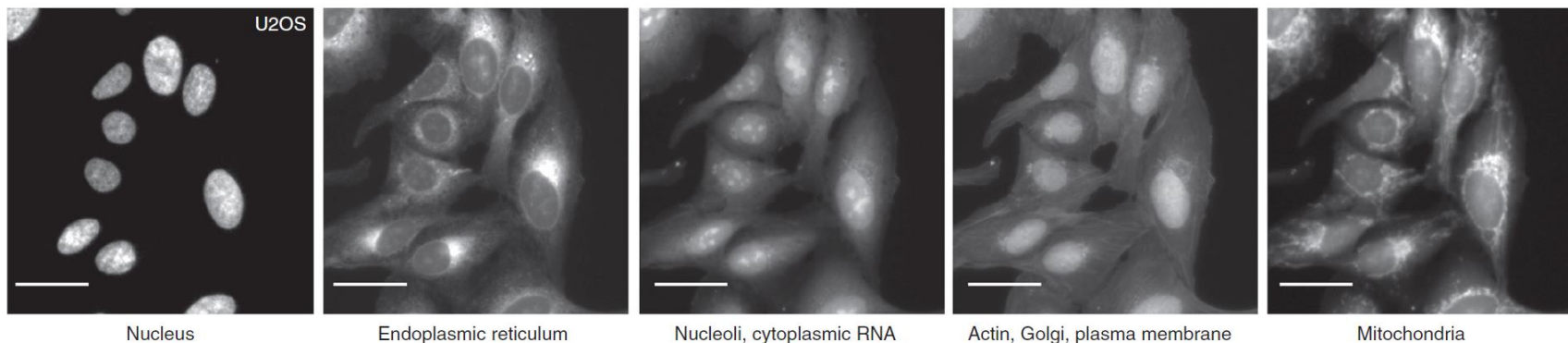
*“... although the number of HCS experiments published each year continues to grow steadily, the information content lags behind. We find that a **majority of high-content screens** published so far (60–80%) **made use of only one or two image-based features** measured from each sample and **disregarded the distribution of those features among each cell population.**”*

Image-based cell profiling

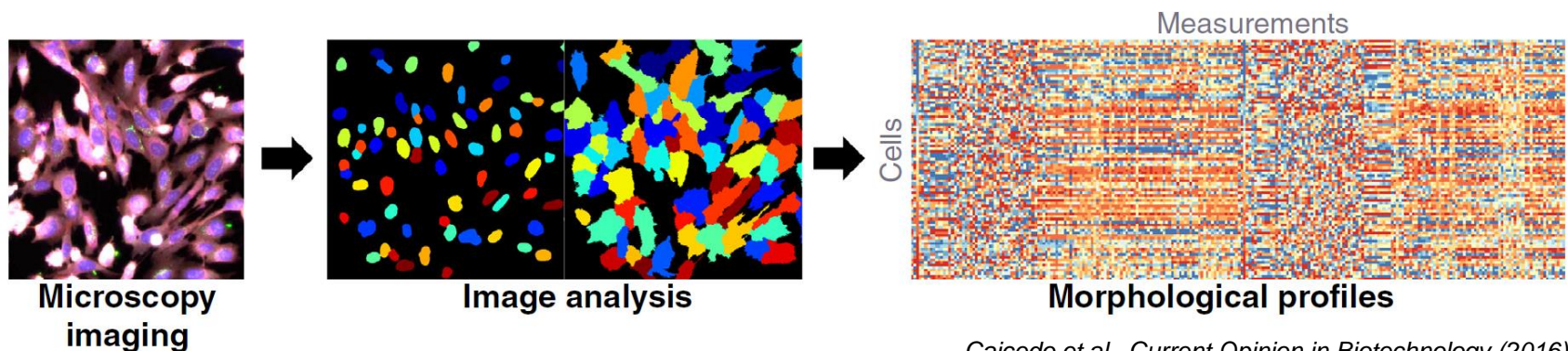


- Global profiling of (genetic, chemical) perturbations based on multiparametric analysis of cell morphology
- Typically involves multiplexed labeling (fluorescent dyes, antibodies) of subcellular structures/organelles

Cell Painting



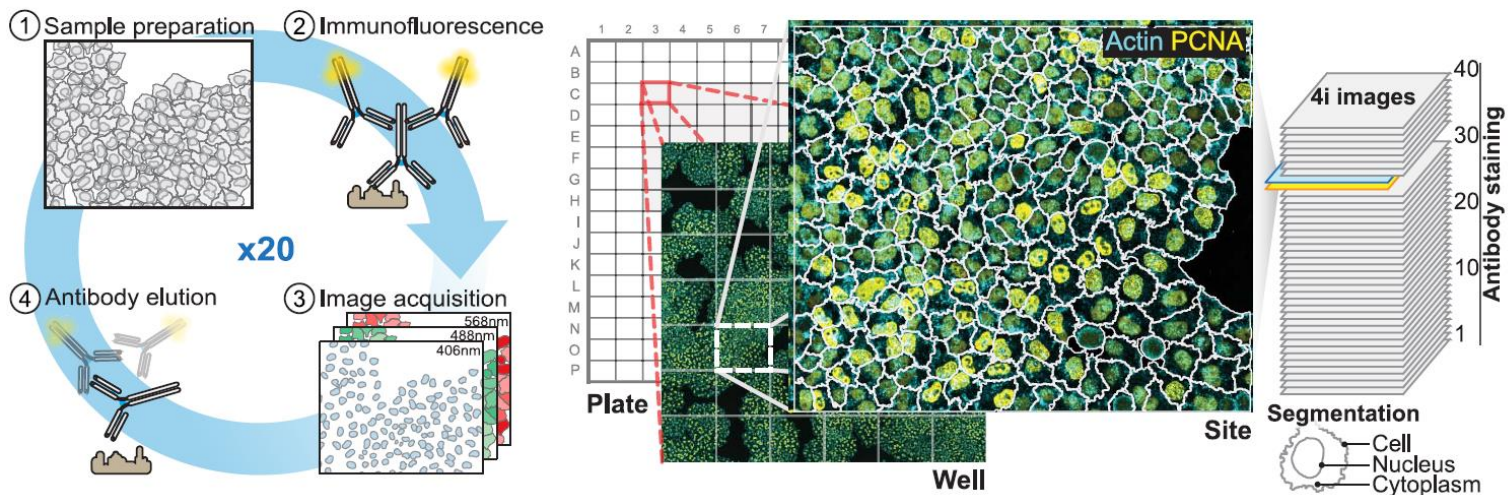
Bray et al., Nature Protocols (2016)



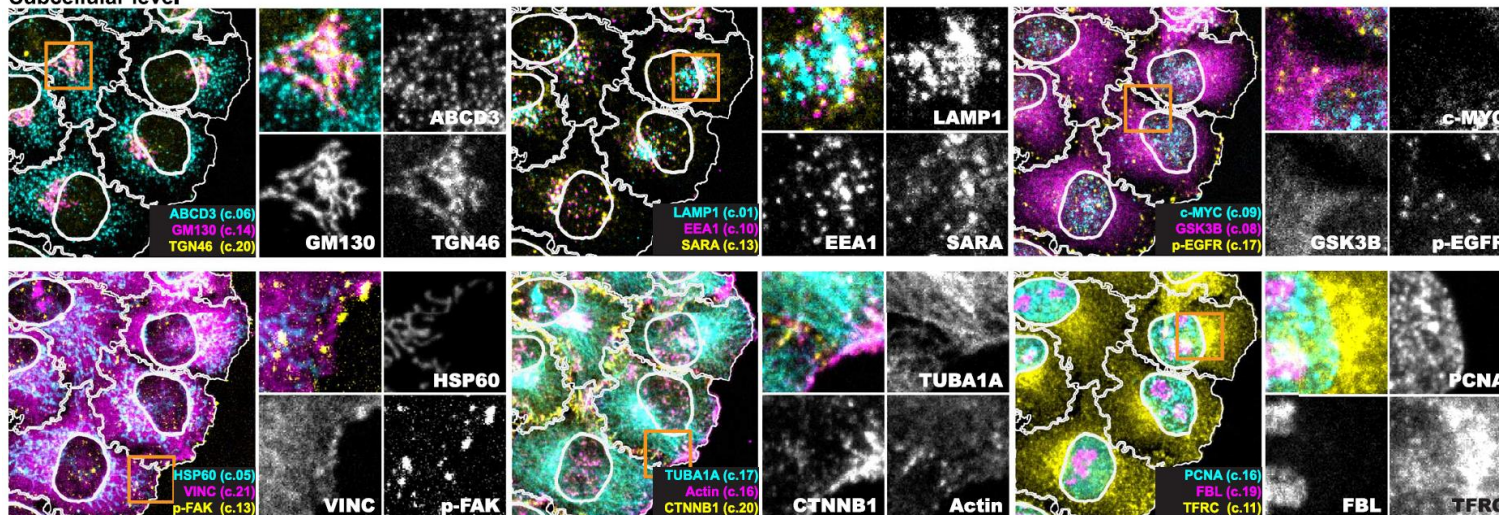
Caicedo et al., Current Opinion in Biotechnology (2016)

- 6 fluorescent dyes (no antibodies)
- 8 subcellular structures/organelles
- 5 channels
- ~1,500 morphological measurements (features) per cell

High-throughput multiplexed protein readouts interactive indirect immunofluorescence imaging



Subcellular level



Gut et al., Science (2018)

40 off-the-shelf antibodies (20 cycles); ~4h per cycle

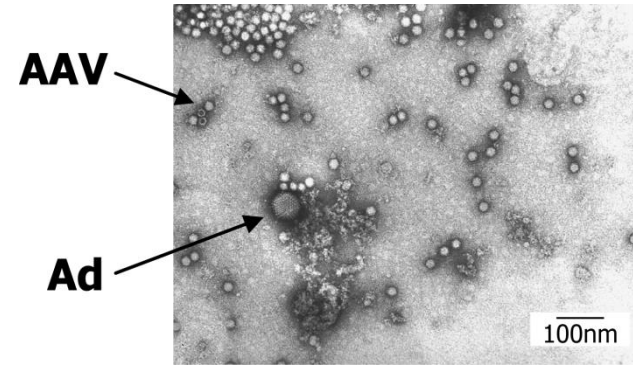
A fluorescence microscopy image showing a dense population of cells with bright green fluorescence. The signal is distributed throughout the field of view, with some brighter spots and some dimmer areas, suggesting a heterogeneous expression of the marker. The background is dark, making the green signal stand out.

GENOME-WIDE siRNA SCREENING

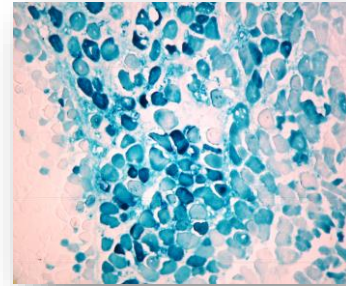
**Identification of host cell factors
controlling transduction of vectors
based on Adeno-Associated Virus (AAV)**

Adeno-Associated Virus (AAV) vectors

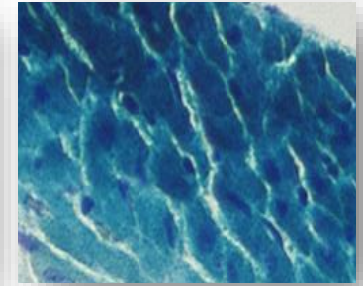
- Vectors based on AAV are efficient gene transfer tools
- AAV vectors transduce differentiated, post-mitotic tissues with high efficiency
- AAV vectors permit a sustained, long term persistence of the transgene in vivo without integrating into the host genome
- AAV vectors can be prepared at high titers and purity
- Expression of the gene of interest can be driven by any desirable promoter
- **Outcome of transduction is critically dependent on the interplay between vectors and the host cell**



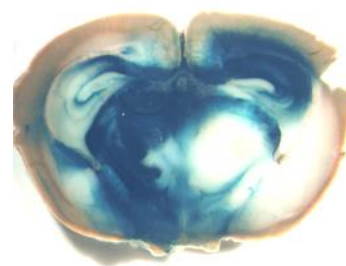
Skeletal muscle



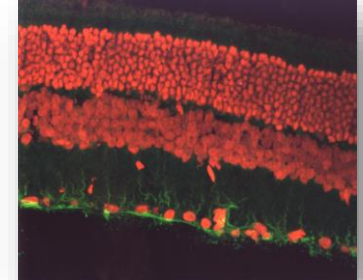
Heart



Brain



Retina



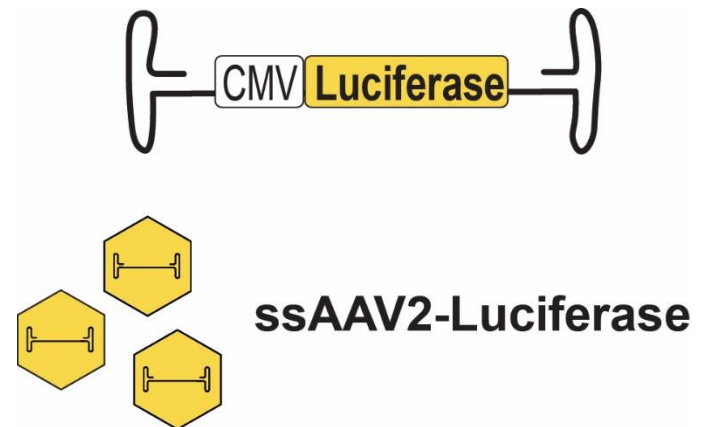
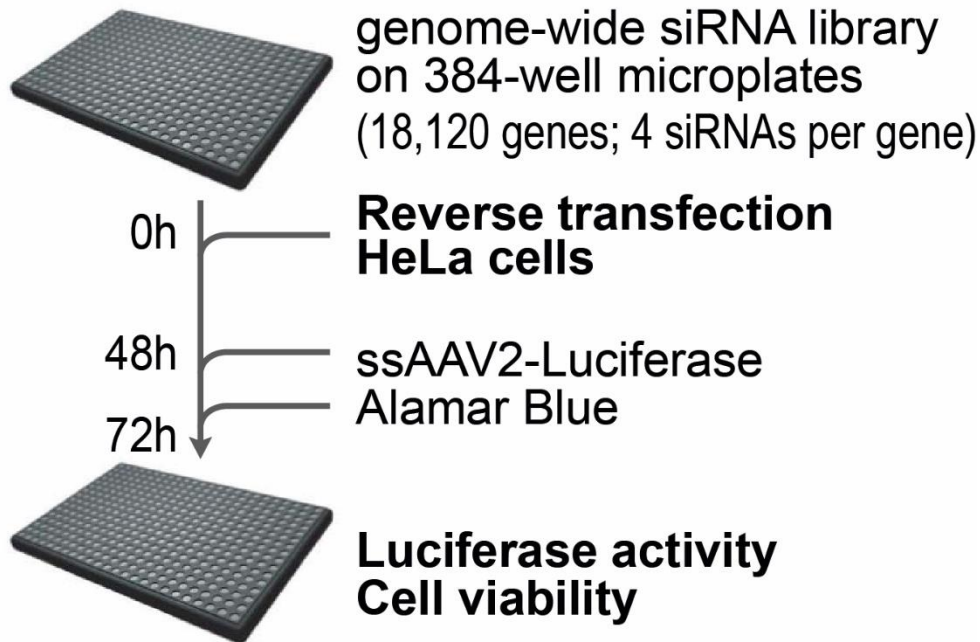
Genome-wide siRNA screening for AAV transduction

Main Goals

- **Identify host cell factors controlling transduction by AAV vectors**
- **Improve current knowledge of the intracellular processing of AAV vectors**
- **Improve AAV as vectors for gene therapy**
 - Broaden the cell/tissue tropism of AAV vectors
 - Increase efficiency of AAV vectors
 - Decrease AAV vector dosage

