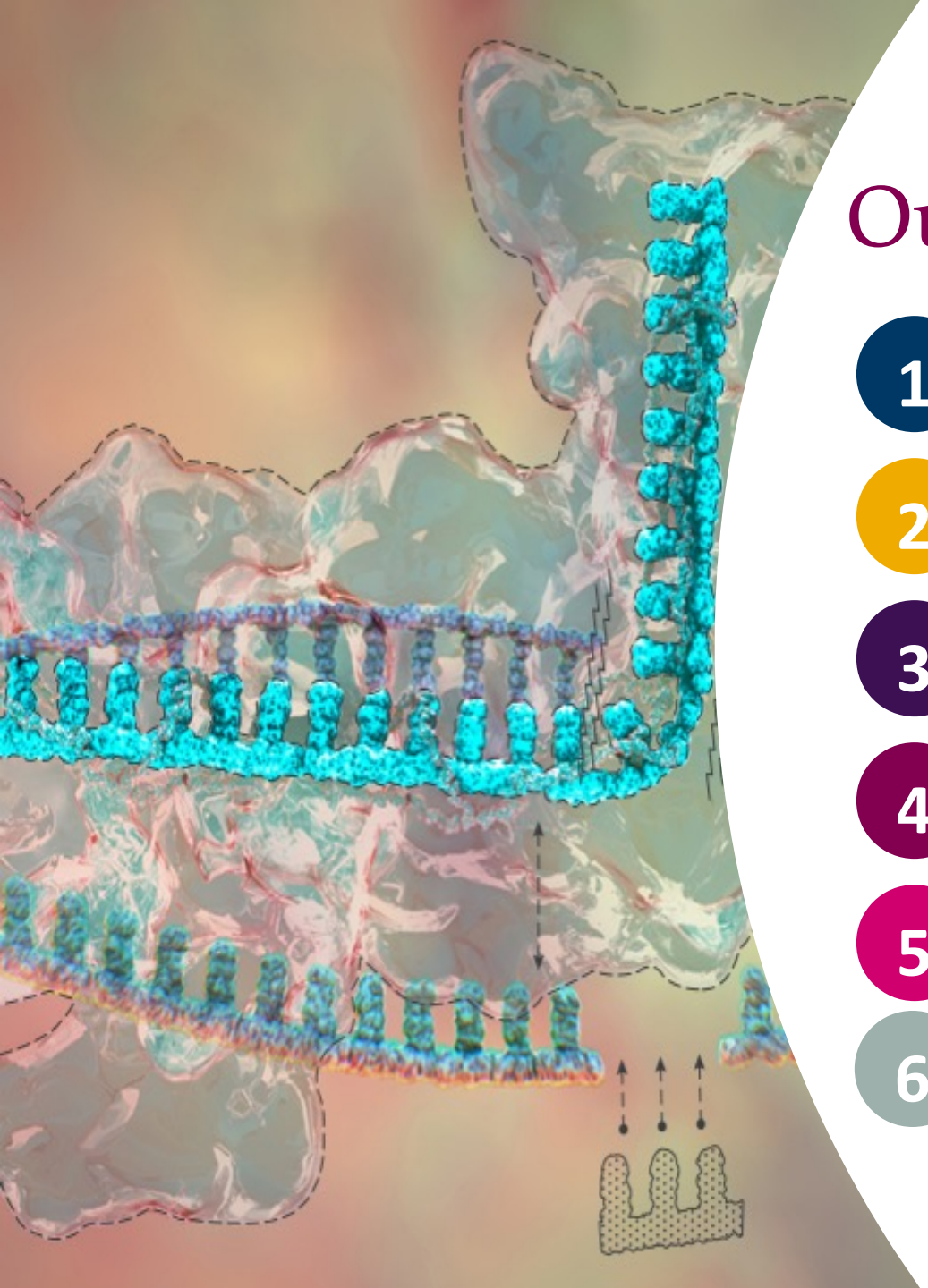
AstraZeneca

CRISPR and Applications of Genome Editing: Bioanalytical Strategies & Challenges

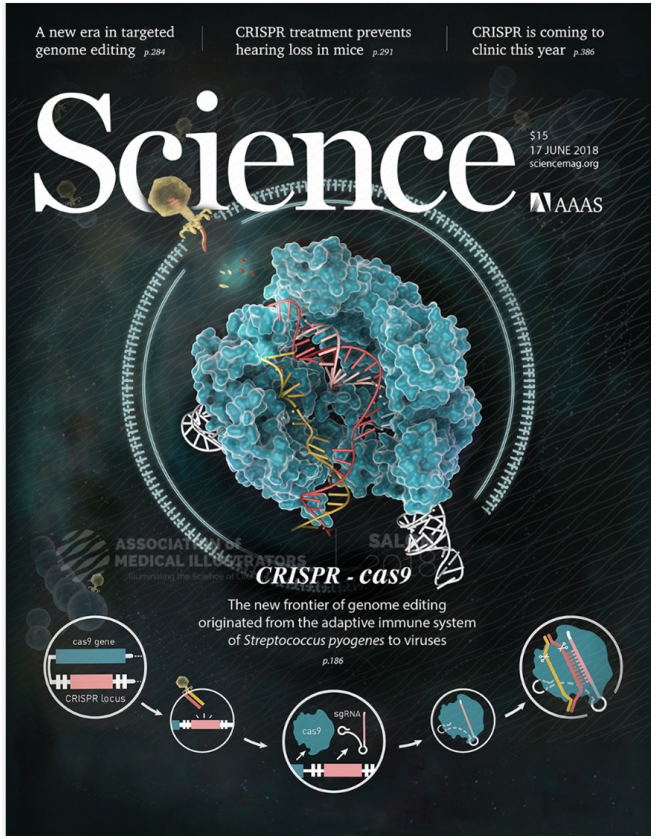
Dr Neil Henderson, Associate Director, Discovery BioAnalysis Europe, Integrated Bioanalysis, CPSS, AstraZeneca Gothenburg



Outline

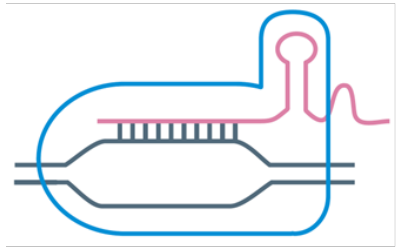
1. Introduction to CRISPR
2. Role of bioanalysis & the BioA toolbox
3. Cas9 Discovery bioanalysis
4. Quantification of gRNA & mRNA
5. Immune measurements
6. Closing thoughts

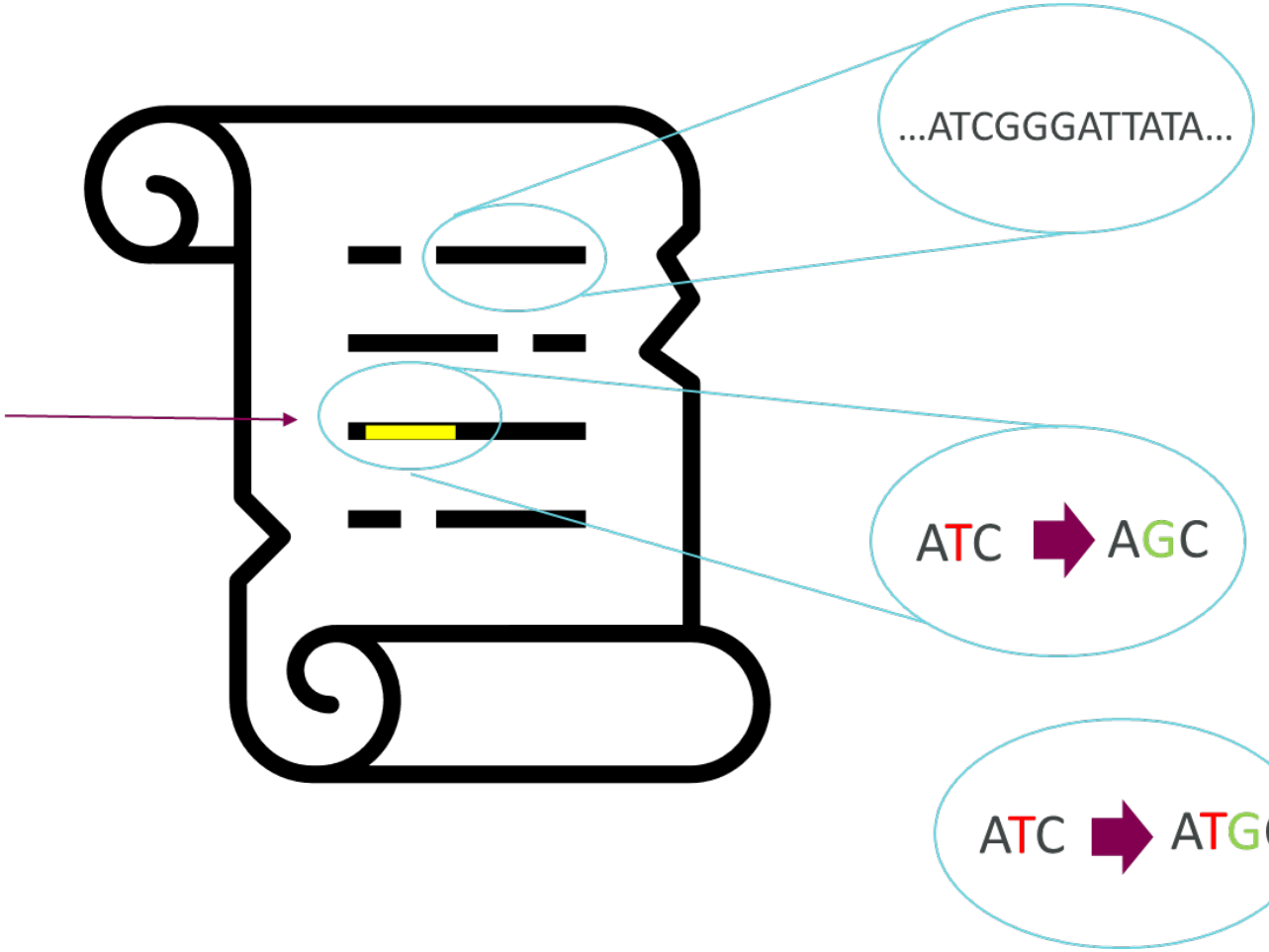
What is so important about CRISPR and Genome Editing?