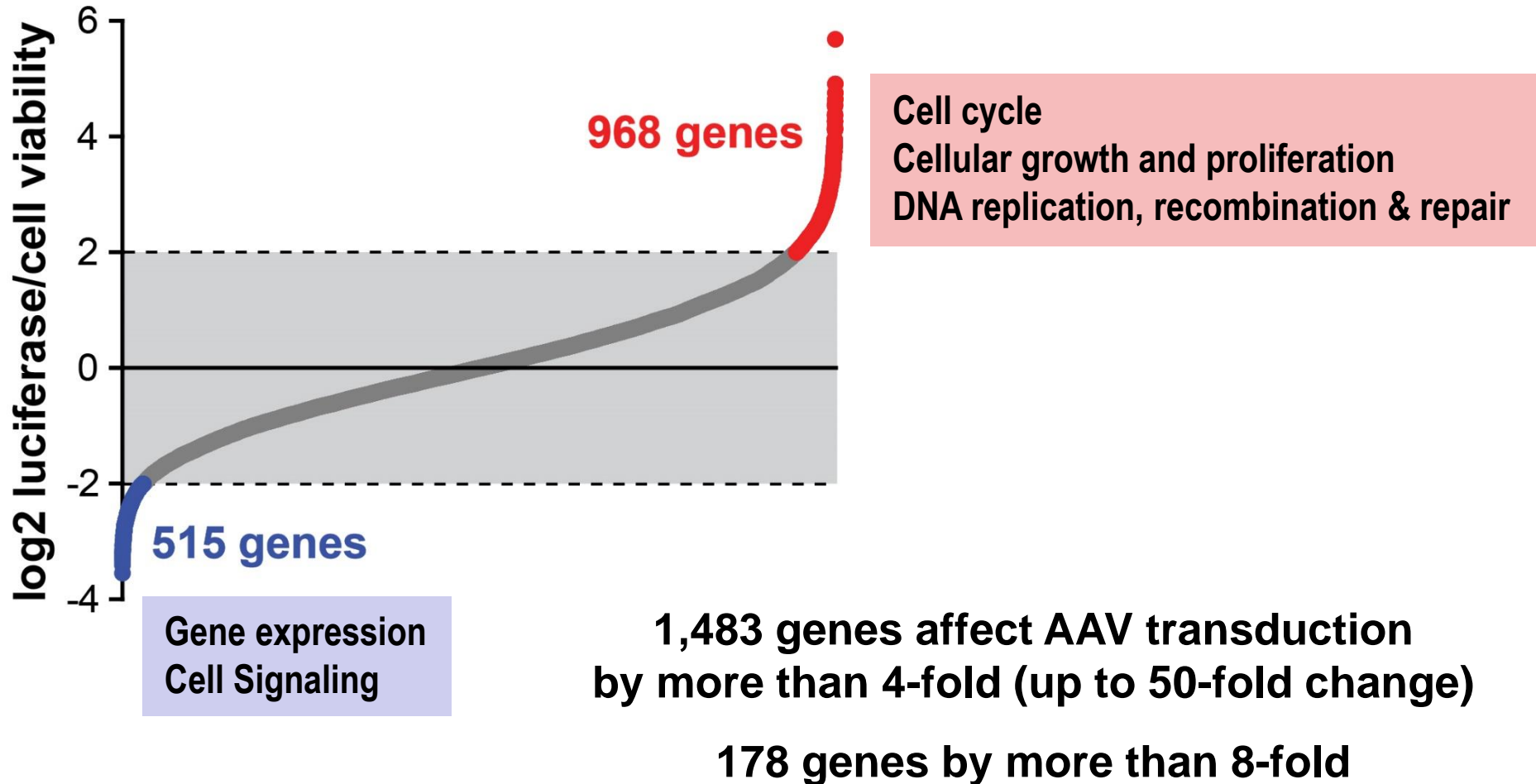
Genome-wide siRNA screening for AAV transduction

Experimental Workflow

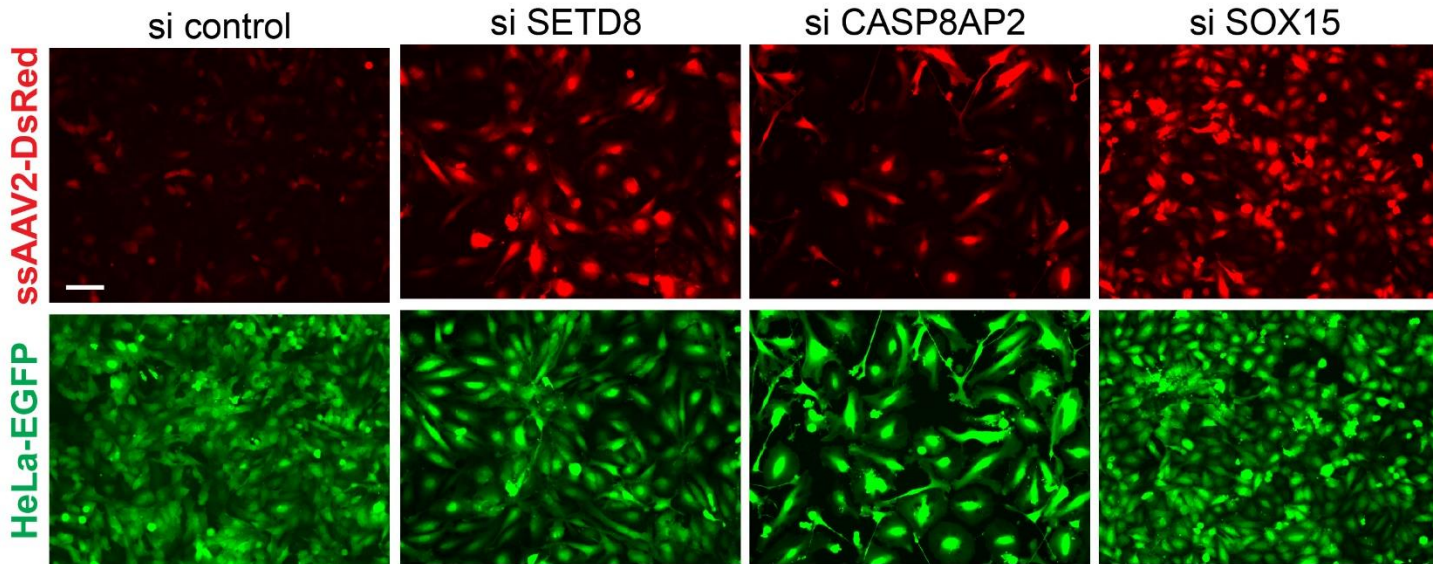
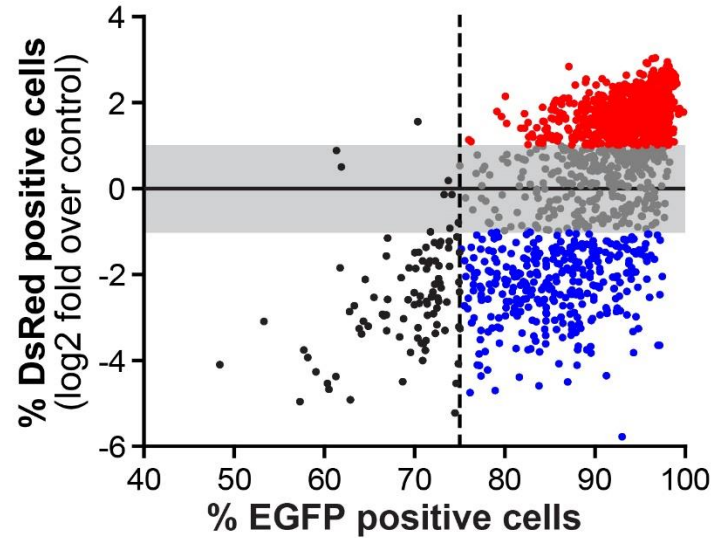
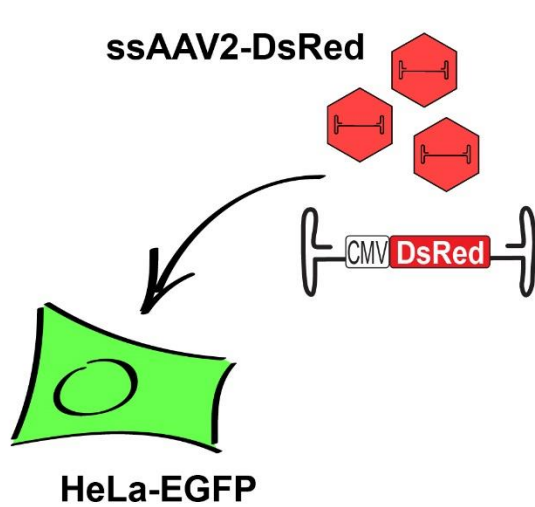


Genome-wide siRNA screening for AAV transduction

18,120 GENE TARGETS

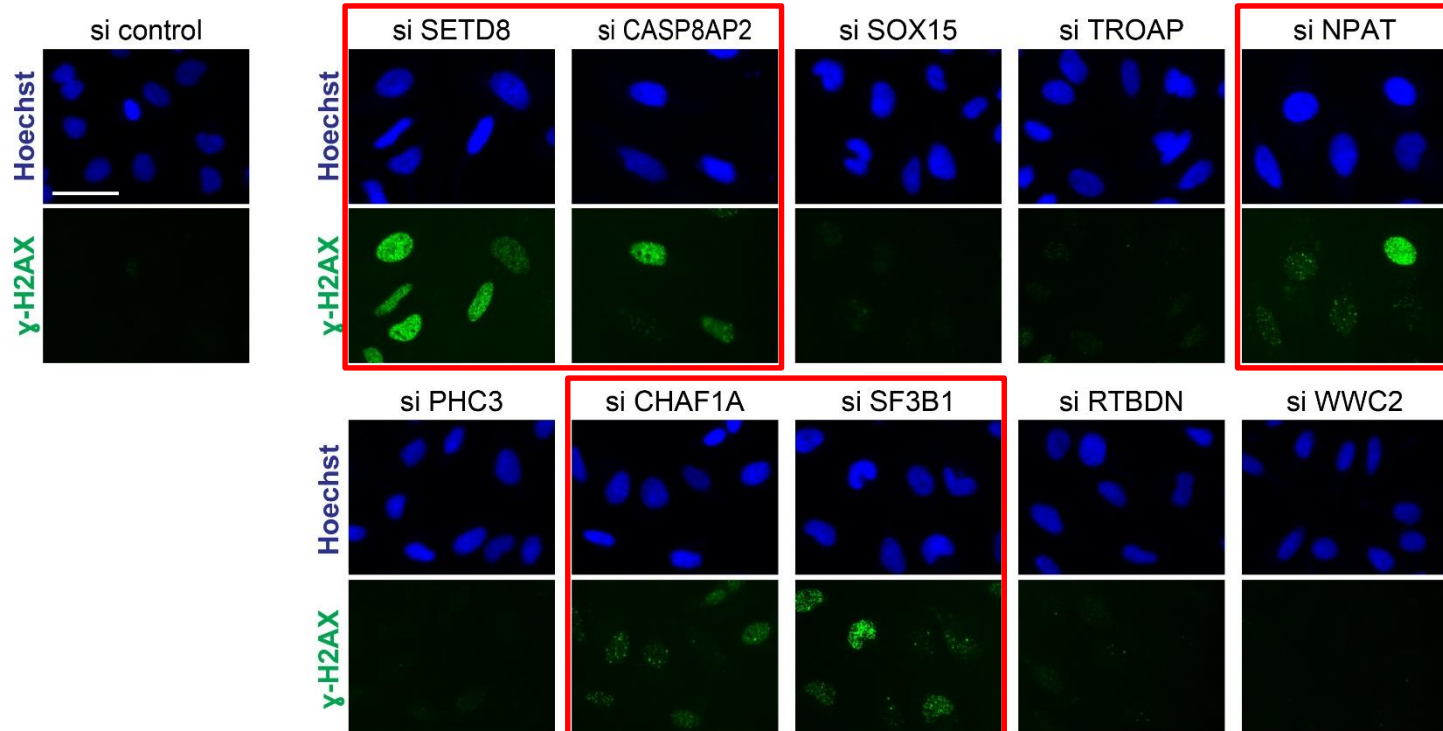
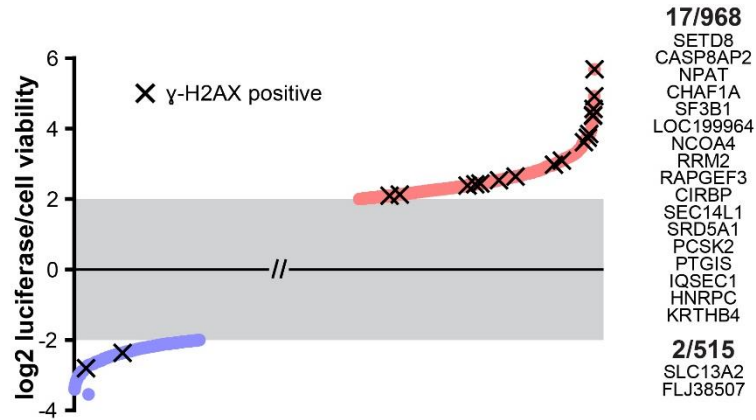


Secondary screening – Refinement for AAV-specific targets



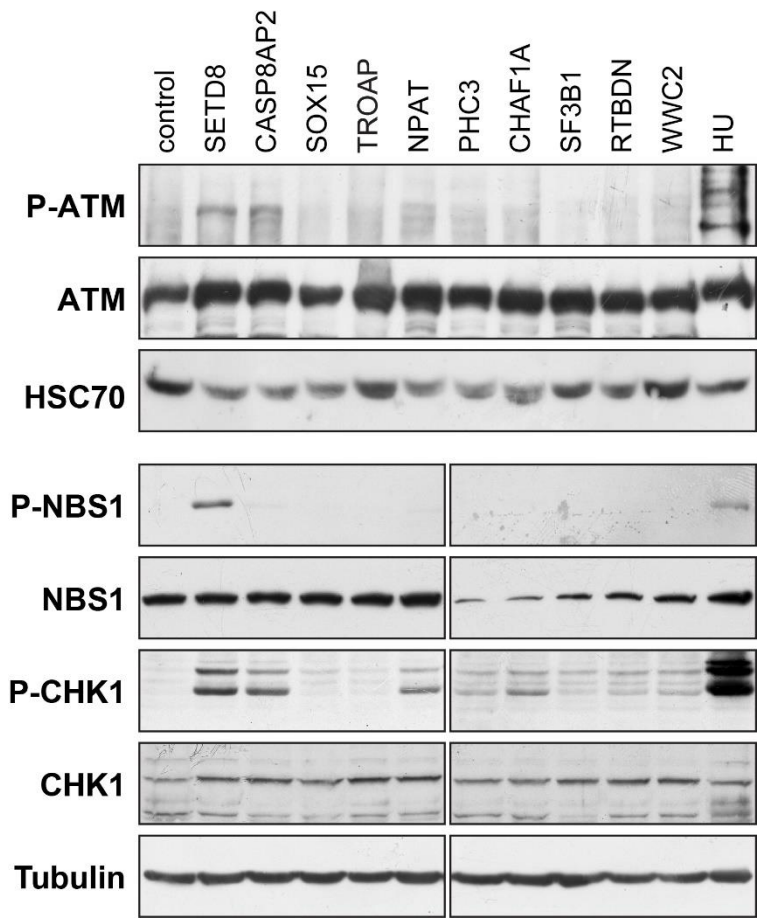
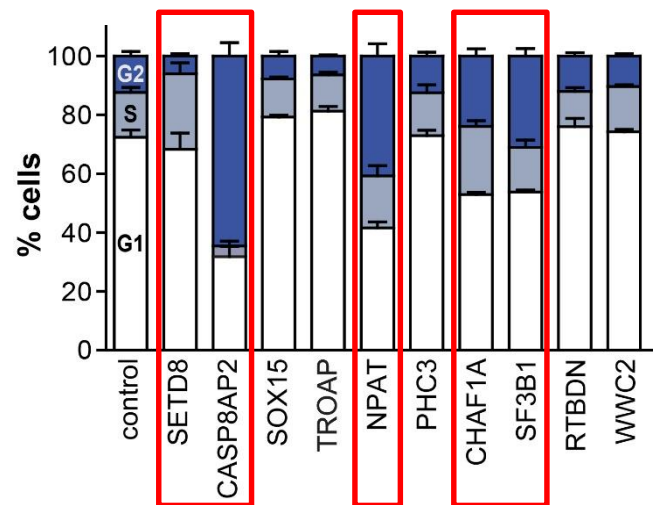
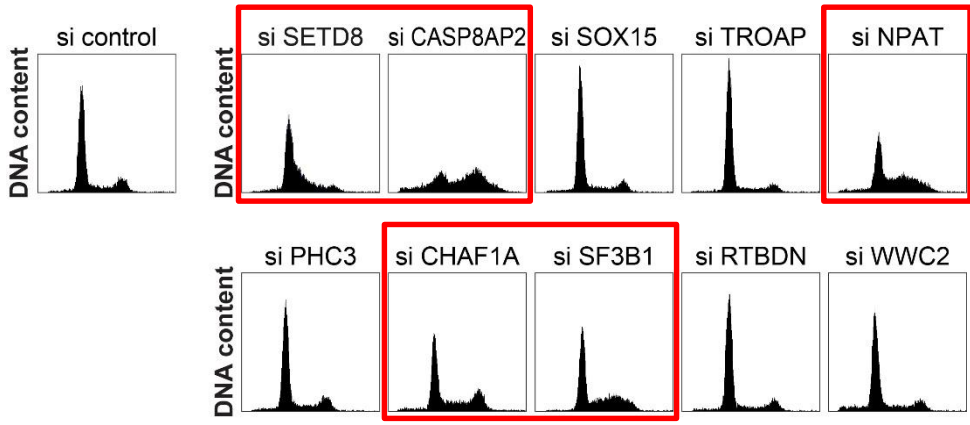
4 fields per well, 10x magnification, average 1,500 cells analyzed per well