”[CRISPR] is a technology that has exciting implications for clinical use, [particularly for cancer therapeutics]”
Jennifer A. Doudna, PhD

What is CRISPR and Genome Editing?





Types of Gene Editing

- Base changes
- Insertion
- Deletion

Moving CRISPR from bench to bedside

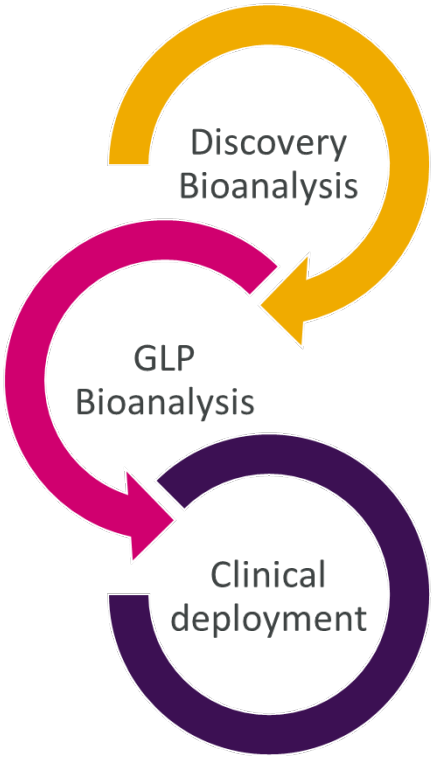
Impactful opportunity

There are many diseases resulting from mutations in single genes

Edits are permanent

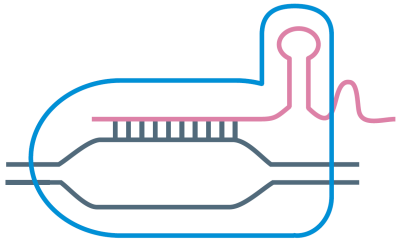
Identifying the therapeutic window poses a challenge

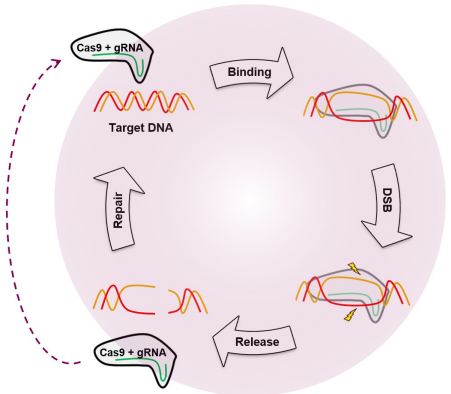
”Bench”

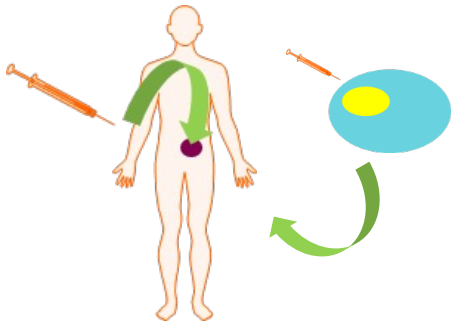


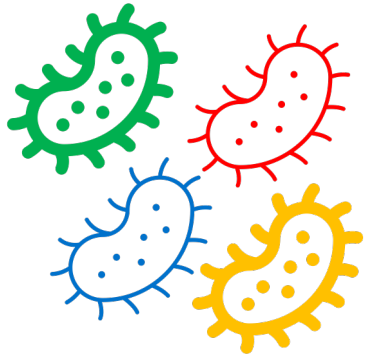
”Bedside”

Complexity of CRISPR









Takes two to tango

Active RNP complex is made up of sgRNA plus CAS9 protein

Edit not guaranted

Active RNP complex will bind & release from target DNA until a misrepair occurs

On- and Off-target

Editing has potential to occur in non-target regions

Context of Use

Approaches can either be ex vivo or in vivo

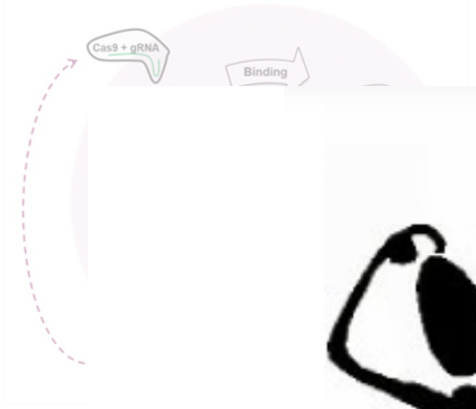
CAS9 origin

Bacterial origin protein recognisable by immune system

Complexity of CRISPR

Takes two to tango

Active RNP complex is made up of sgRNA plus CAS9 protein



Edi

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What is our BioA toolbox?

How can it help us to unravel this complexity to develop safe & effective CRISPR drugs?

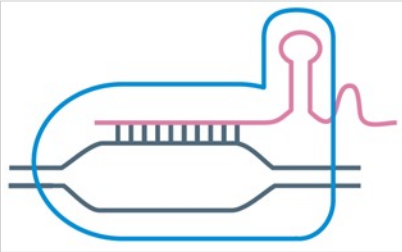
ext of Use

aches can be ex vivo in vivo

CAS9 origin

Bacterial origin protein recognisable by immune system

Answering the complexity of CRISPR



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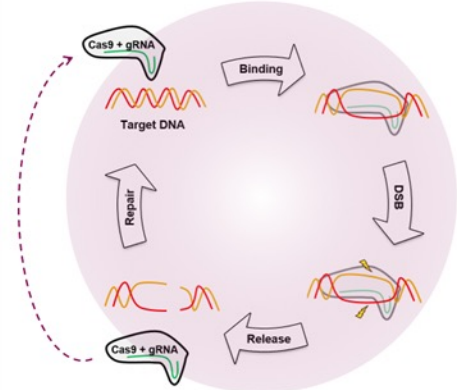

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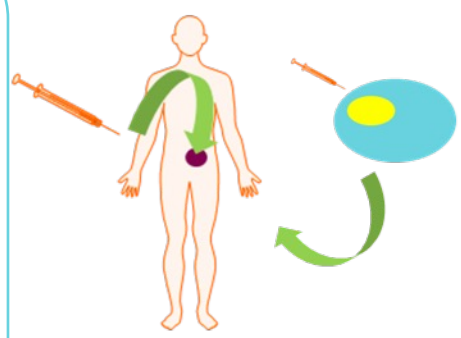