Secondary screening – γ H2AX foci



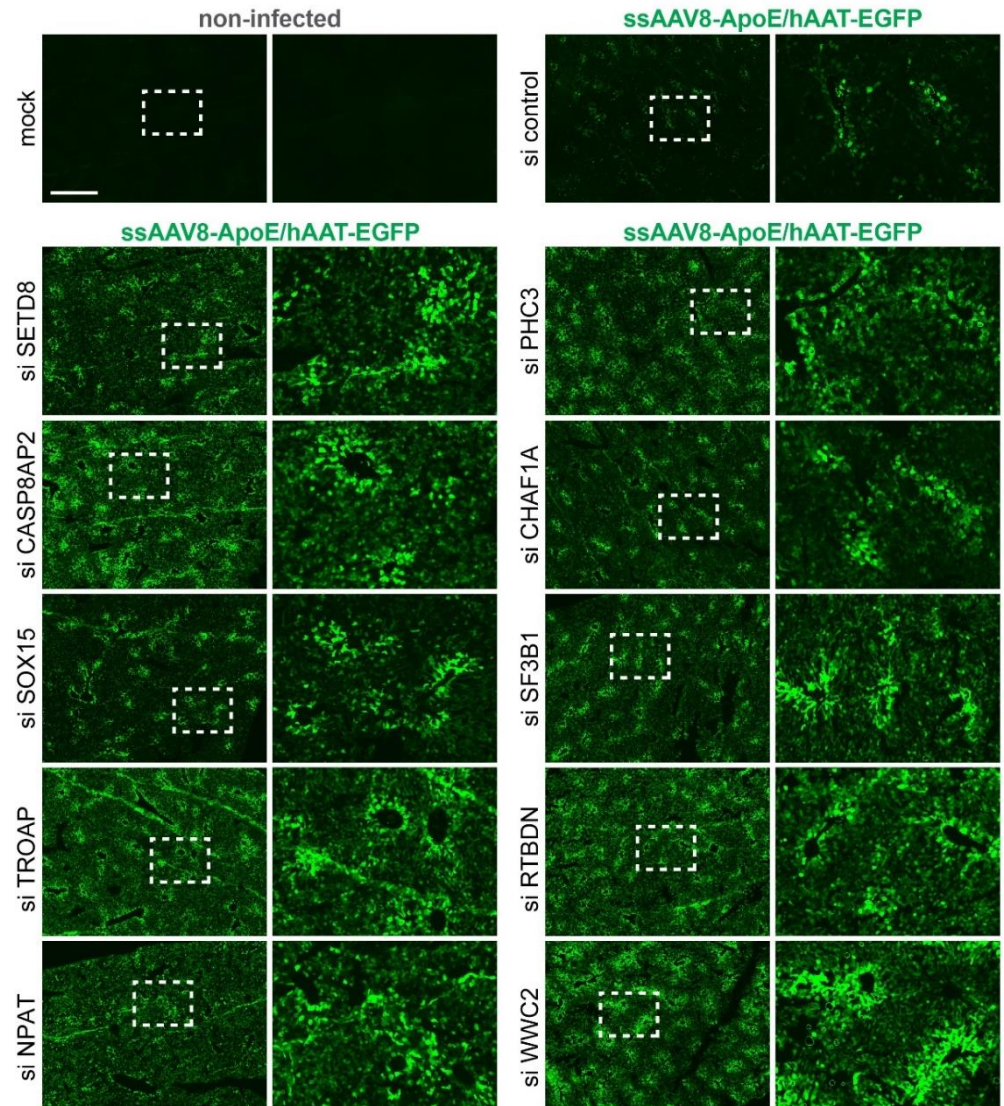
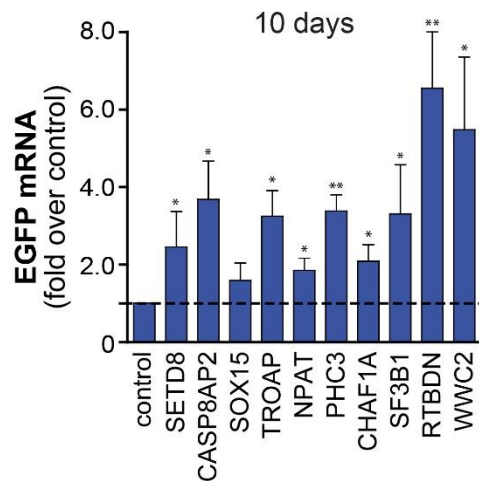
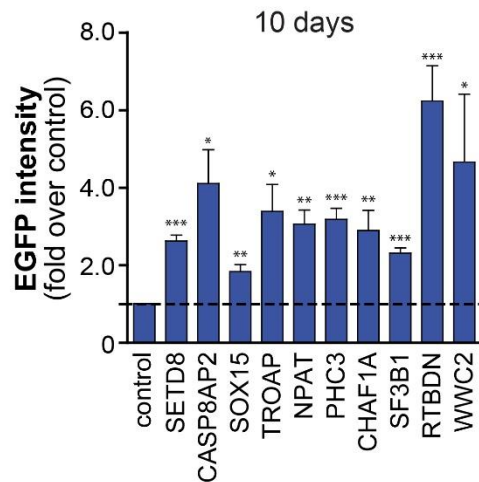
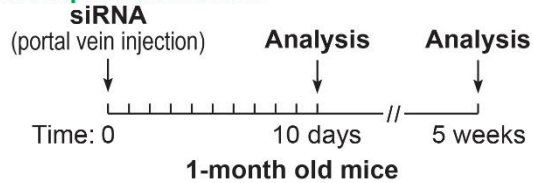
9 fields per well, 20x magnification

DNA Damage Response – Top 10 gene targets



Effect of siRNAs on AAV transduction in vivo (liver)

ssAAV8-ApoE/hAAT-EGFP





microRNA SCREENING

**microRNAs controlling
infection by bacterial pathogens**

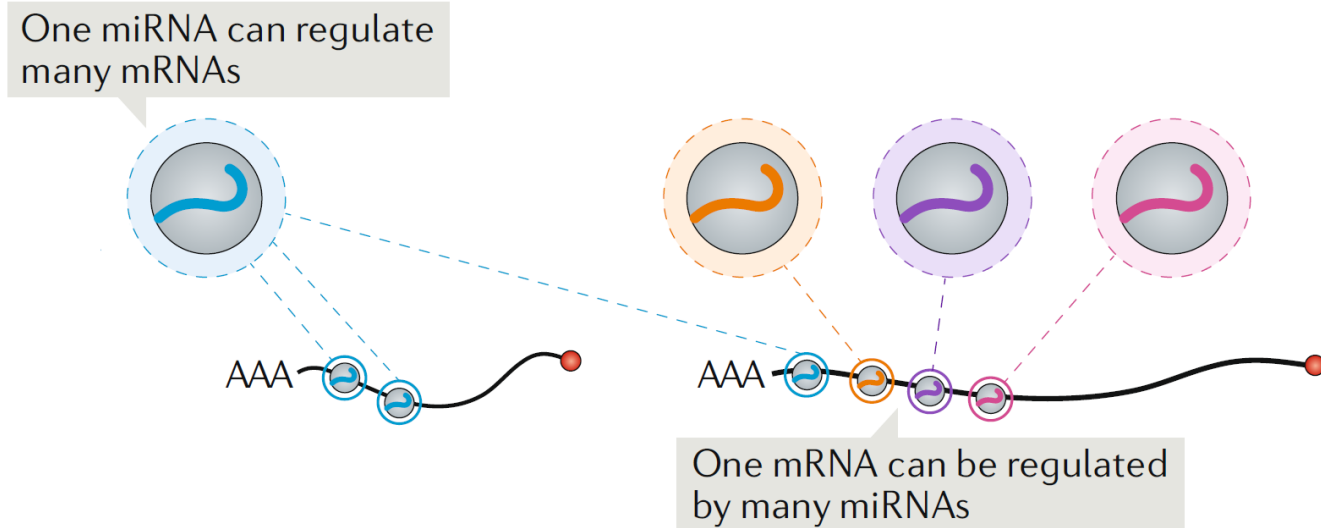
in collaboration with Ana Eulalio lab

Institute for Molecular Infection Biology | University of Würzburg, Germany
Center for Neuroscience and Cell Biology | University of Coimbra, Portugal

MicroRNAs

MicroRNAs are genome-encoded (20-22nt) non-coding RNAs that play a major role in the post-transcriptional regulation of gene expression

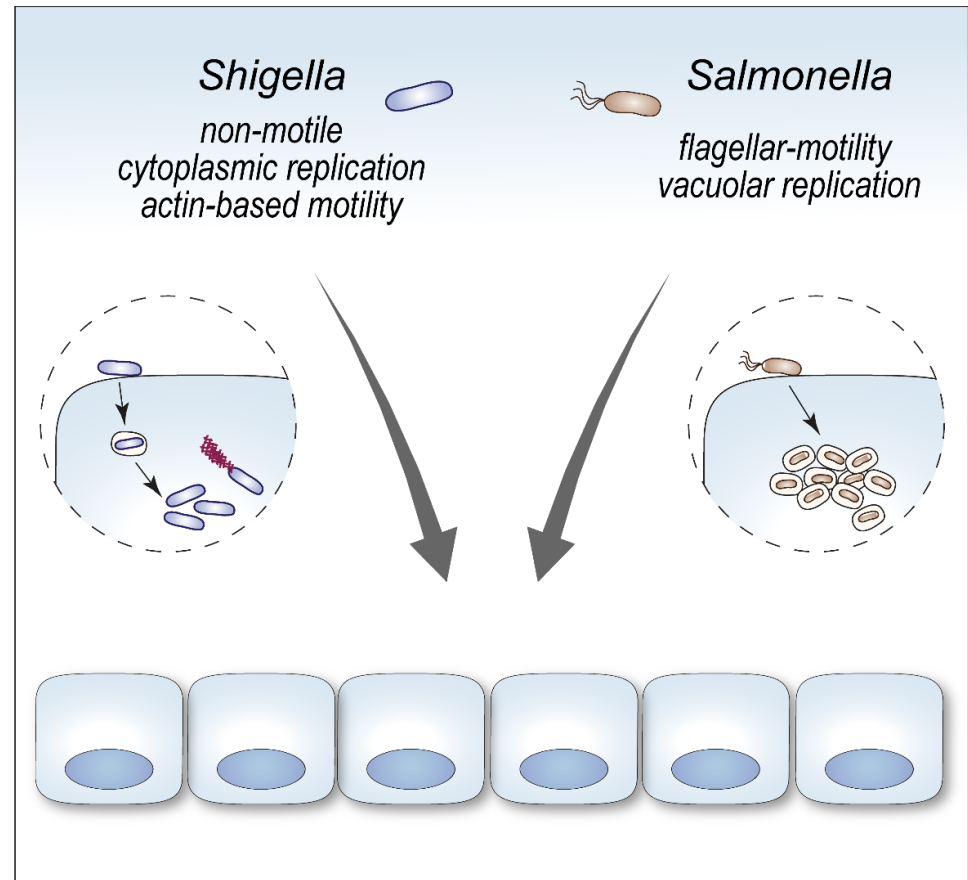
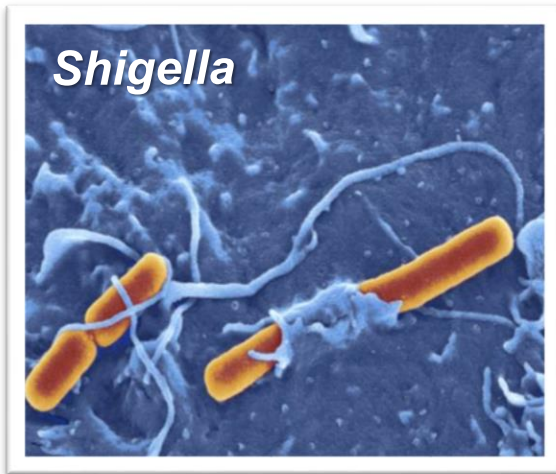
More than 2,500 human mature miRNAs currently annotated



adapted from Gebert & MacRae. 2018. Nat Rev Mol Cell Biol.

MicroRNAs have been shown to be involved in a broad range of biological processes

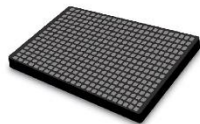
Shigella flexneri and *Salmonella* Typhimurium



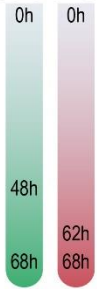
Among the most important bacterial pathogens causing food-borne diseases

Host miRNAs are major regulators of *Salmonella* and *Shigella* infection

library of miRNA mimics
arrayed on 384-well plates
(2,042 mature sequences)



Reverse transfection



Salmonella infection

Fixation and staining
(20h post-infection)

Shigella infection
Fixation and staining
(6h post-infection)

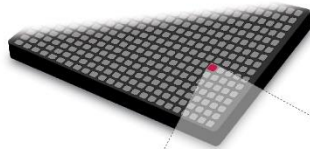
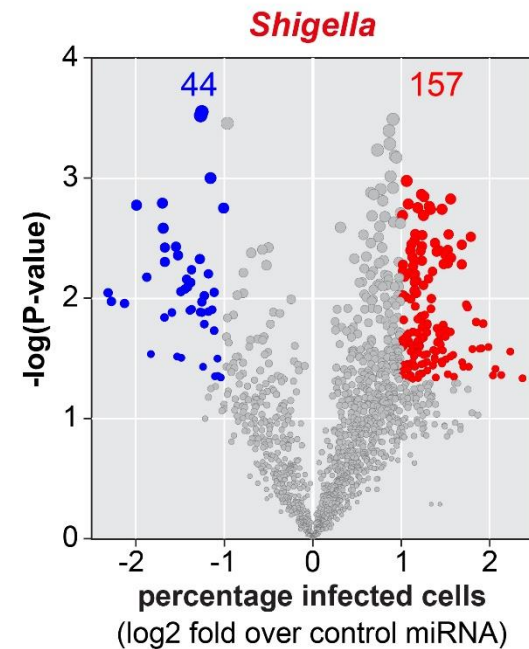
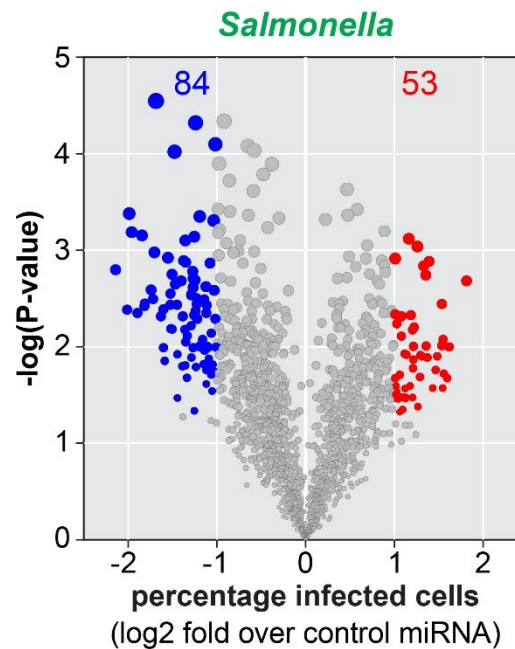
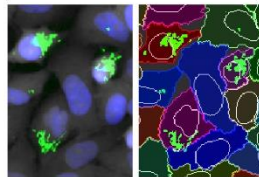
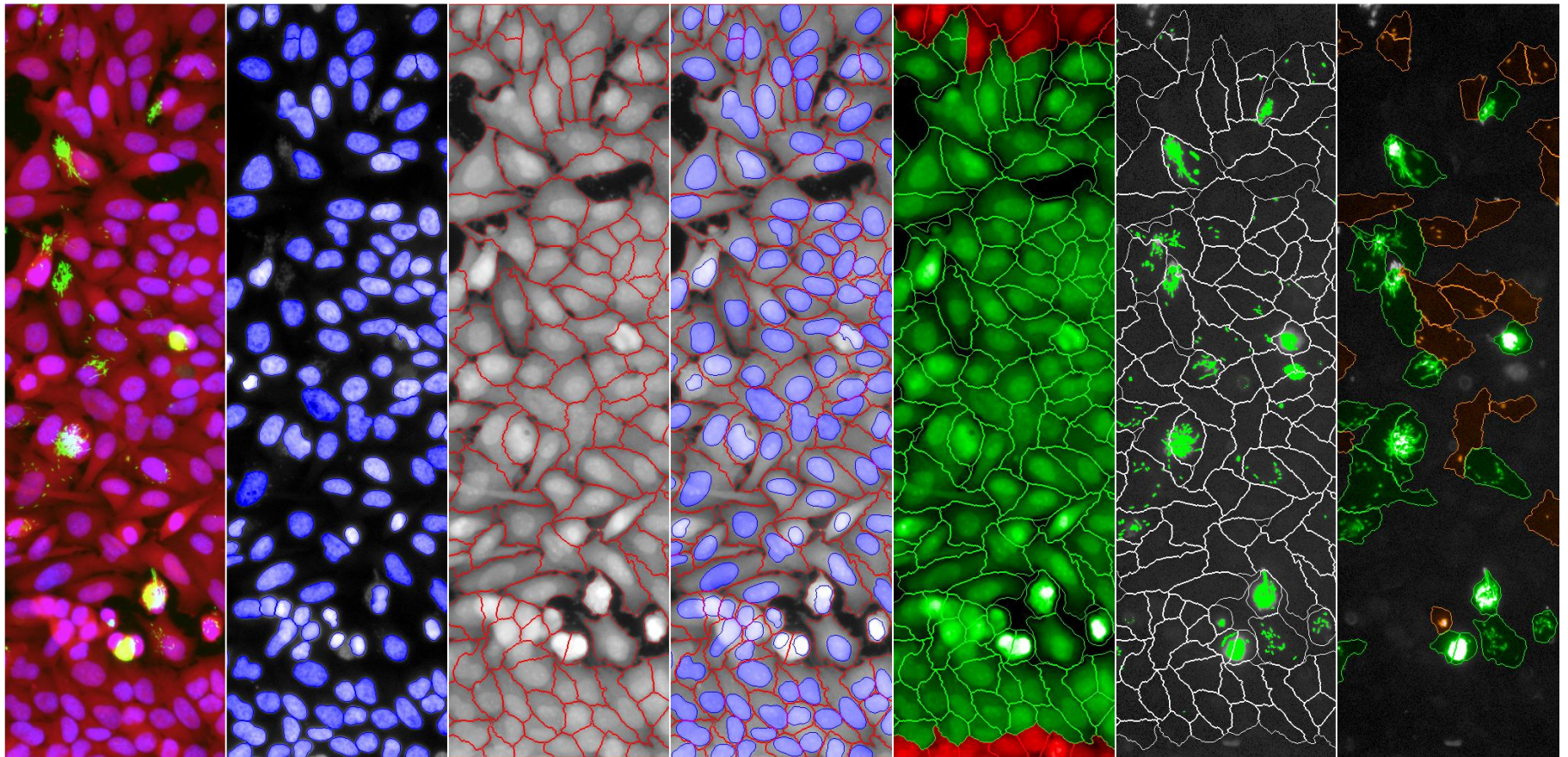


Image acquisition
Image analysis

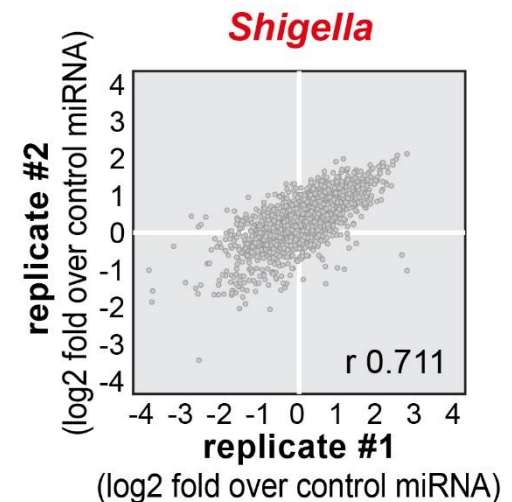
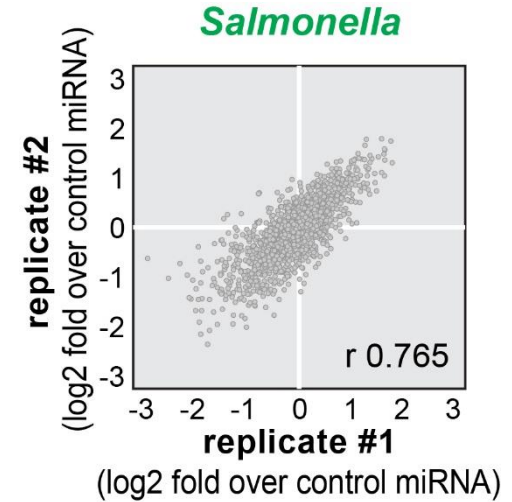
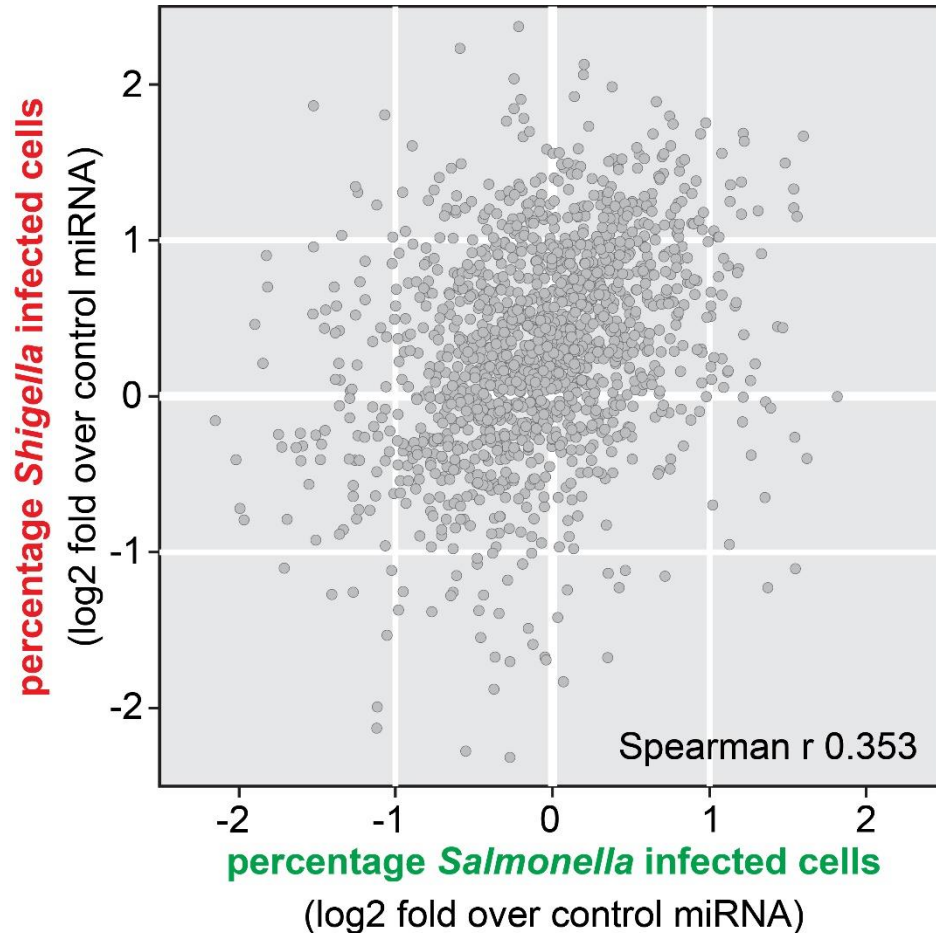


High-Content Screening: analysis of bacterial infection

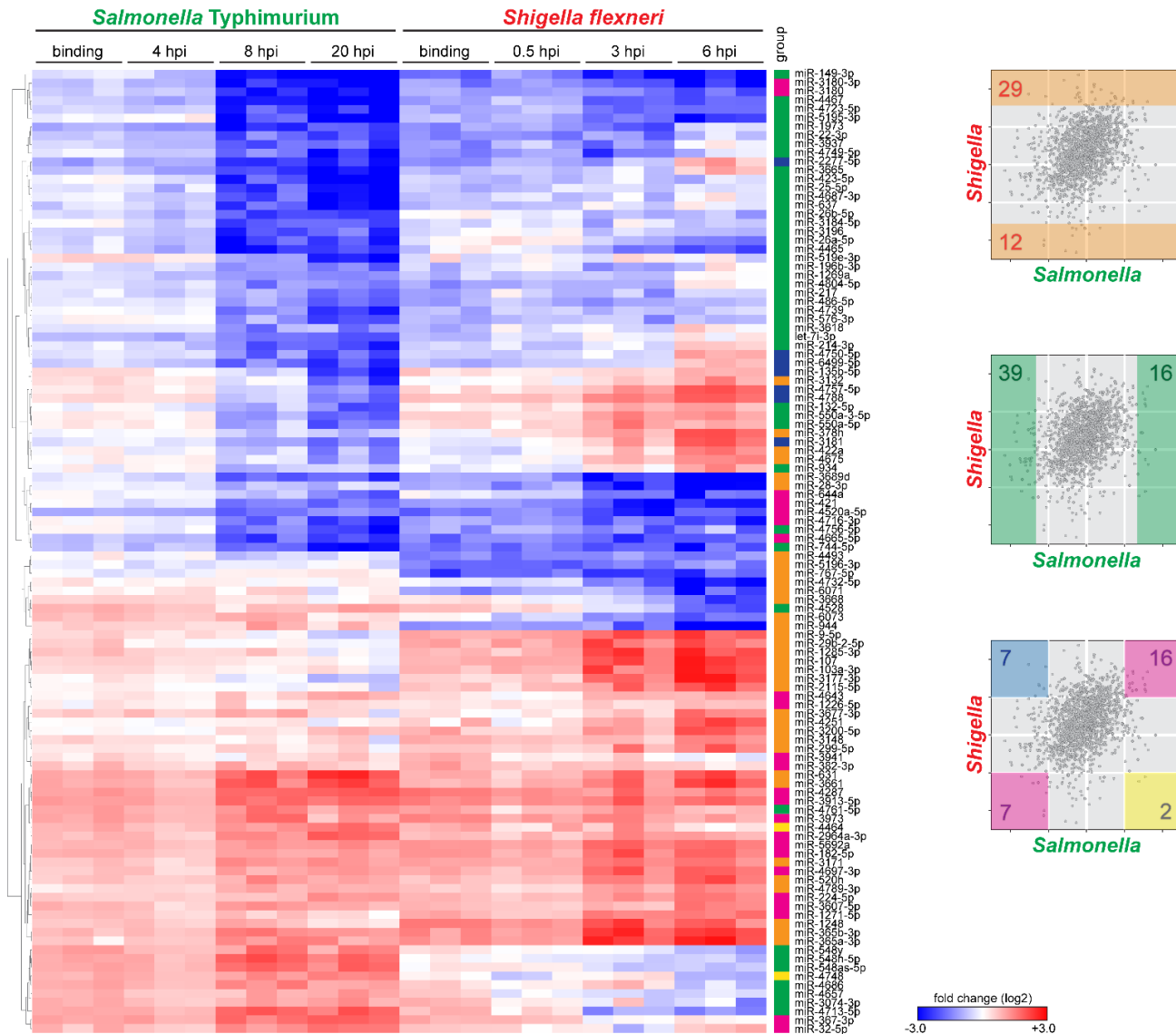
Unbiased extraction of multiparametric numerical data from images
Subcellular resolution, single-cell analysis



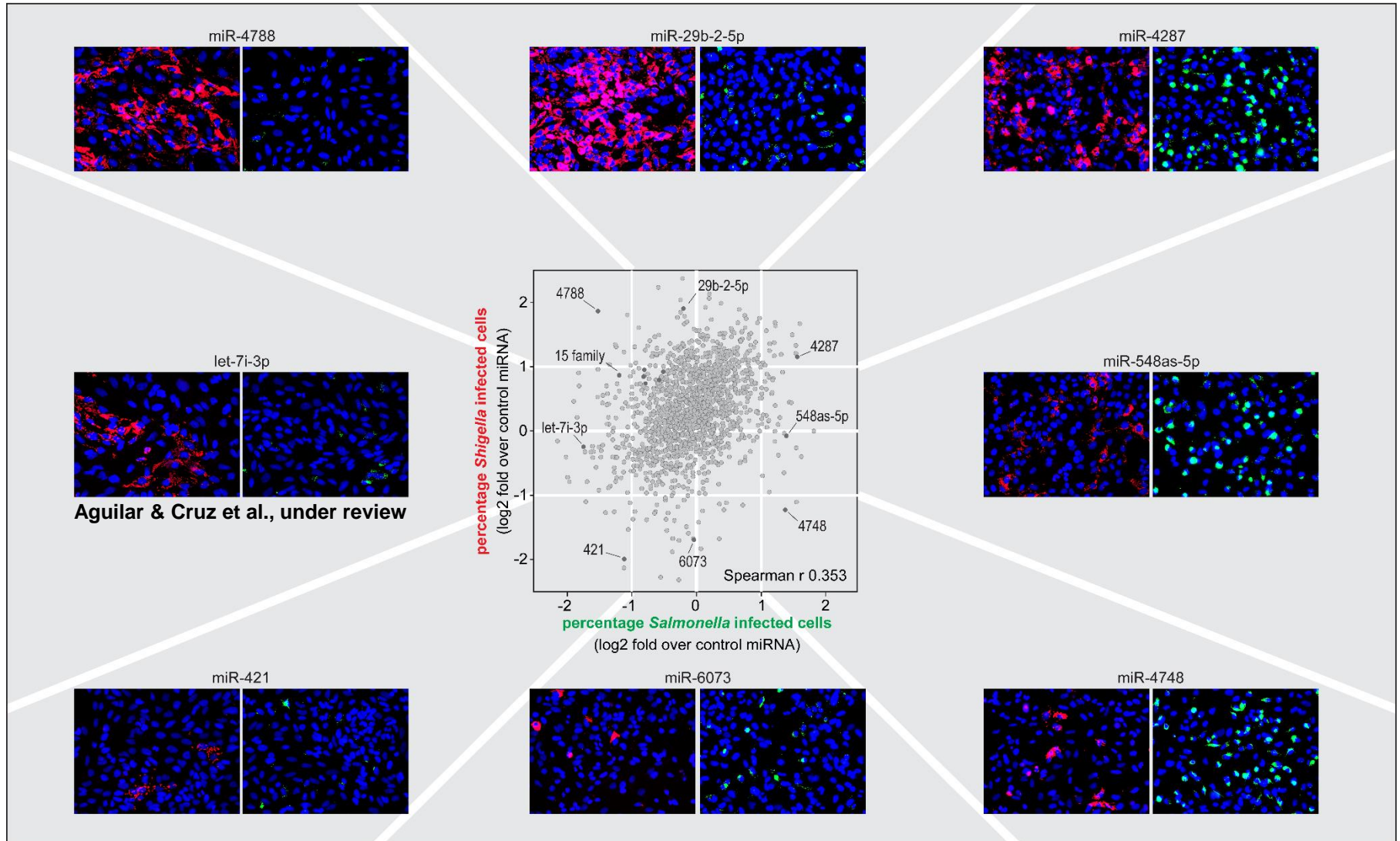
Salmonella and *Shigella* infection are regulated by a different set of miRNAs