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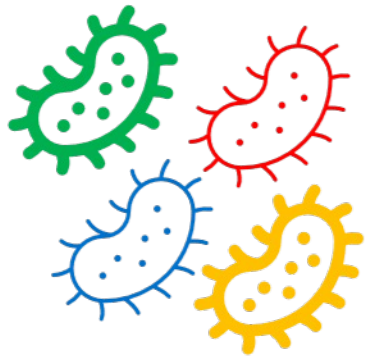
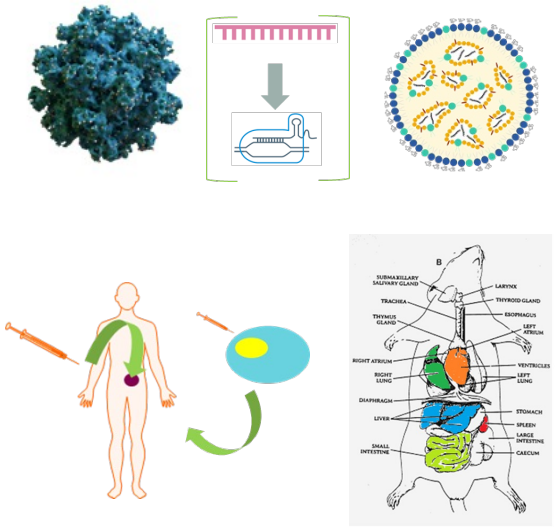
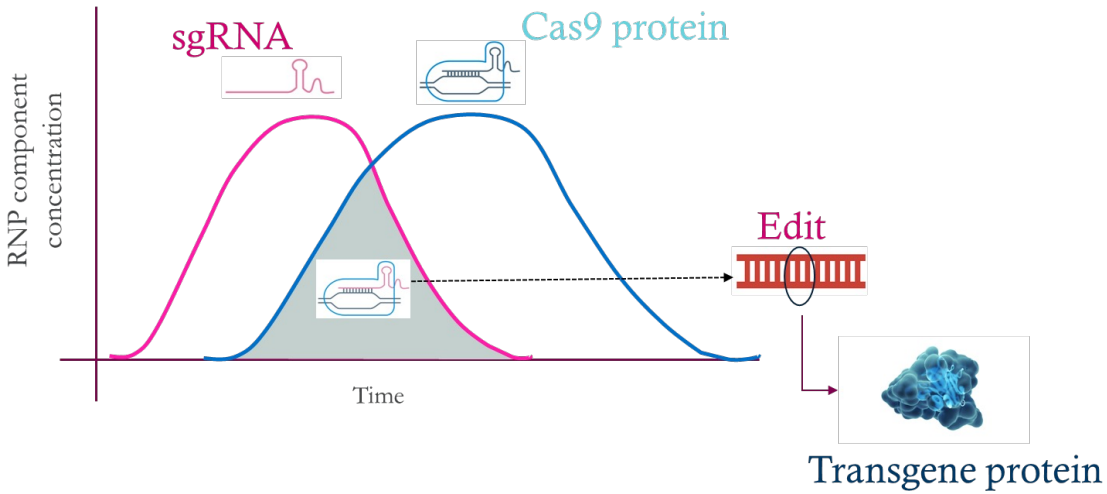


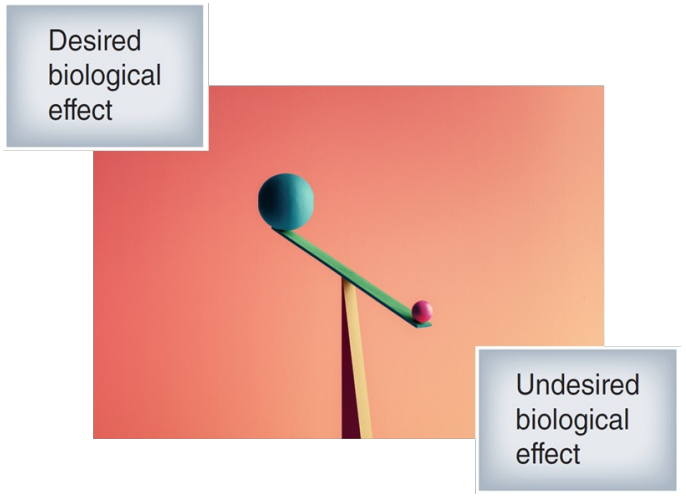
CAS9 origin

Bacterial origin protein recognisable by immune system

Role of Bioanalysis in CRISPR drug development







Dosing

Which delivery approach is best & does it stay at site of action?

Temporal relationship

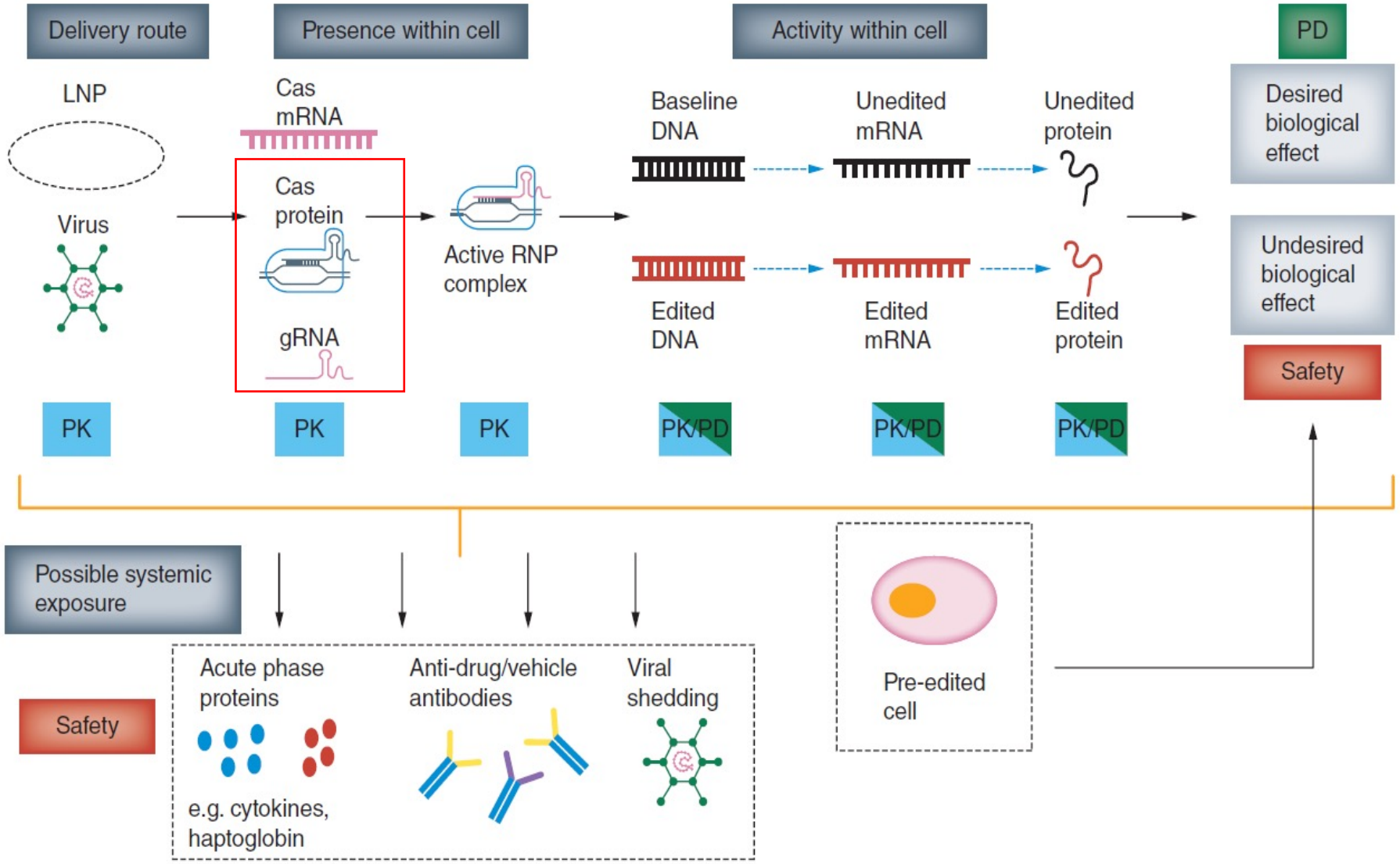
X + Y = (XY)t = E:e = TP = TO

Therapeutic window

What are the safety related issues & severity?