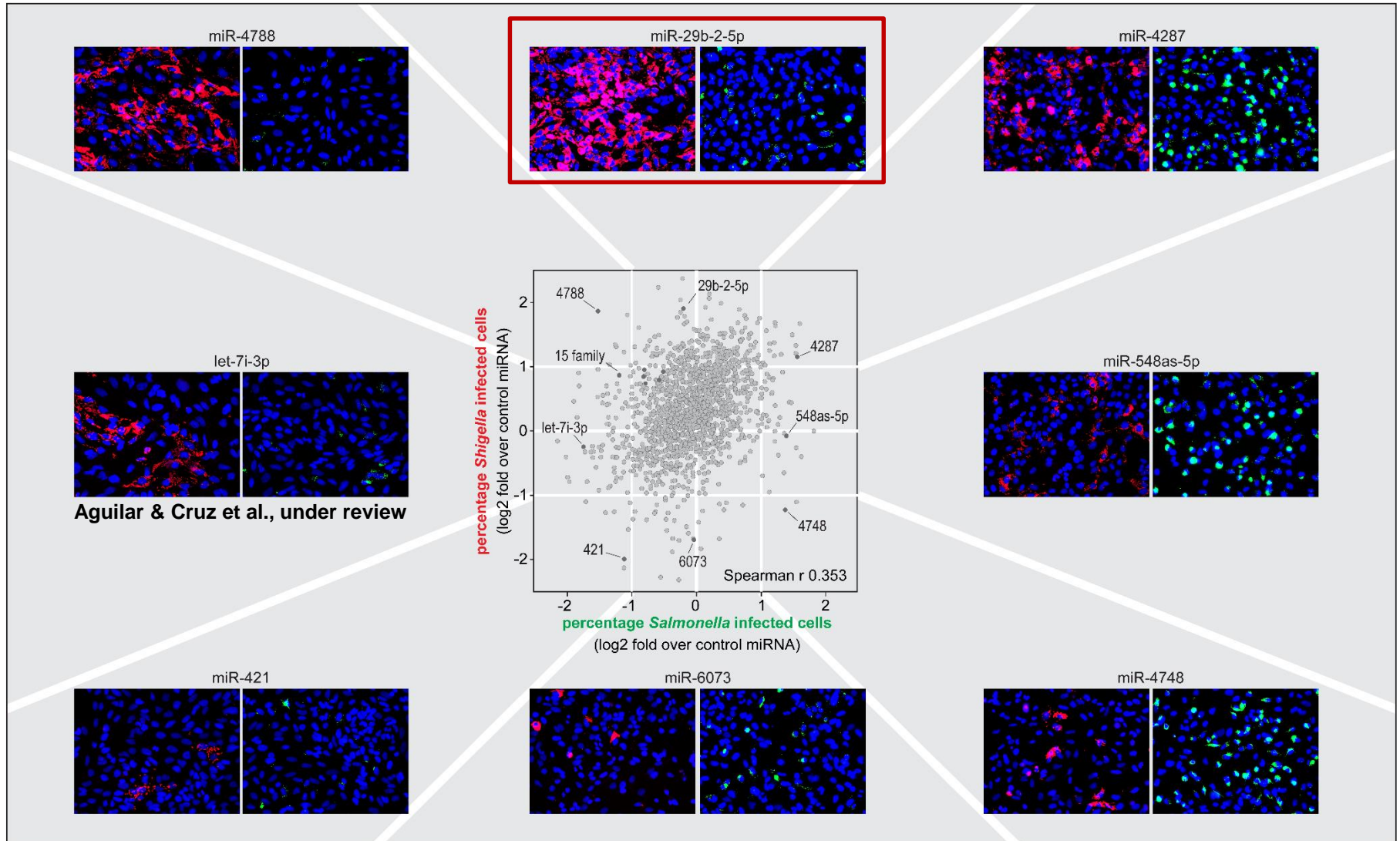
miRNAs interfere with different stages of *Salmonella* and *Shigella* infection



Salmonella and Shigella infection are regulated by a different set of miRNAs

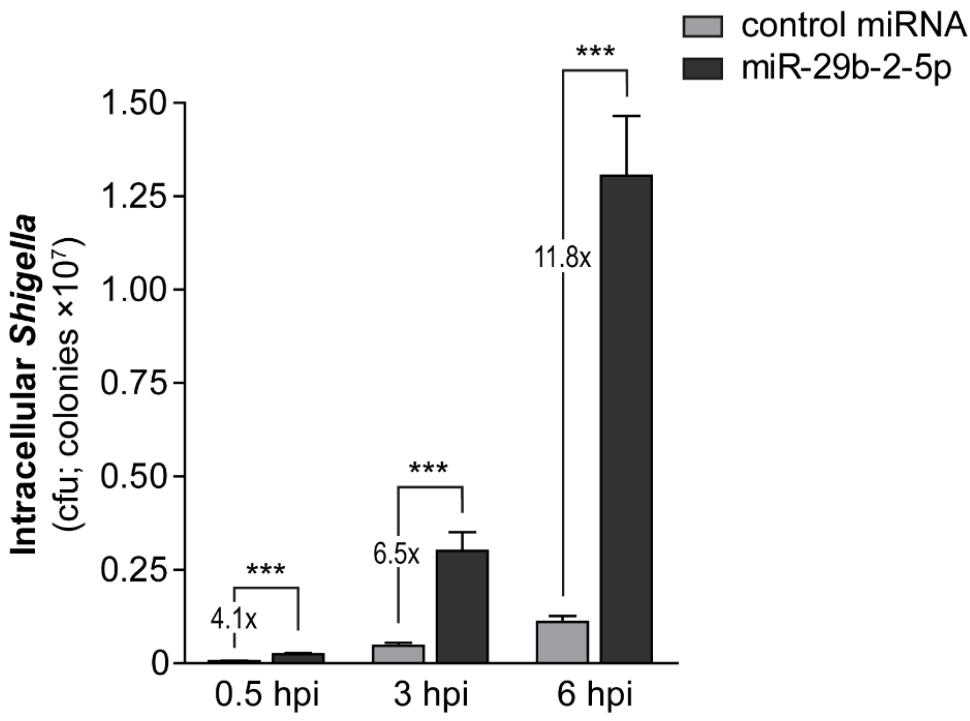


Salmonella and Shigella infection are regulated by a different set of miRNAs



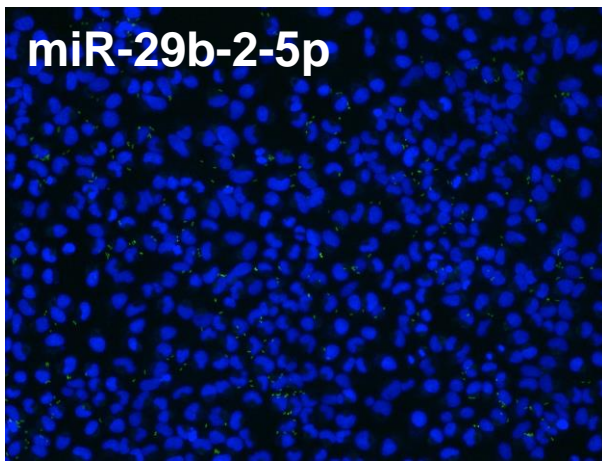
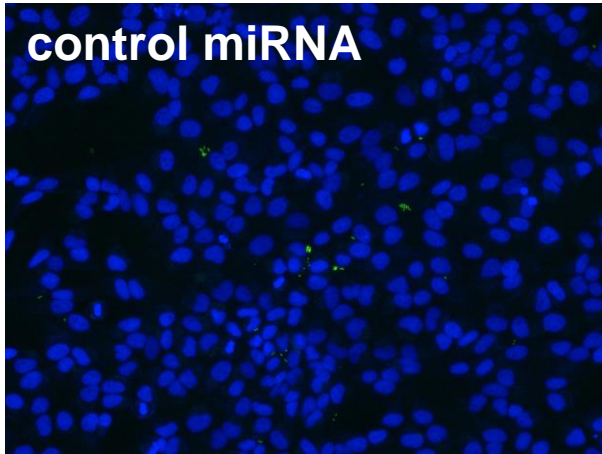
miR-29b-2-5p increases both early and late steps of *Shigella* infection

Hoechst *Shigella flexneri*

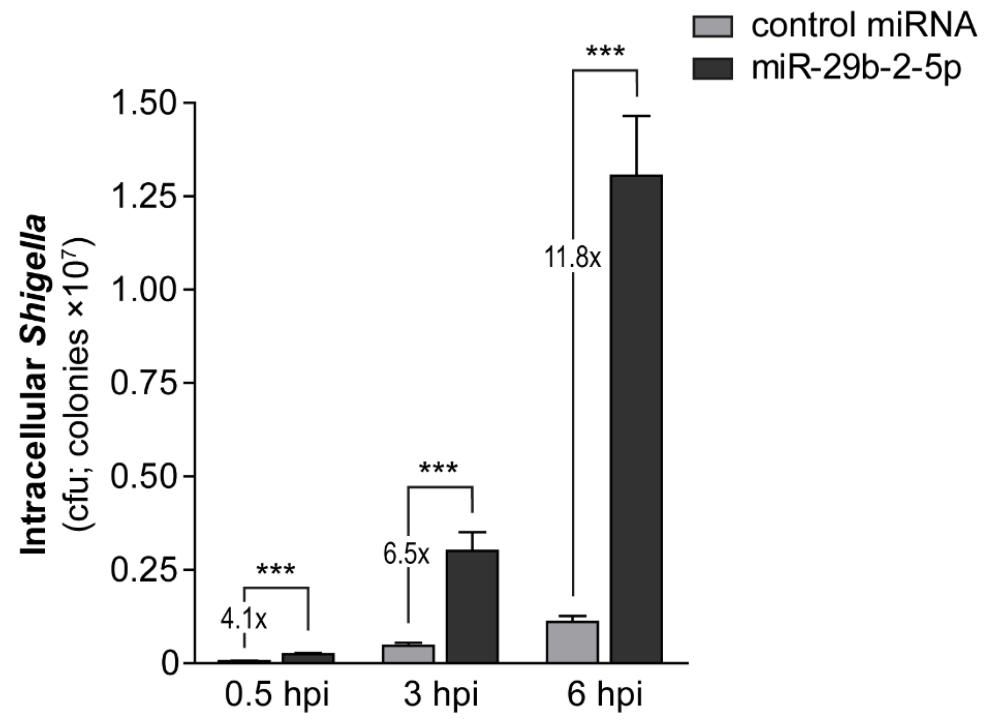


6 h time course

miR-29b-2-5p increases both early and late steps of *Shigella* infection

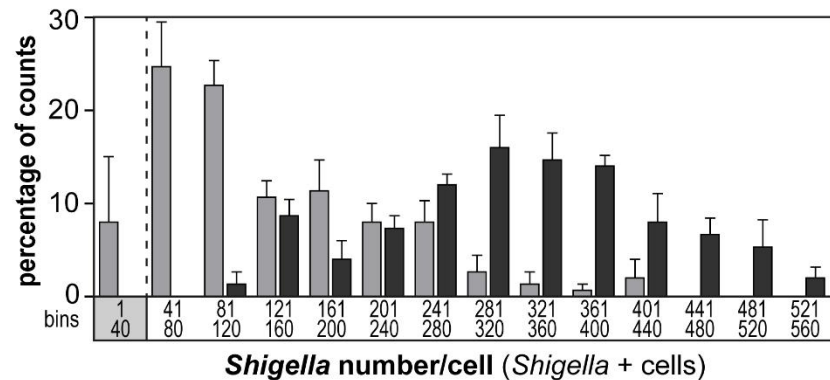
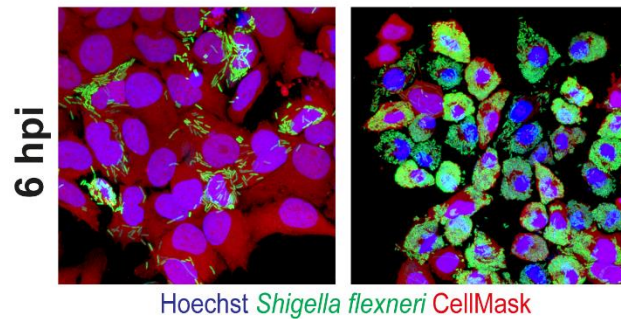
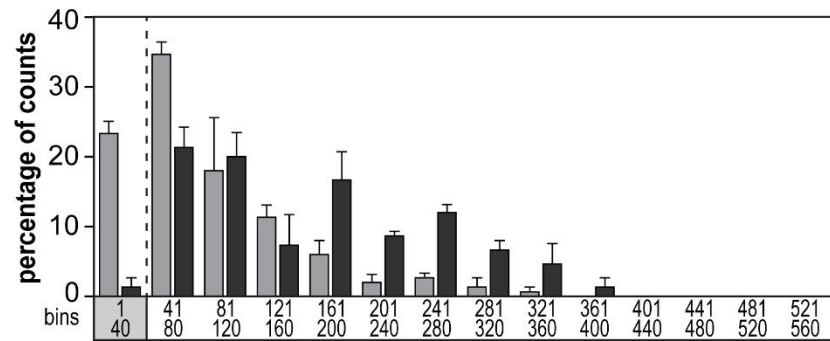
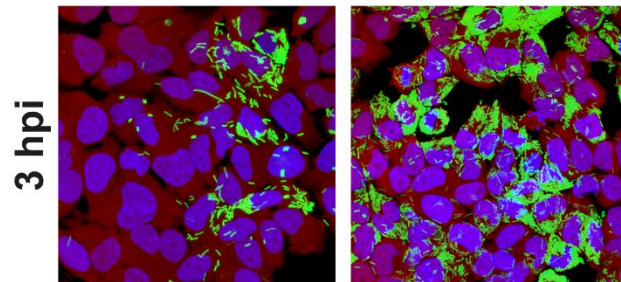
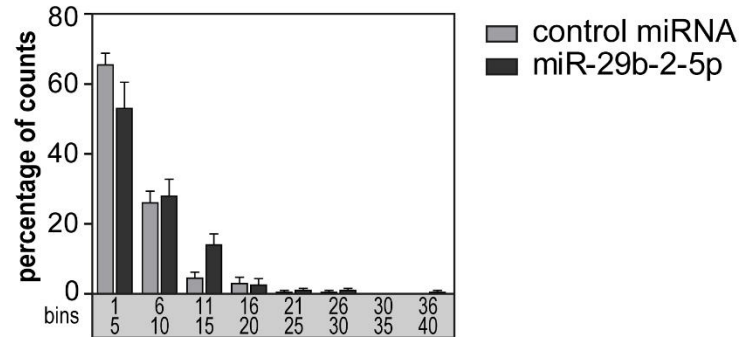
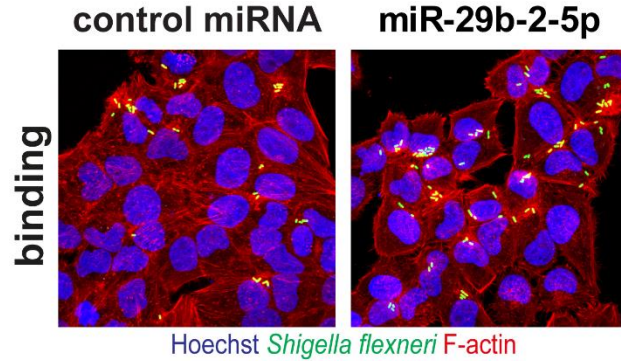


Hoechst *Shigella flexneri*

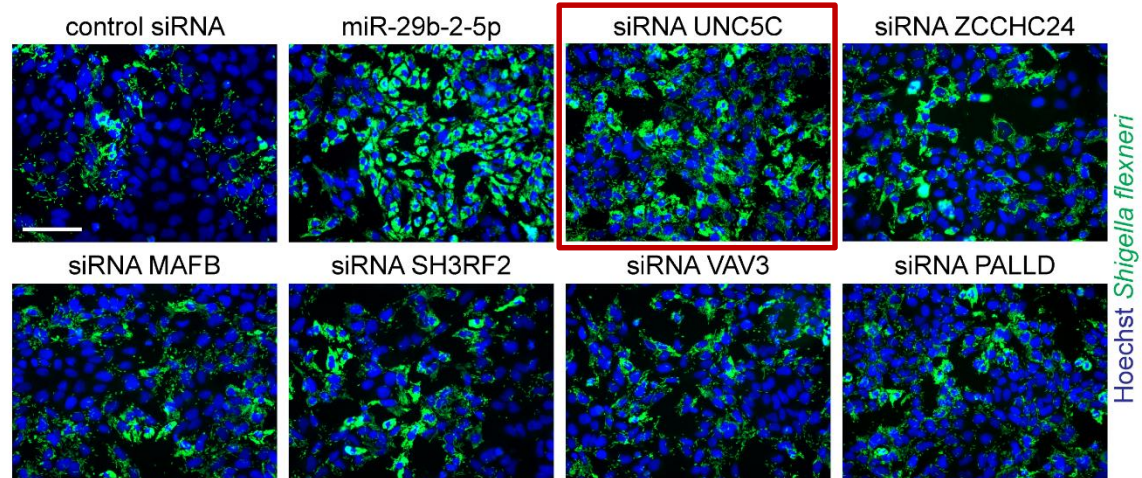
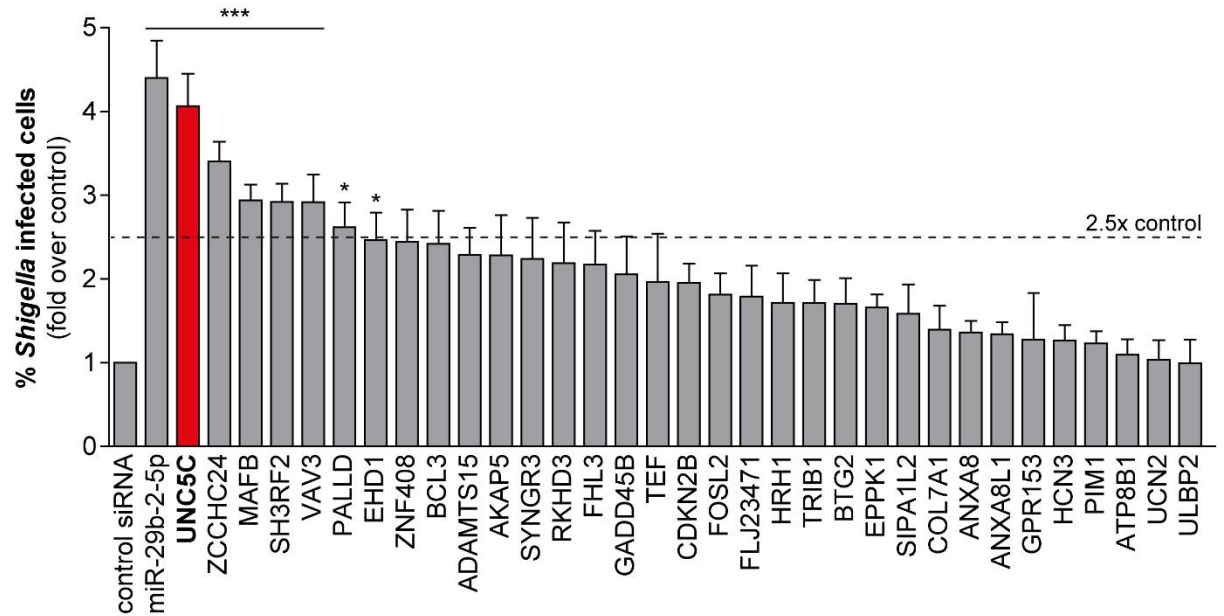
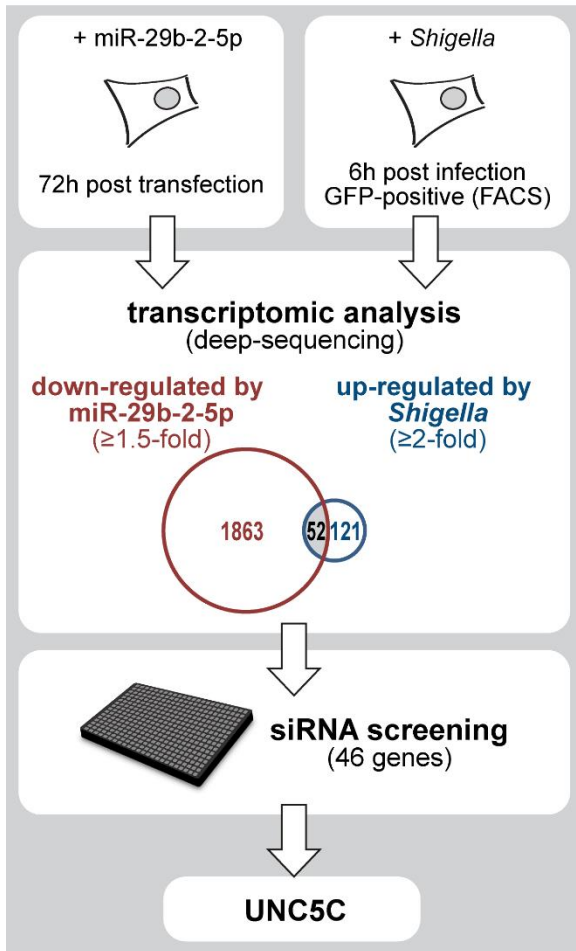


6 h time course

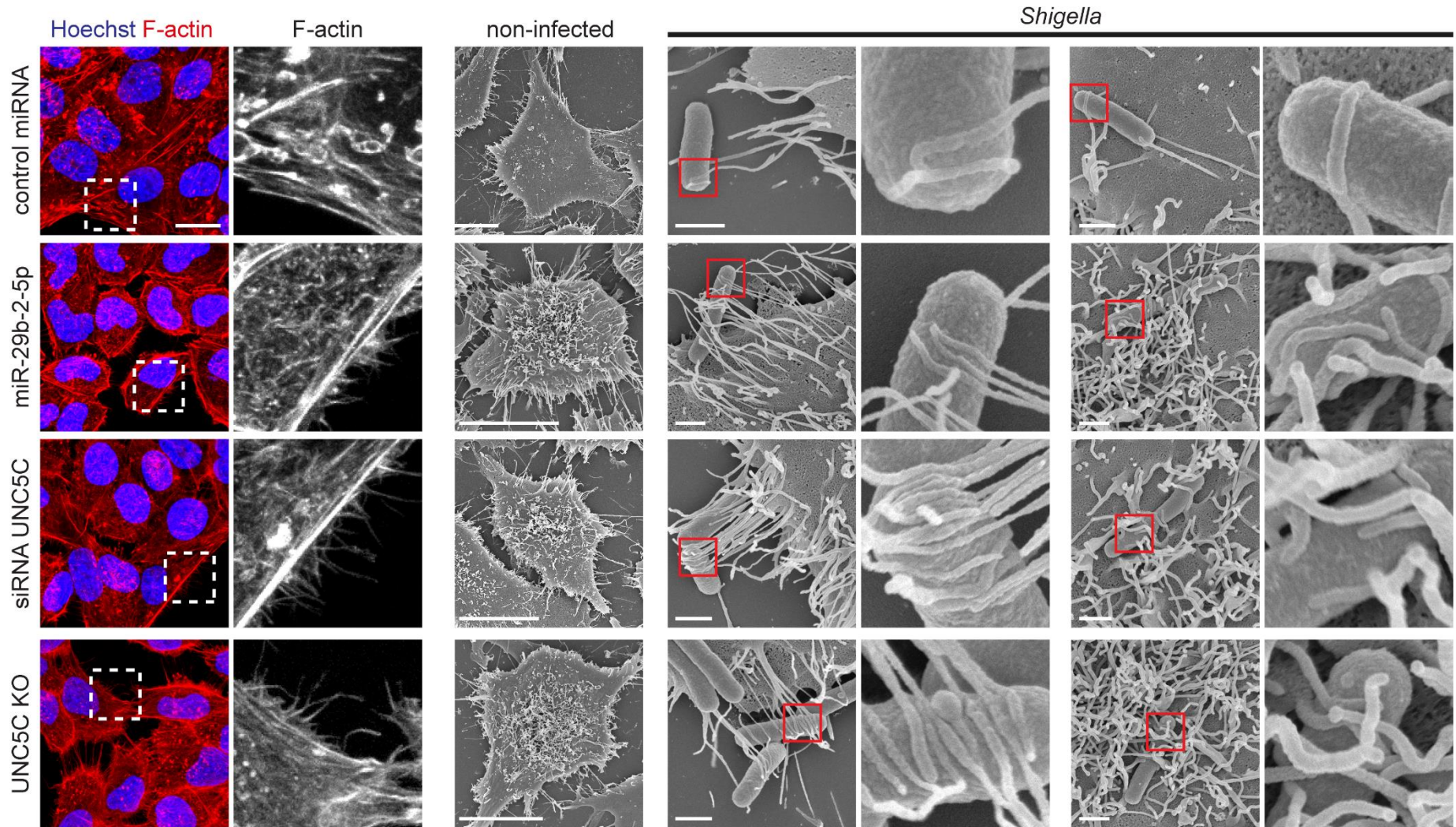
miR-29b-2-5p increases both *Shigella* binding to host cells and intracellular replication



Identification of miR-29b-2-5p targets relevant for *Shigella* infection



miR-29b-2-5p increases filopodia formation and *Shigella* capture by targeting UNC5C



(slides removed, video cut)

Shedding light on microRNA function via microscopy-based screening

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Table 1
Microscopy-based phenotypic screenings using arrayed libraries of synthetic miRNA precursors, mimics and inhibitors.

Phenotype	Library	Species	Cell line	Readout	Reference
<i>Infection</i>					
Salmonella infection	875 human miRNA mimics (Dharmacon)	Human	HeLa	Hoechst, Salmonella-GFP	Maudet et al. (2014) [17]
Shigella infection	2042 human miRNA mimics (Dharmacon)	Human	HeLa	Hoechst, CellMask, Shigella	Sunkavalli et al. (2017) [18]
Flavivirus replication (DENV, WNV, JEV)	1200 human miRNA mimics (Dharmacon)	Human	HeLa	DAPI, Flavivirus envelope	Smith et al. (2017) [19]
<i>Cancer</i>					
Cell proliferation	328 human miRNA precursors (Ambion)	Human	Human primary mammary epithelial cells (HEMC)	DAPI, BrdU	Borgdorff et al. (2010) [20]
Cell migration	904 human miRNA mimics (GenePharma)	Human	HeLa, HepG2, U2OS	area occupied by cells	Zhang et al. (2011) [21]
Cell proliferation and apoptosis	319 human precursor miRNAs (Ambion)	Human	HCT116, LS174T TR4, DLD1 TR7, HT29, Caco2, SW480	Ki-67, cPARP, DAPI	Christensen et al. (2014) [22]
Cell migration	646 human miRNA mimics (GenePharma)	Human	SK-Hep-1	wound healing	Zha et al. (2014) [23]
Apoptosis	895 human miRNA mimics (GenePharma)	Human	HeLa	Hoechst, Annexin V, FITC	Ma et al. (2017) [24]
Fibroblast pro-tumorigenic activity	988 human miRNA mimics (Dharmacon)	Human	Immortalized cancer associated fibroblasts	Hoechst, A549-GFP cells	Andriani et al. (2018) [25]
<i>Cardiovascular</i>					
Cell proliferation	875 human miRNA mimics (Dharmacon)	Rat	Primary neonatal rat cardiomyocytes	Hoechst, α -actinin, Ki67, Edu	Eulalio et al. (2012) [26]
Hypertrophy	230 human miRNA precursors (Ambion)	Rat	Primary neonatal rat cardiomyocytes	DAPI, α -actinin, P4HB	Jentzsch et al. (2012) [27]
SERCA2a expression	875 human miRNA precursors (Ambion)	Human	HEK293	EGFP-SERCA2a-3'UTR	Wahlquist et al. (2014) [28]
Cell proliferation	875 human miRNA mimics (Applied Biosystems)	Human	hiPSC derived cardiomyocytes	DAPI, α -actinin, Edu	Diez-Cuñado et al. (2018) [29]
<i>Cell reprogramming</i>					
iPSC colony formation	379 human miRNA precursors (Ambion)	Mouse	MEF	Oct4-GFP	Pfaff et al. (2011) [30]
Germ layer formation	875 human miRNA precursors (Ambion)	Mouse	mESC	Myh6-EGFP	Colas et al. (2012) [31]
Dedifferentiation of fibroblasts to iPSCs	570 mouse miRNA mimics (Dharmacon)	Mouse	MEF	Oct4-GFP	Judson et al. (2013) [32]
Muscle cell differentiation	870 human miRNA inhibitors (Exiqon)	Human	LHCN	Hoechst, MHC	Poleskaya et al. (2013) [33]
Reprogramming of epithelial cells to organoids	328 human miRNA precursors (Ambion)	Human	Human primary mammary epithelial cells (HEMC)	CD44, CD24, DAPI	Delom et al. (2018) [34]
<i>Others</i>					
Lipid droplet formation	327 human miRNA precursors (Ambion)	Human	Huh7	nuclei, lipid droplets	Whittaker et al. (2010) [35]
Improved protein expression	875 human miRNA mimics (Qiagen)	Human	T-REX-293	Hoechst, NTSR1-GFP	Xiao et al. (2015) [36]
Low-density lipoprotein receptor pathway activity	1719 miRNA mimics (mirVana)	Human	Huh7	Hoechst, DiI-LDL	Goedeke et al. (2015) [37]

Table 3

High-content microscopy screening platforms.

Manufacturer/Model	Camera Technology, Bit-depth, pixel number, FOV@10×*	Confocal	Illumination	Autofocus	Transmitted light	Special hardware features
<i>Acquifer</i> Imaging Machine	https://www.acquifer.de/screening/ sCMOS 16-bit, 4.2 MP, 1.8 mm ²	no	LED	laser + image	yes	static sample holder, moving optics block
<i>GE Healthcare</i> IN Cell Analyzer 2200	https://www.gelifesciences.com/en/au/shop/cell-imaging-and-analysis/high-content-analysis-systems/instruments sCMOS 16-bit, 5.5 MP, 1.3 mm ²	no	LED	laser + image	yes	optional O ₂ control
IN Cell Analyzer 2500 HS	sCMOS 16-bit, 4.2 MP, 1.3 mm ²	no	LED	laser + image	yes	optional O ₂ control
IN Cell Analyzer 6000	sCMOS 16-bit, 5.5 MP, 1.3 mm ²	IRIS line scanning	laser	laser + image	yes	optional O ₂ control
IN Cell Analyzer 6500 HS	sCMOS 16-bit, 4.2 MP, 1.3 mm ²	IRIS/EDGE line scanning	laser	laser + image	yes	optional O ₂ control
<i>Molecular Devices</i> ImageXpress Micro 4	https://www.moleculardevices.com/products/cellular-imaging-systems#High-Content-Imaging sCMOS 16-bit, 4.6 MP, 2.0 mm ²	optional	LED	laser + image	optional	optional on-board liquid handling
ImageXpress Micro Confocal	sCMOS 16-bit, 4.2 MP, 2.0 mm ²	AgileOptix spinning disk	LED	laser + image	optional	optional on-board liquid handling
<i>Perkin Elmer</i> Operetta CLS	http://www.perkinelmer.com/category/high-content-screening-instruments-microscopes sCMOS 16-bit, 4.6 MP, 1.7 mm ²	optional	LED	laser	yes	automated water immersion objectives
Opera Phenix	sCMOS 16-bit, 4.6 MP, 1.7 mm ²	Nipkow spinning disk	laser	laser	yes	automated water immersion objectives; up to 4 cameras
<i>ThermoFisher Scientific</i> CellInsight CX5	https://www.thermofisher.com/pt/en/home/life-science/cell-analysis/cellular-imaging/high-content-screening/high-content-screening-instruments.html CCD 14-bit, 4.9 MP, 1.0 mm ²	no	LED	Image	yes	
CellInsight CX7	CCD 14-bit, 4.9 MP, 1.0 mm ²	CrEST spinning disk	LED	laser + image	yes	
CellInsight CX7 LZR	CCD 14-bit, 4.9 MP, 1.0 mm ²	CrEST spinning disk	laser	laser + image	yes	
<i>Yokogawa</i> CQ1	https://www.yokogawa.com/solutions/products-platforms/life-science/high-content-analysis/ sCMOS 16-bit, 5.5 MP, 2.4 mm ²	Nipkow spinning disk	laser	laser + image	optional	
CellVoyager CV8000	sCMOS 16-bit, 4.0 MP, 1.7 mm ²	Yokogawa spinning disk CSU	laser	laser + image	yes	automated water immersion objectives; up to 4 cameras; built-in stage incubator for live cell imaging
<i>Zeiss</i> CellDiscoverer 7	https://www.zeiss.com/microscopy/int/products/imaging-systems.html CCD 14-bit, 6 MP, 0.6 mm ²	no	LED	laser + image	yes	automated water immersion objectives; up to 2 cameras; optional liquid handling; 12 MP external camera

* FOV@10× – Field of view with a 10× objective.