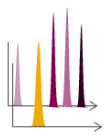
Where x = sgRNA, Y = CAS9 protein, t = time, E = on-target edit, e = off-target edit, TP = transgene protein, TO = therapeutic outcome

Understanding of the pharmacokinetics of the RNP components will facilitate Dose Setting and Optimal Safety Profiles



The BioA toolbox required to support gene editing projects

Delivery system	Pre-IVEB	IVEB	Edit (on / off)	PD efficacy	Immune activation
LCMS qPCR	bDNA qPCR ddPCR	LBA qPCR Hybrid-LCMS	NGS qPCR CRISPR-seq	Bioassays LBA Flow cyt qPCR Hybrid-LCMS	Bioassays LBA Flow cyt qPCR Fluorospot

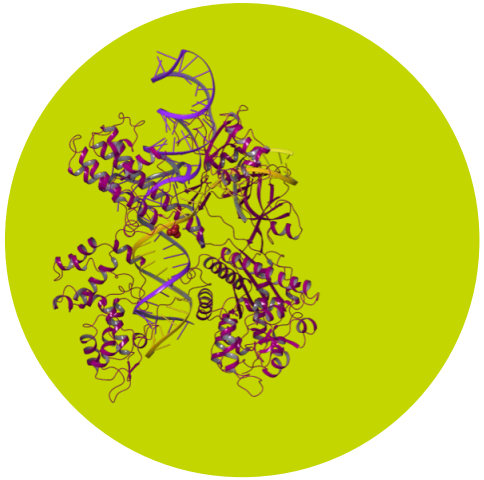






A mix of classical and novel bioA approaches will need to be used

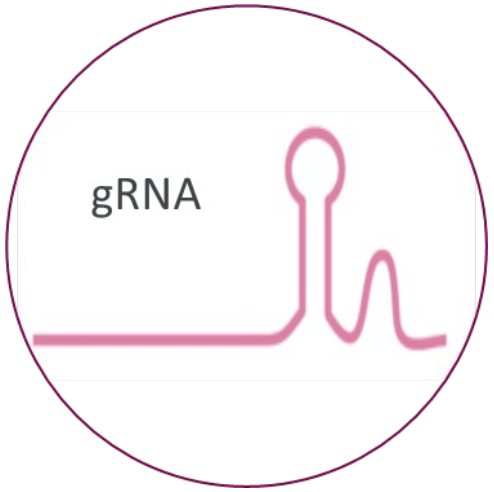
Evolving bioanalytical strategies for quantification of CRISPR components



Cas9 protein

LBA

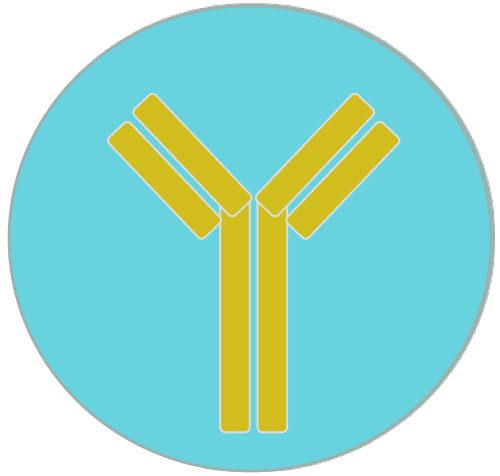
LC/MS



sgRNA / mRNA

Branched-DNA (bDNA)

qPCR



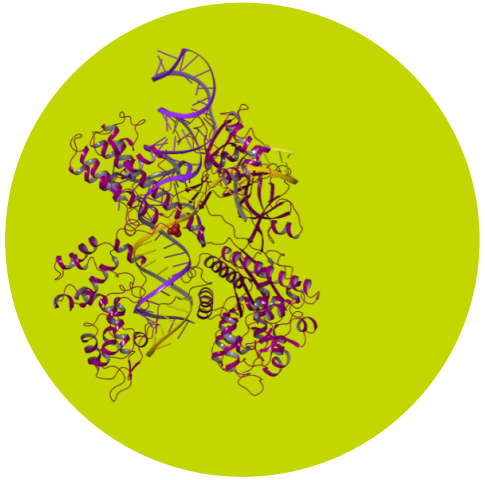
Immune response

LBA

Flow cytometry

Elispot/fluorospot

Evolving bioanalytical strategies for quantification of CRISPR components



Cas9 protein

LBA

LC/MS

sgRNA / mRNA

Branched-DNA (bDNA)

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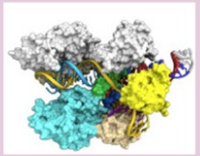
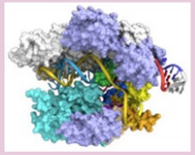
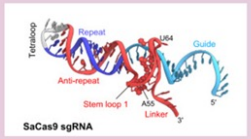
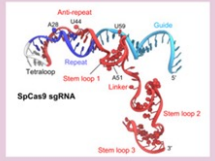
Measuring CAS 9 protein

	SaCa9	SpCas9	Comments
Size	1053aa 	1368aa 	Different bacterial origin
PAM	NNGRRT	NGG	Frequency
gRNA	~120nt 	~100nt 	Complexity
Immunogenicity	High	Mid	In pre-exposed humans

Types of Cas9 proteins

Two predominantly used Cas proteins are SaCas9 (staphylococcus aureus) and SpCas9 (streptococcus pyrenes)

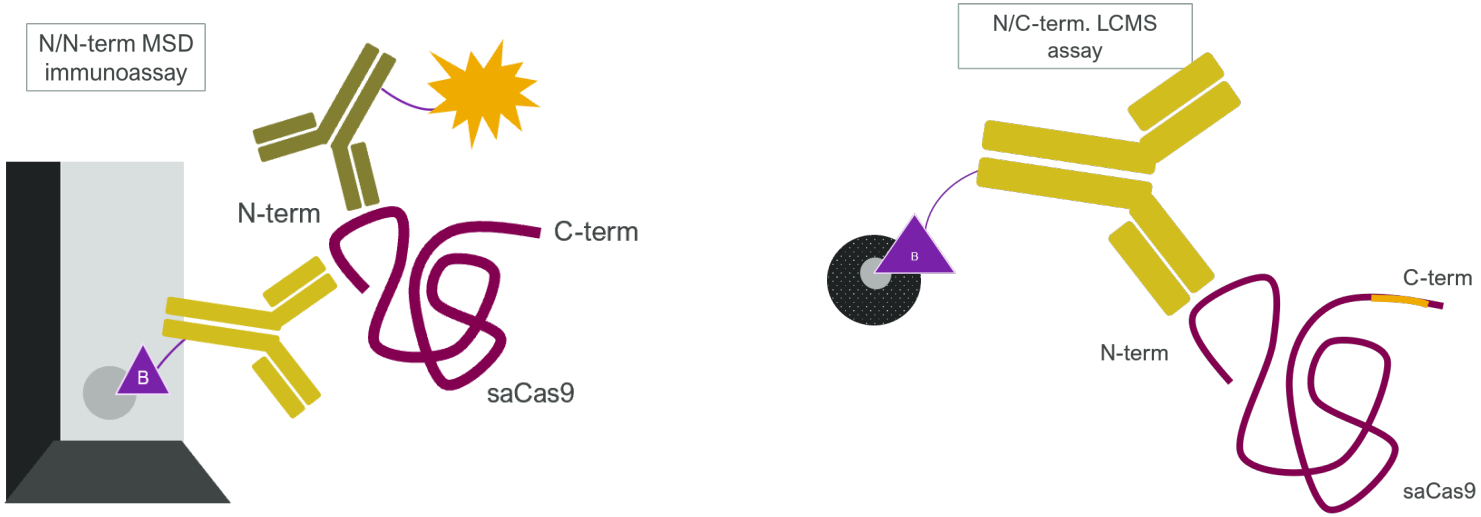
Developing assays

Right technology, right reagents, right sample processing, right performance

Is it answering the right question?

https://doi.org/10.1016/i.cell.2015.08.007

Need for flexible BioA approaches



	MSD N/N-term assay	IA/LCMS
Measures	saCas9 N-terminal region	"intact" saCas9 (N/C terminus)
Assay range in mouse brain	0.018-1.11 ng/mg tissue (Matrix-matched)	0.087-11.1 ng/mL tissue (Matrix-matched)
Neat sample amount	5 µL homogenate	50 µL homogenate
Time	1 day	~2 days

Fit for purpose assays

Using the available tools in the toolbox to develop SaCas9 protein PK methods

Creative solutions

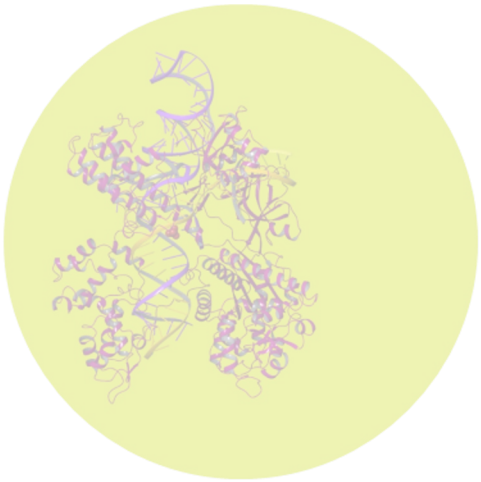
Factors such as reagent availability steers innovation in the toolbox

Future thinking

How to improve upon the assays developed?