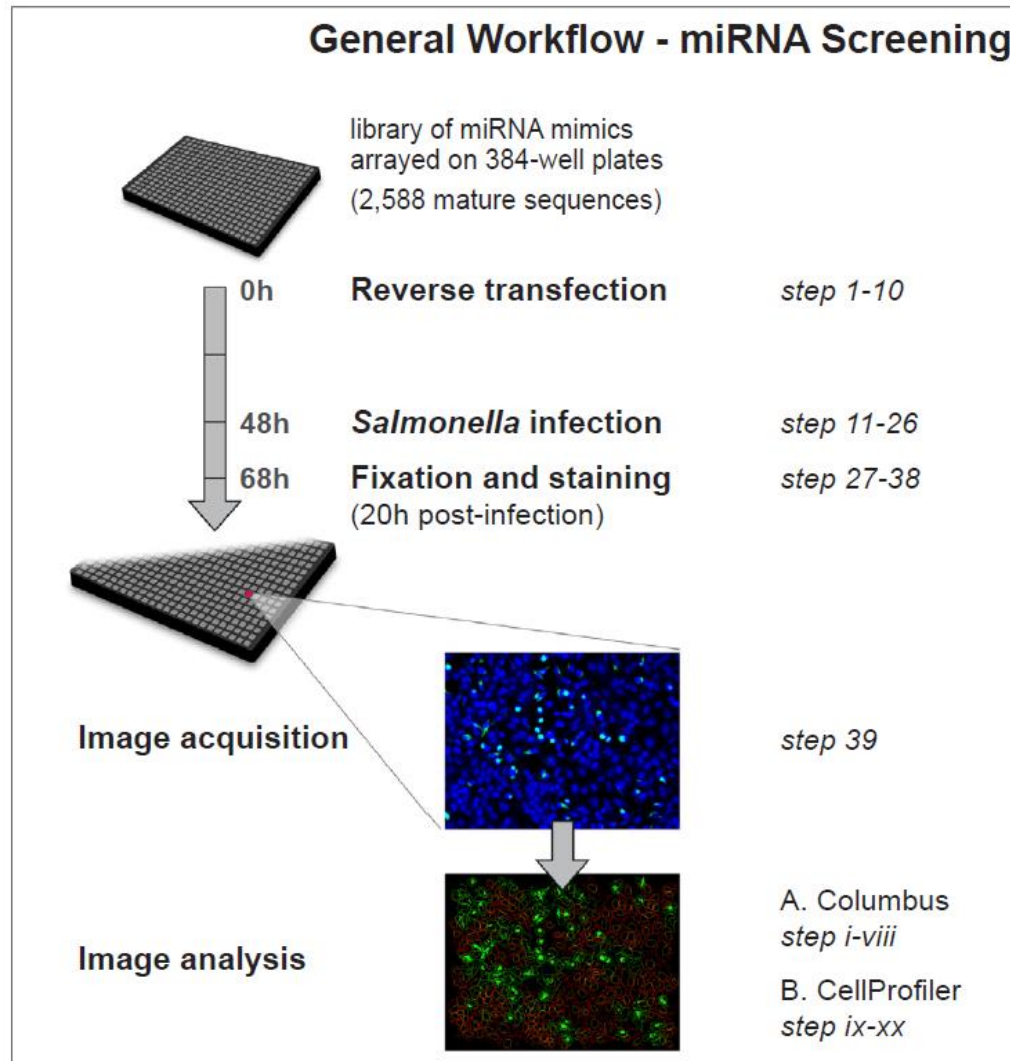


Fig. 1. Overview of a general workflow for miRNA screening. Procedures for reverse transfection, *Salmonella* infection and cell fixation and staining, as well as image acquisition and analysis using a commercial and an open-source image analysis software platform are detailed in [Section 3](#).

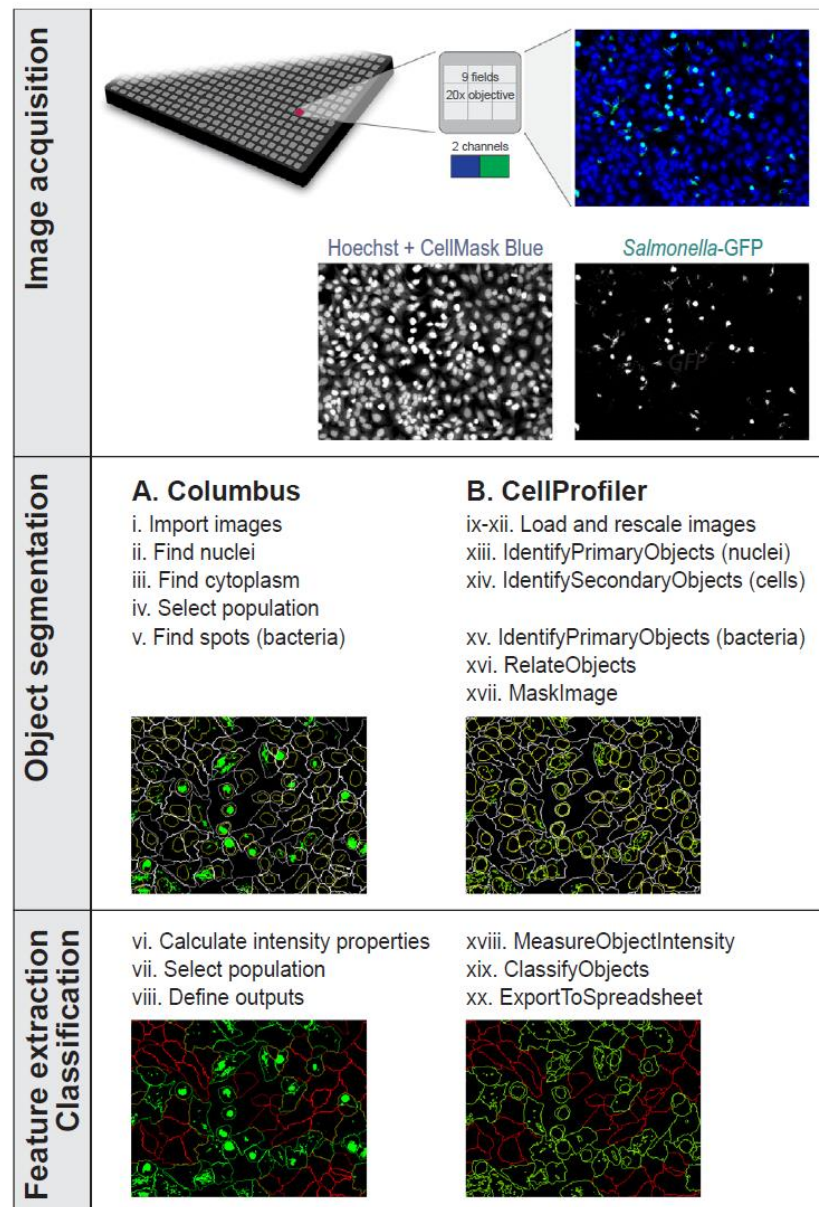


Fig. 3. Overview of image analysis workflows in Columbus (PerkinElmer) and CellProfiler (open-source) image analysis software platforms. Details of each step related to object segmentation, feature extraction and classification are provided in [Section 3.5](#).

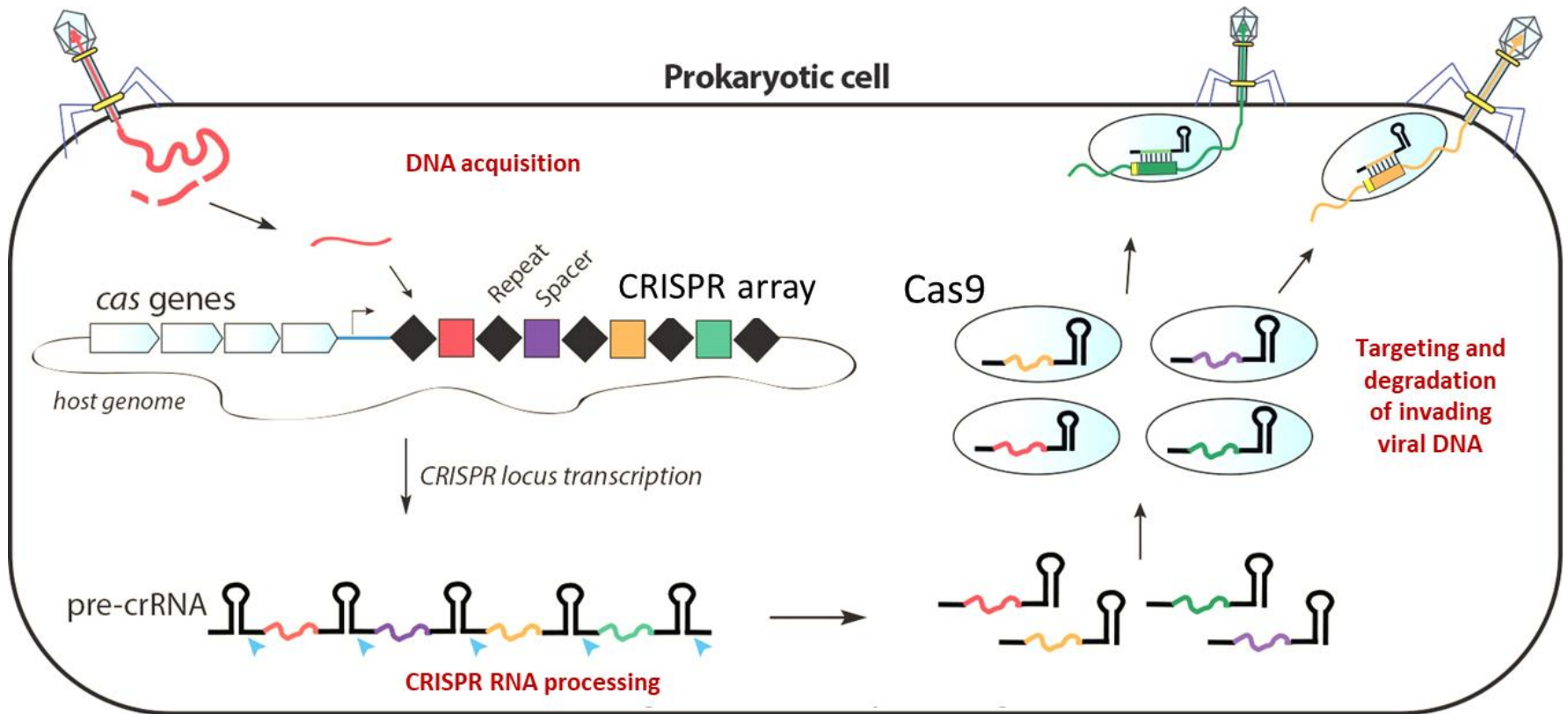
CRISPR/Cas9 technology

Precise genome editing and beyond

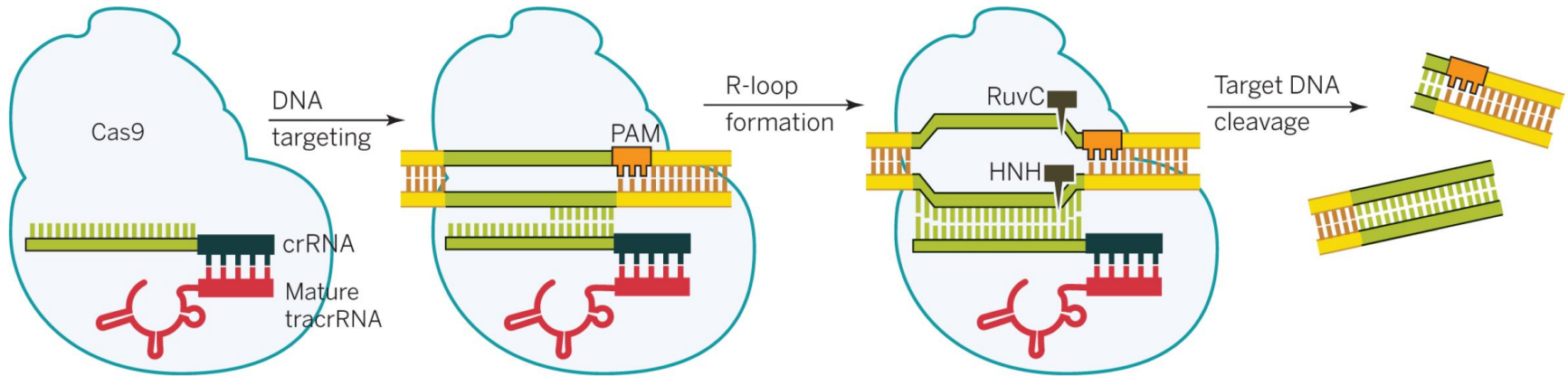
The CRISPR/Cas System

Bacterial and archaea adaptive immunity

Clustered Regularly Interspaced Short Palindromic Repeats / CRISPR-associated proteins



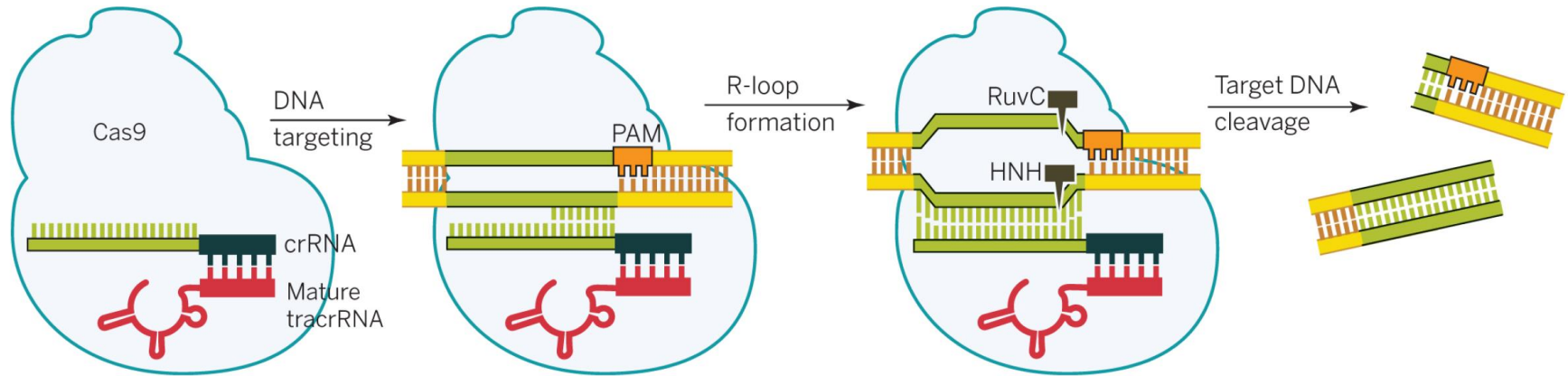
The CRISPR/Cas System



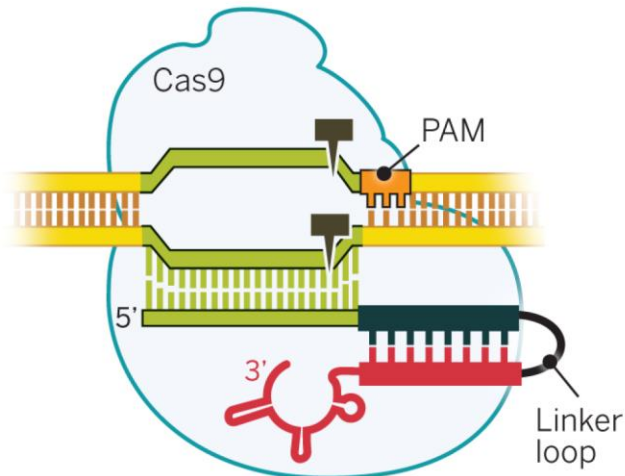
CRISPR (clustered regularly interspaced palindromic repeats)

Cas (CRISPR-associated proteins).

The CRISPR/Cas System



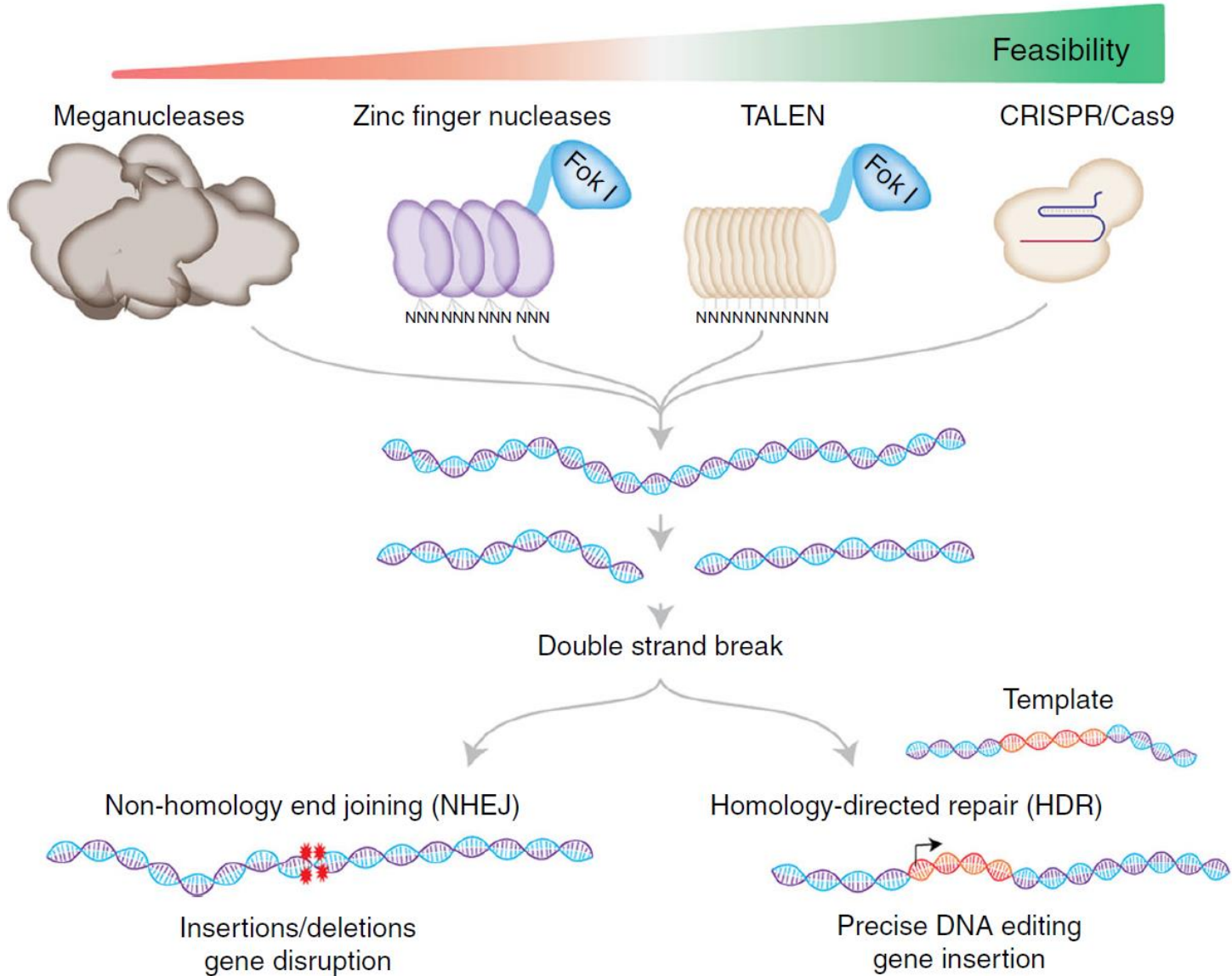
Cas9 programmed by single guide RNA



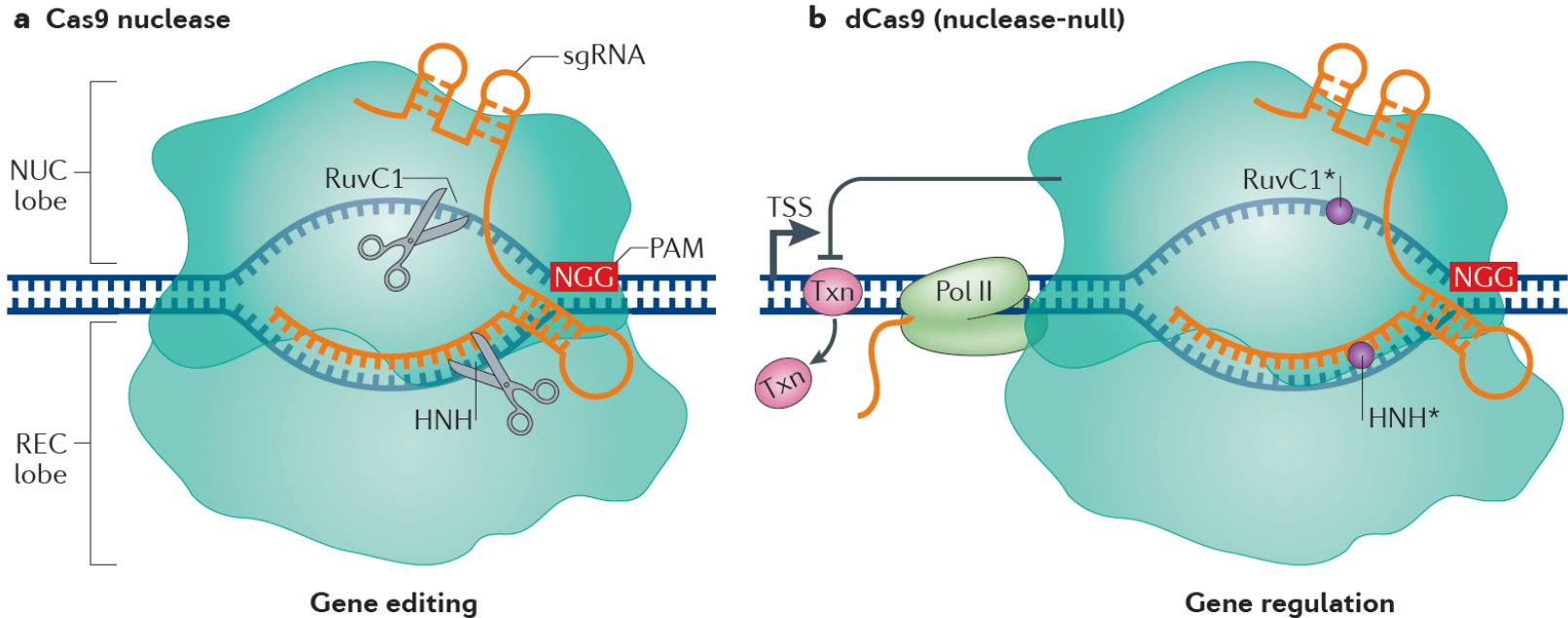
CRISPR (clustered regularly interspaced palindromic repeats)

Cas (CRISPR-associated proteins).

Nuclease-induced genome editing



CRISPR/Cas system: beyond cutting

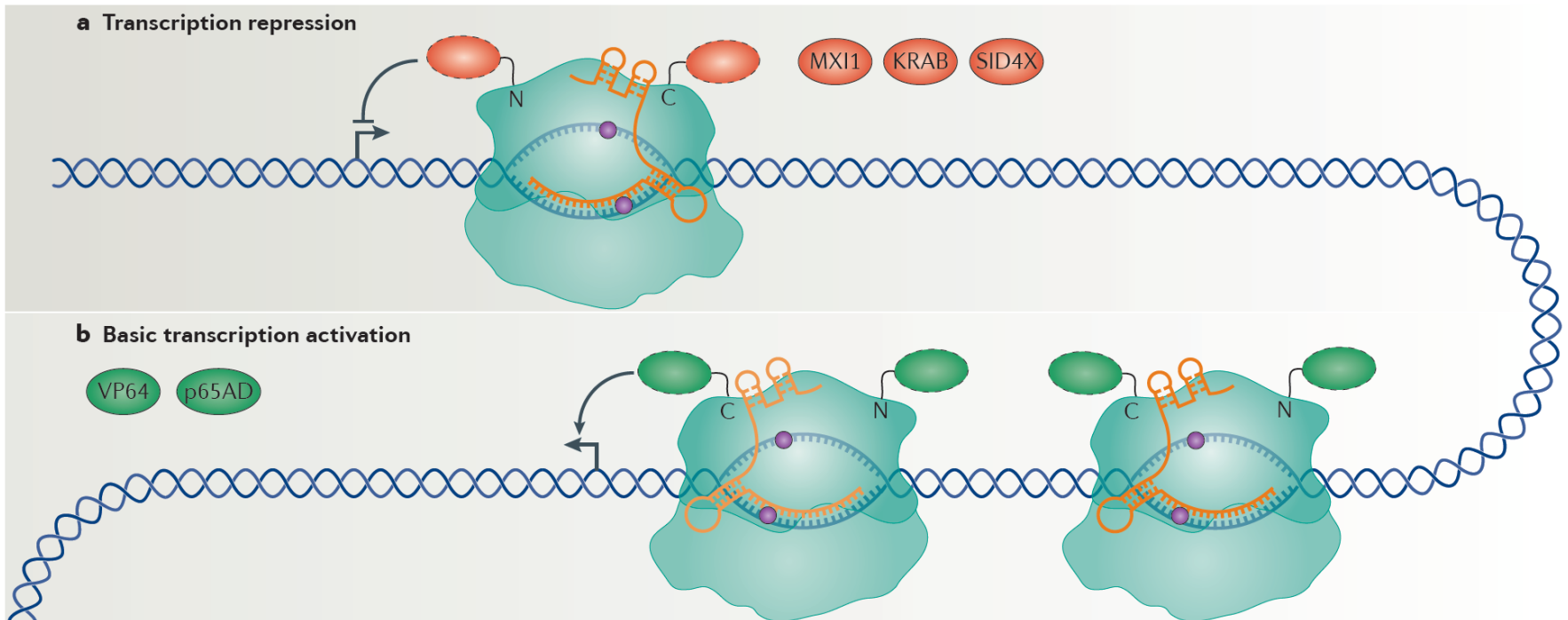


Dominguez et al., Nat Rev Mol Cell Biol (2016) 1:5-15

- **dCas9 maintains DNA targeting specificity, without endonuclease activity**
(Cas9 with nickase activity induces single-strand DNA cuts)

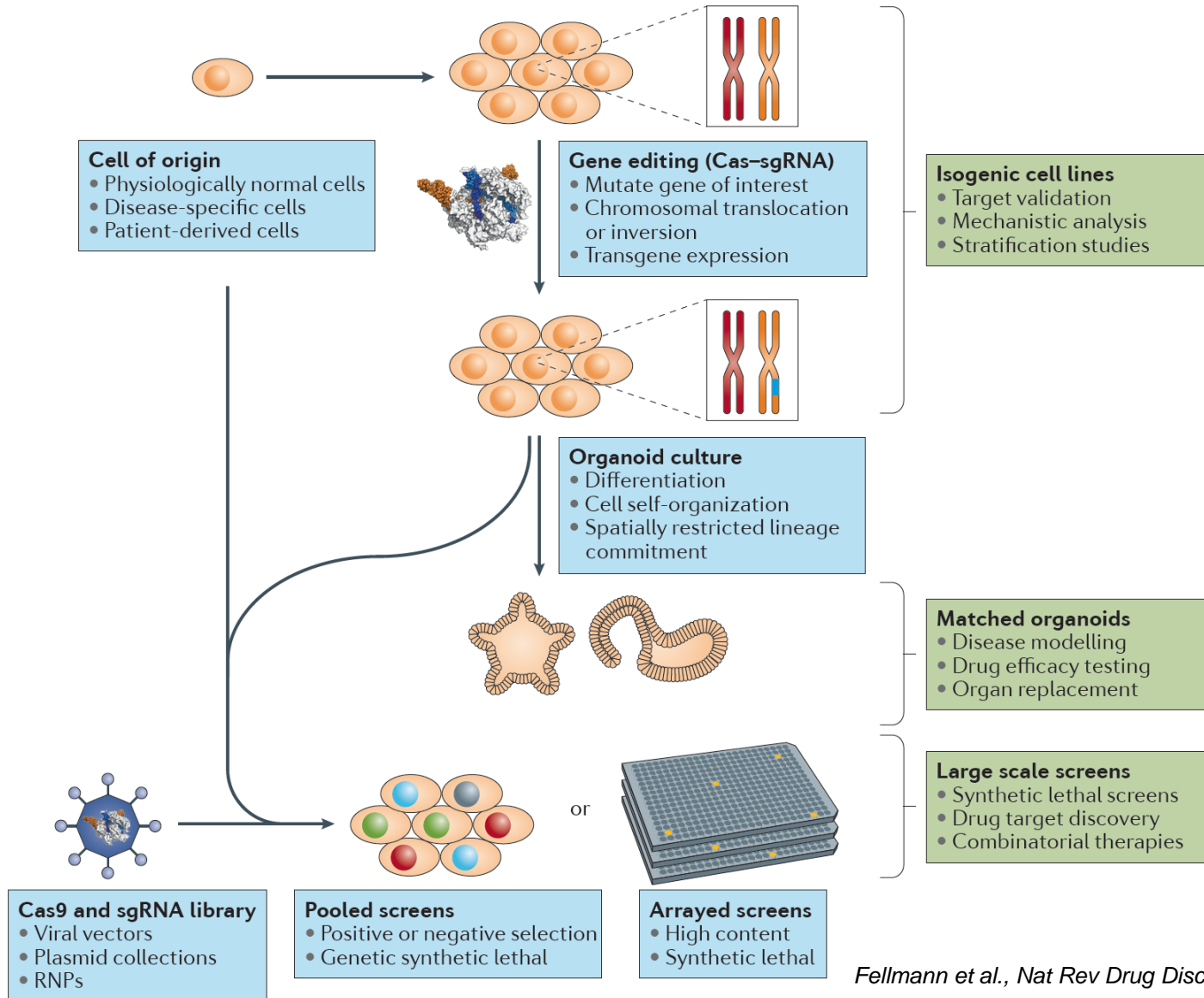
CRISPR/Cas system for gene regulation

CRISPRi



CRISPRa

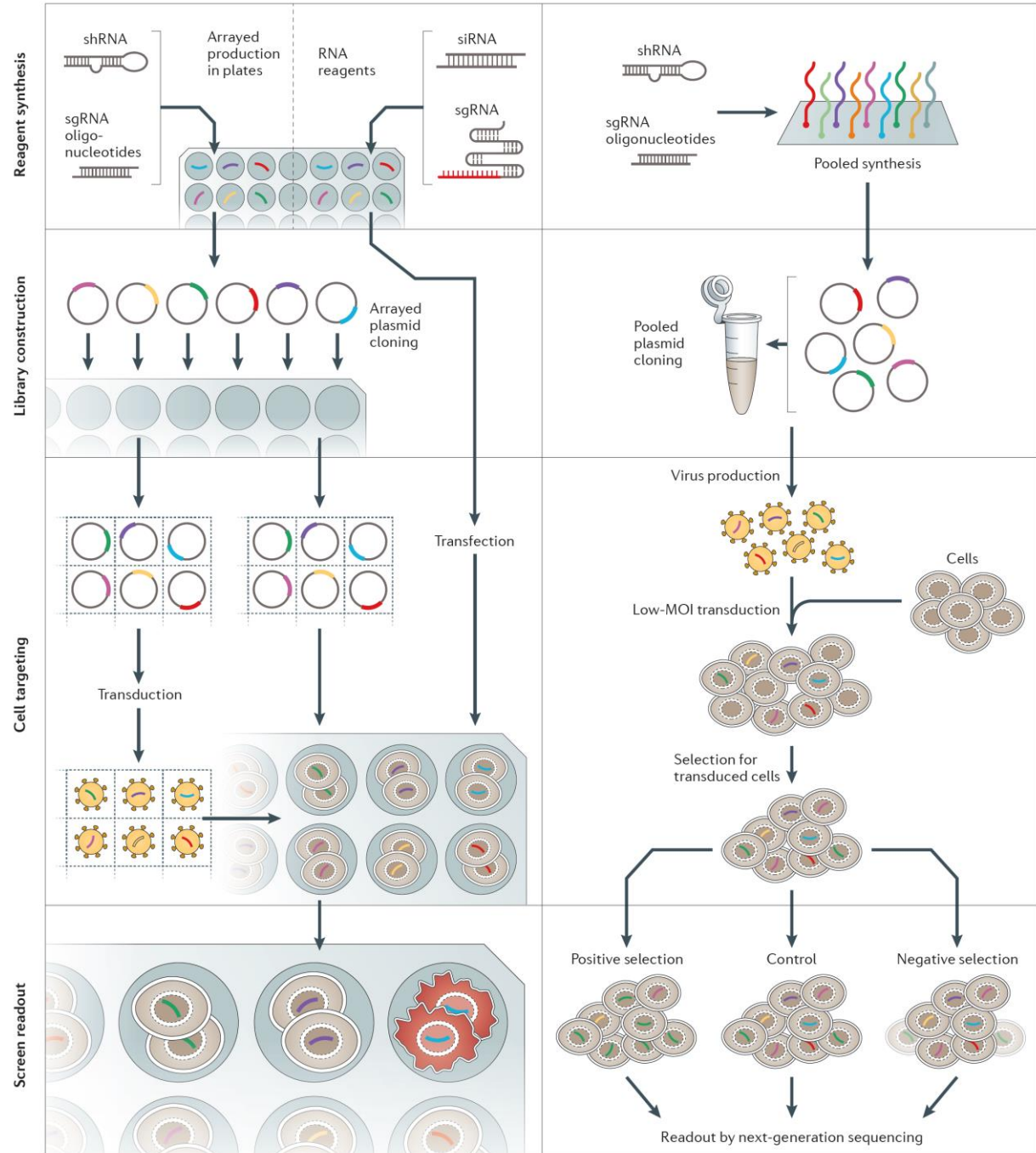
CRISPR/Cas for disease modelling and large-scale screening



Screening approaches

Arrayed screens

Pooled screens





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Drug combinations for T. cruzi infection

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iPSC-derived cardiomyocytes



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