SaCas9 methods developed by Mikko Holtta

Evolving bioanalytical strategies for quantification of CRISPR components



Cas9 protein

LBA

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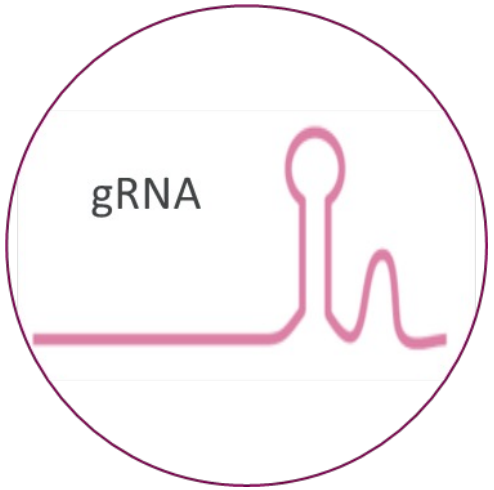

sgRNA / mRNA

Branched-DNA (bDNA)

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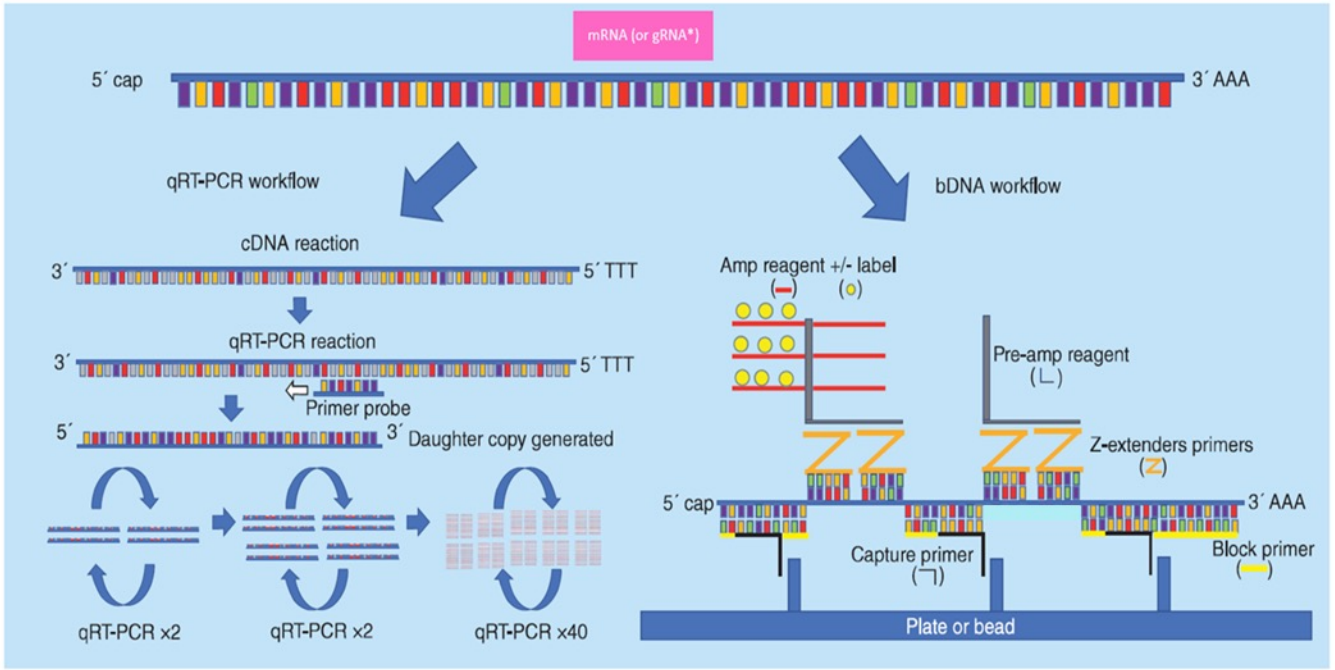
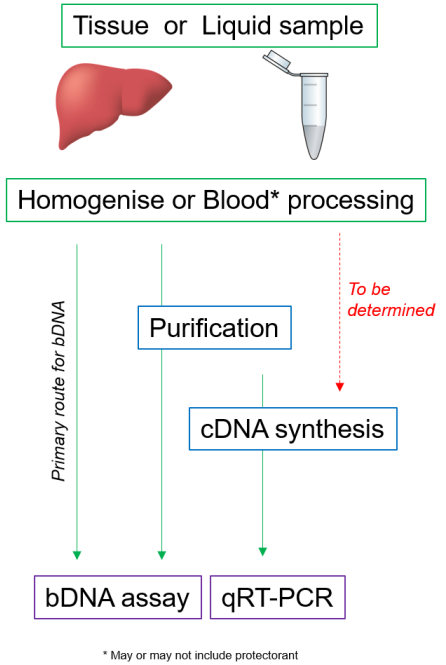
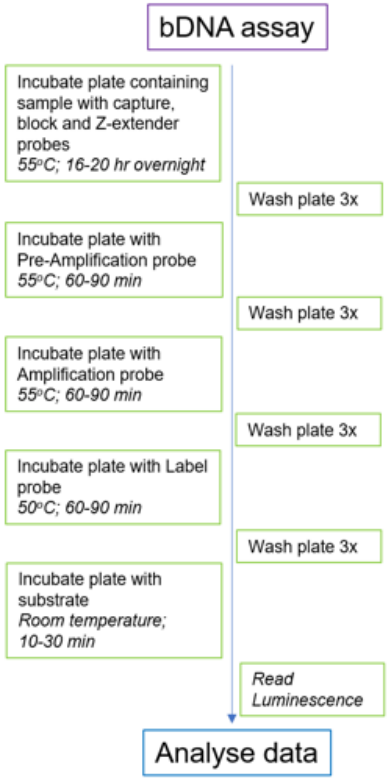
Measuring sgRNA (or mRNA)







qRT-PCR

works by amplifying copy number of target in a sample using specific probes

Branched DNA (bDNA)

works by amplifying the signal intensity of a captured analyte

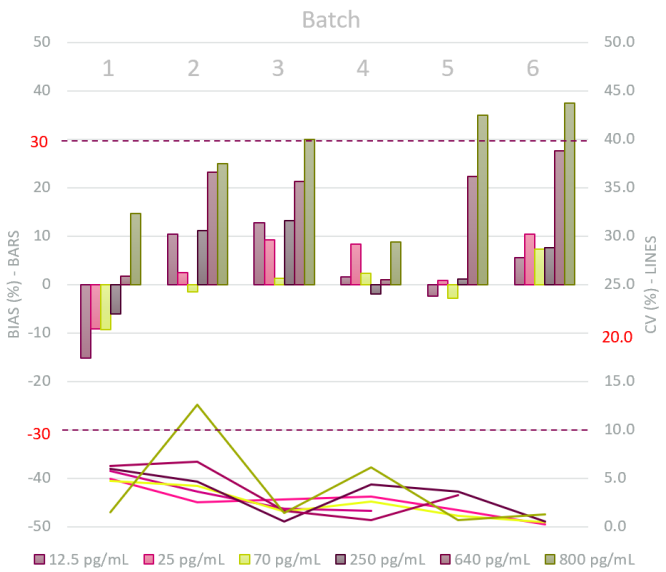
Sample

bDNA can work on non-purified samples; a purified sample is often required/prefered for qRT-PCR workflows

Assay

Specialist equipment to run assay is an incubator and a luminometer

Measuring sgRNA (or mRNA)



Performance

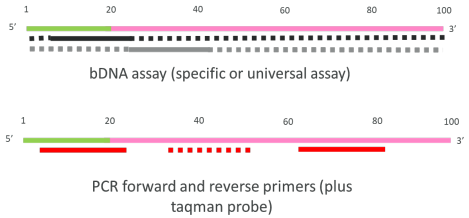
Assay can perform with acceptable precision (<20%) and bias (<30%)p to 640 pg/mL

Guide RNA is 100 base long*

20 base targeting region is unique sequence

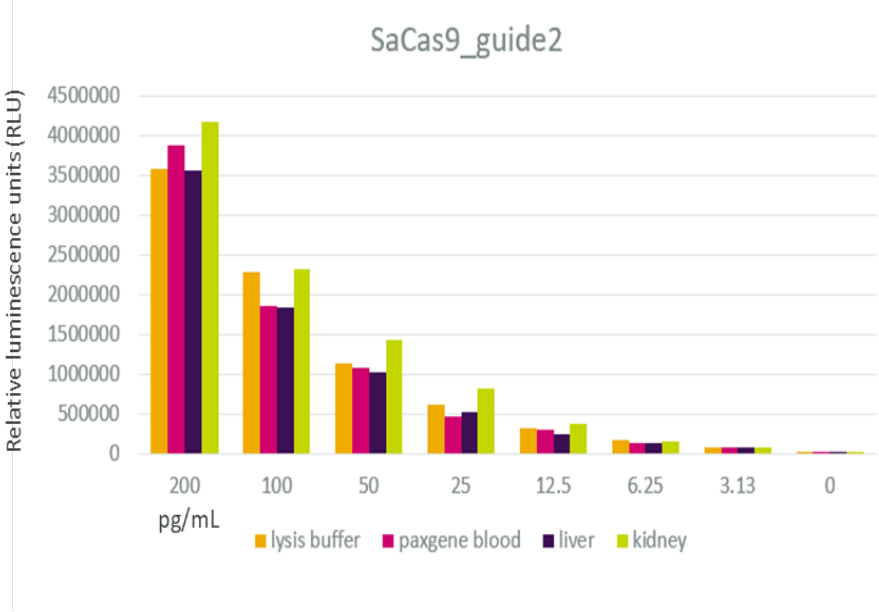
80 base scaffold region is conserved

Guide RNA (with secondary structure)



High probe specificity

Bespoke probes cover the entire analyte

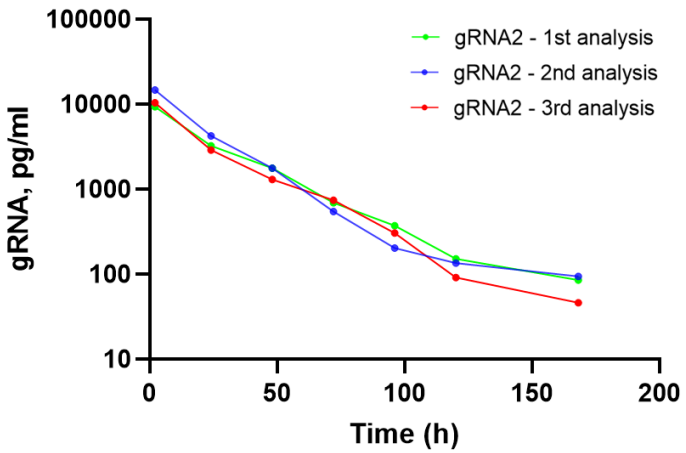


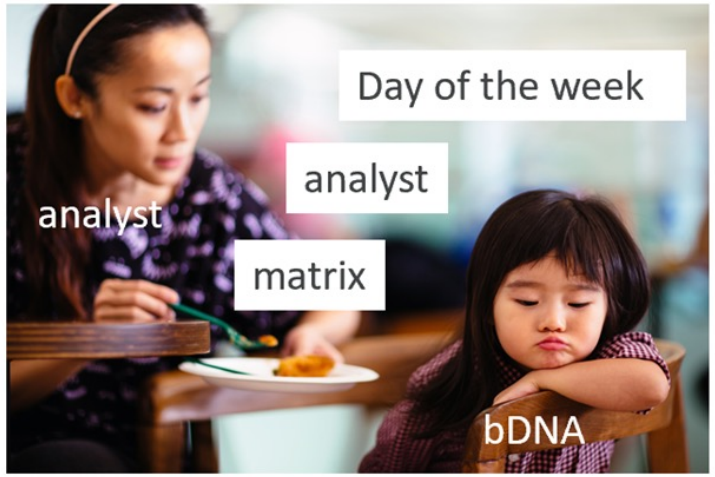
sgRNA measurable

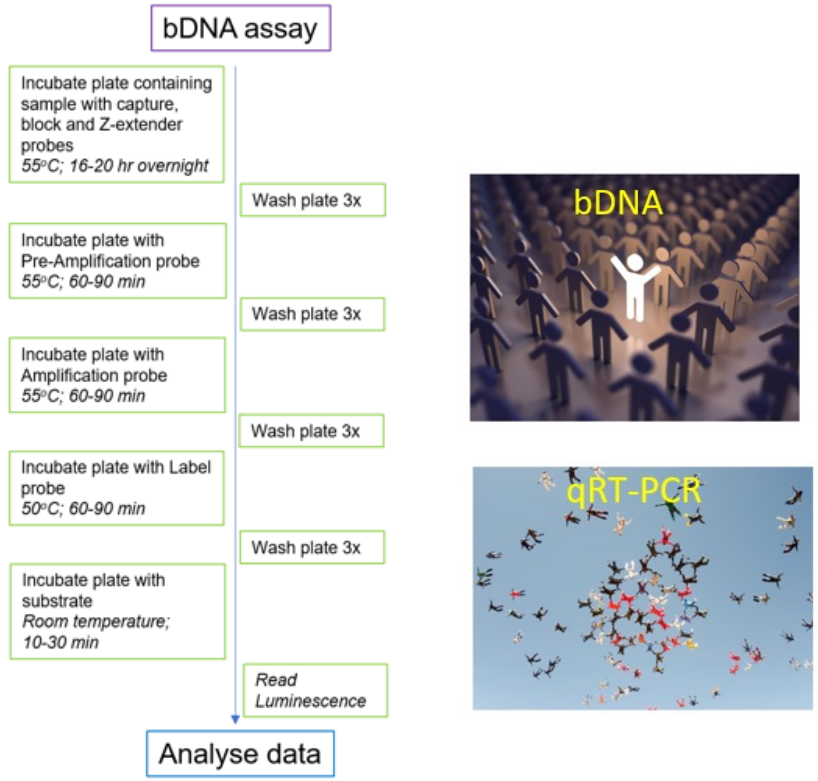
bDNA can be used to measure sgRNA but is more suited to mRNA measurement

Example shown is mRNA spiked into liver samples

Measuring sgRNA (or mRNA)







Failed batches

A number of “performance-failed” runs (>30% A&P), yet samples consistent.

Temperamental assay

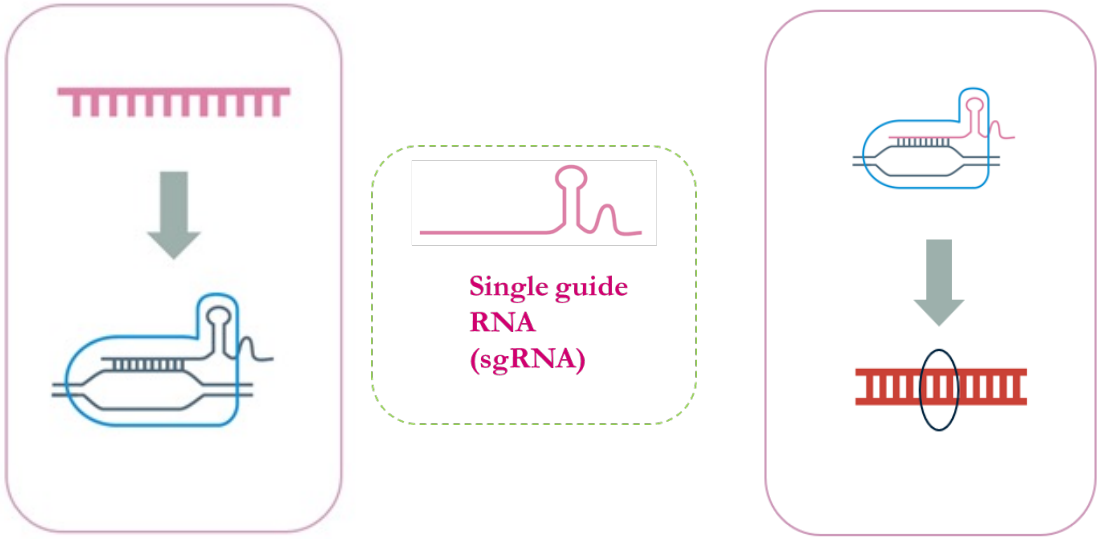
Assay can be problematic. Only suitable for discovery BioA

Format flexibility

bDNA is only available from a single vendor and the workflow has minimal wiggle room for refinement

gRNA data courtesy of Jakob Alhede

Measuring sgRNA (or mRNA)



	Advantage	Challenge
Branched DNA (singleplex)	• No requirement for purification of sample	• Probe design (analyte specificity) • Sample throughput
qRT-PCR	• Greater sensitivity* • Less material required	• Probe design (analyte specificity) • Background

qRT-PCR

Currently being used in discovery bioanalysis to measure CAS-9 mRNA, sgRNA and target edit mRNA

bDNA vs qRT-PCR

Head to head comparrison

* qRT-PCR is a more sensitive method, however with bDNA greater amount of sample can be added to the well to increase detectable signal without loss of assay performance; ddPCR is more sensitive and absolutely quantative

Evolving bioanalytical strategies for quantification of CRISPR components

Cas9 protein

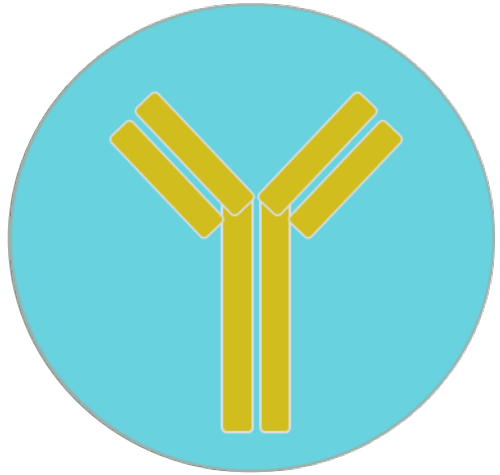
LBA

LC/MS

sgRNA / mRNA

Branched-DNA (bDNA)

qPCR



Immune response

LBA

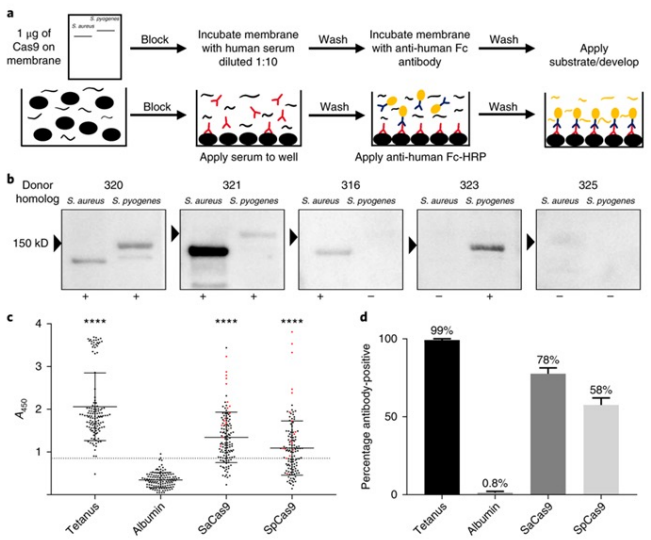
Flow cytometry

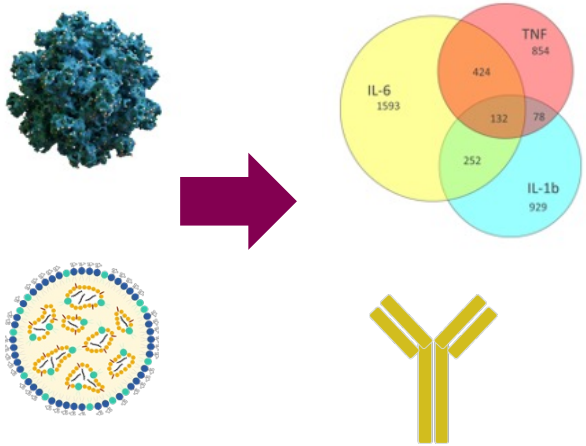
Elispot/fluorospot

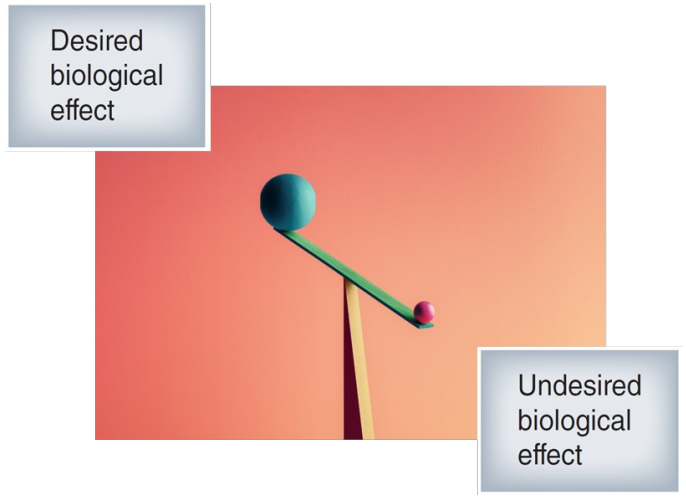
Measuring immune activation

Fig. 1: Identification of preexisting humoral immunity to Cas9.

From: Identification of preexisting adaptive immunity to Cas9 proteins in humans







Preexisting immunity

Many individuals have antibodies against Cas9 protein

Delivery systems

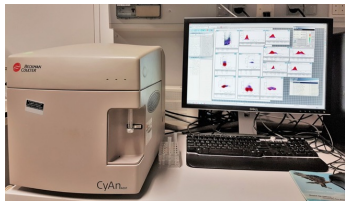
AAVs and LNPs also induce immune activation (e.g., cytokines, anti-PEG ADAs)

Identifying safety profiles

Understanding how individuals respond to drug:delivery components may facilitate deployment

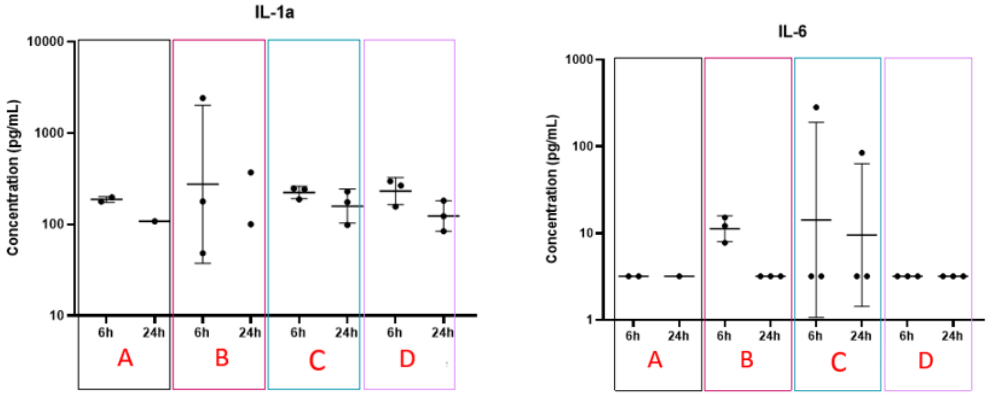
Charlesworth et al (2019), Nature Medicine (25), pg 249-254

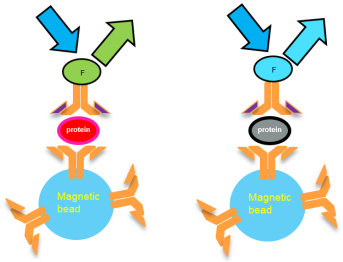
Methods for the measurement of immunogenicity and immune response activation



	FluoroSpot	Flow cytometry	Immunoassay
ANALYTES	Secreted	Cell surface or intracellular	Secreted or cell associated
SAMPLE TYPE	Cells	Cells	Medium or tissue extract
SAMPLE SIZE	Low ~10^5 cells per well	Medium ~10^6 cells per sample	Low to high, assay dependent
SENSITIVITY	Very sensitive single positive cell detected	Sensitive	Sensitive, assay dependent
QUANTITATIVE	Positive cell frequency and analyte abundance	Positive cell frequency and analyte abundance	Analyte concentration
MULTIPLEXING	Yes, easy, up to 4 analytes	Yes, but larger panels require more optimization	Possible, assay dependent
KINETICS	Independent sum of all events throughout stimulation	Dependent phenotype at given moment	Dependent affected by autocrine/paracrine signalling, protease processing
HIGH-THROUGHPUT	Yes, easy	Possible, but difficult	Yes, easy

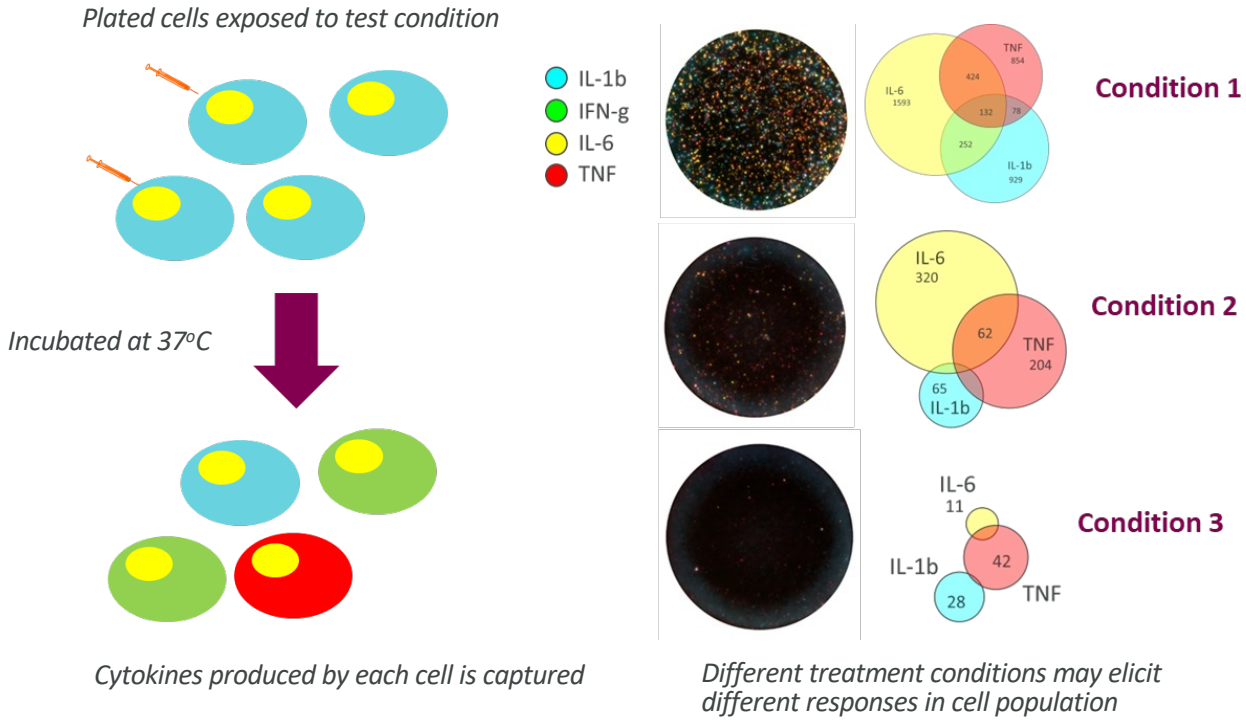
Exploring new approaches to answer questions differently





Immune reaction

Historically greater focus has been placed upon whole-matrix cytokine profiles from dosed subjects



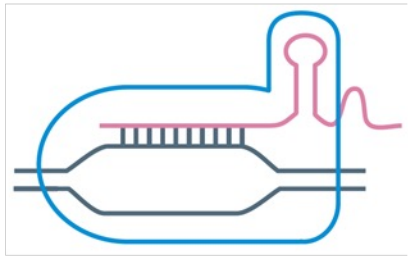
Increased granularity

Using Fluorospot to capture immune reaction differences between drug:delivery components on in vitro or ex vivo stimulated cells

Does more granularity on immune reactions lead to better drug design?

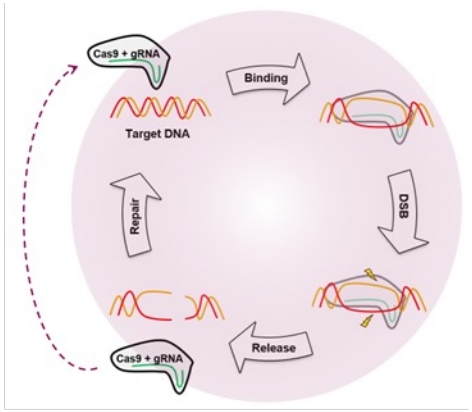
Luminex plots by Johnny Lindqvist and Åsa Sandelius; Fluorospot images by Anna Marzeda

Answering the complexity of CRISPR



Takes two to tango

Active RNP complex is made up of sgRNA plus CAS9 protein

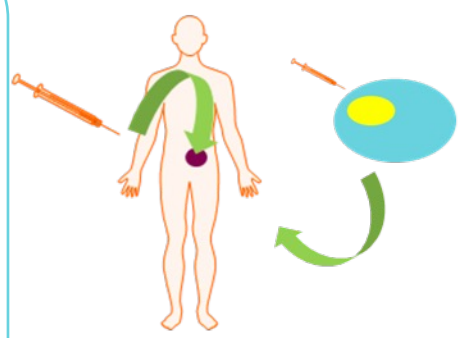


Edit not guaranted

Active RNP complex will bind & release from target DNA until a misrepair occurs

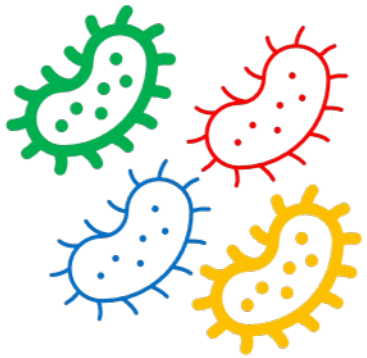
On- and Off-target

Editing has potential to occur in non-target regions



Context of Use

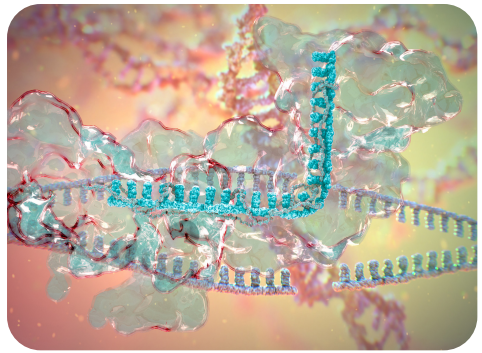
Approaches can either be ex vivo or in vivo



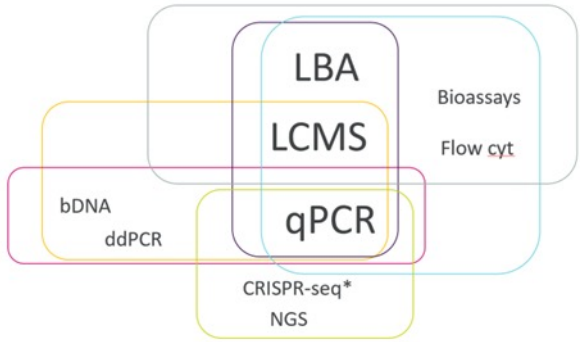
CAS9 origin

Bacterial origin protein recognisable by immune system

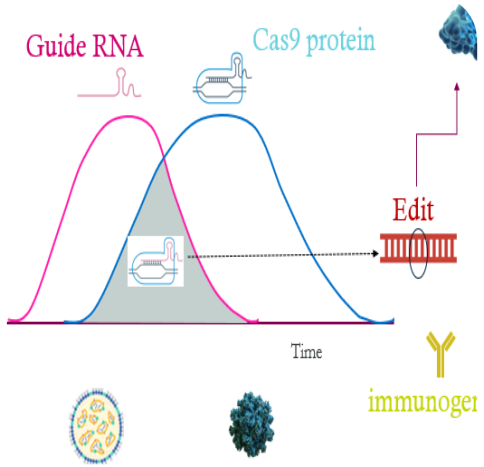
CRISPR bioA summary



CRISPR technology offers us an opportunity to treat disease that are dependent on a single point mutations



The Bioanalytical toolbox consists of classical and novel techniques



Fit for purpose discovery approaches are needed for multiple component understanding

What adds value to progressing molecules will depend upon project-bespoke factors

Acknowledgements

Anna Marzeda

Jakob Alhede

Johnny Lindqvist

Åsa Sandelius

Liselotte Björsson

Amanda Wilson

Mikko Hölttä

Stefanie Krambeck

Roberto Nitsch

Craig Stovold

Do not use layouts in this section appearing after this point.

Delivering CRISPR-Cas9 RNP complex into a cell