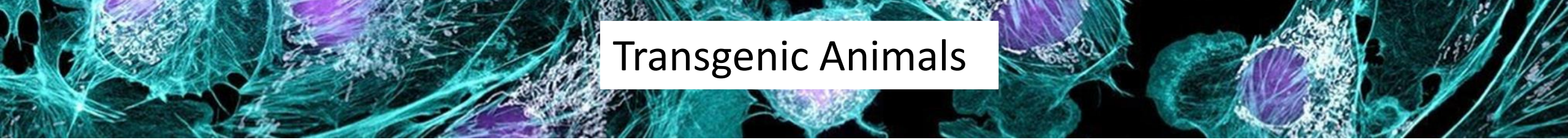
Advanced Cell Biology and Biotechnology

ACBB 2021/22

…the lecture of November 17th is about to begin…



Transgenic Animals

1- DNA microinjection
- random insertion

2- embryonic stem cell-mediated gene transfer
- random insertion
- homologous recombination (double selection)
- Rosa26 locus
- genomic analysis to identify genetically modified animals

- knock-out animals
- knock-in animals
- conditional knock-out (cre-lox technique, inducible systems)
- siRNA
- CRISPR-CAS9

siRNA

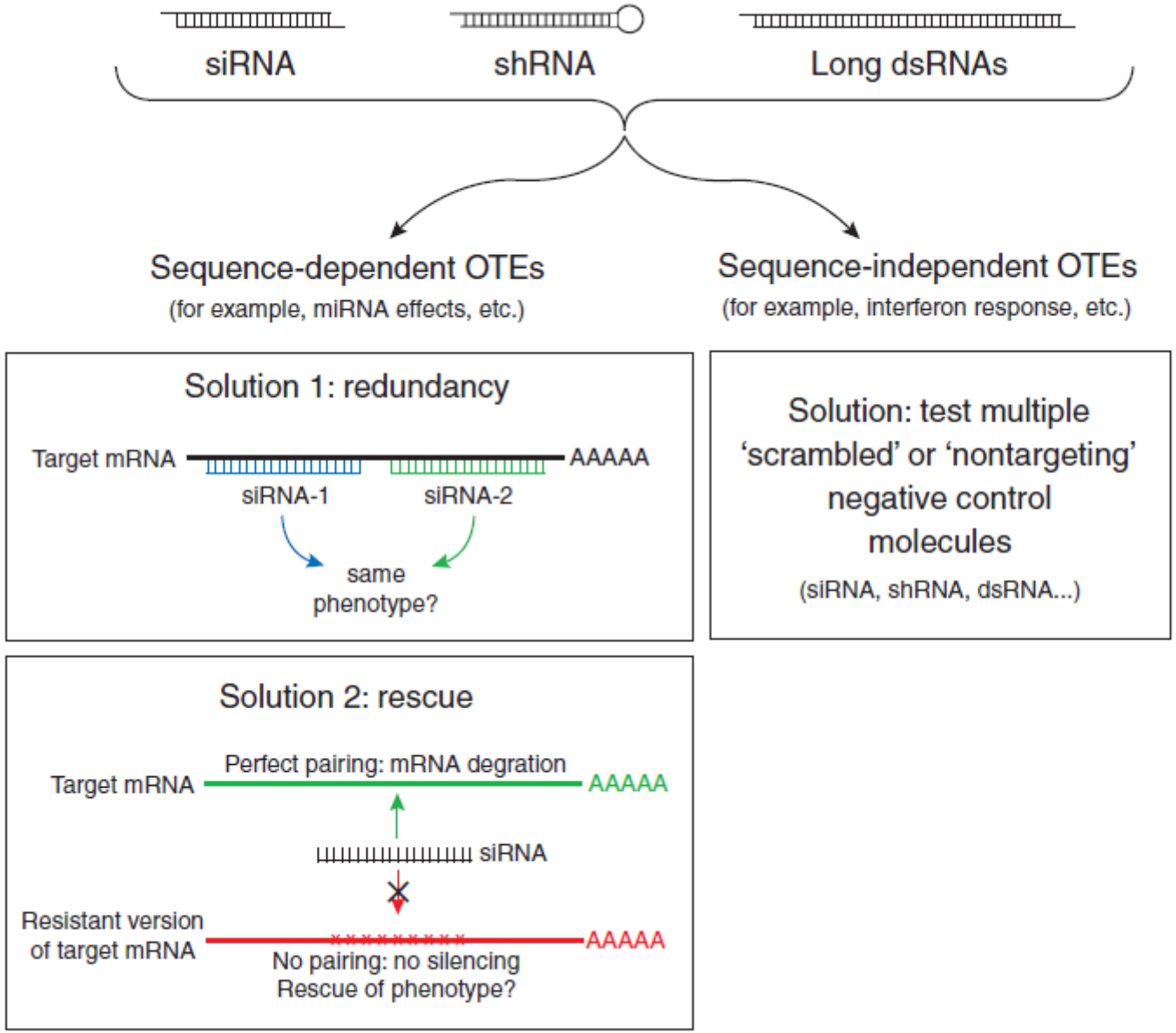


Figure 1 | Appropriate experimental controls to minimize risks of misinterpretation of RNAi data due to off-target effects (OTEs). siRNA-like molecules, vector-based shRNAs and long dsRNAs trigger detectable off-target effects in all major systems studied to date, from mammalian cells to *D. melanogaster* and *C. elegans*. Simple solutions are available to minimize the risk that an observed phenotype may arise from an off-target effect rather than the targeted gene's loss of function.

NATURE METHODS | VOL.3 NO.10 | OCTOBER 2006 |

Minimizing the risk of reporting false positives in large-scale RNAi screens

Christophe J Echeverri[1], Philip A Beachy[2], Buzz Baum[3], Michael Boutros[4], Frank Buchholz[5], Sumit K Chanda[6], Julian Downward[7], Jan Ellenberg[8], Andrew G Fraser[9], Nir Hacohen[10,11], William C Hahn[10,12], Aimee L Jackson[13], Amy Kiger[14], Peter S Linsley[13], Lawrence Lum[15], Yong Ma[2], Bernard Mathey-Prévôt[16], David E Root[8], David M Sabatini[8,17], Jussi Taipale[18], Norbert Perrimon[16,19] & René Bernards[20]

Large-scale RNA interference (RNAi)-based analyses, very much as other 'omic' approaches, have inherent rates of false positives and negatives. The variability in the standards of care applied to validate results from these studies, if left unchecked, could eventually begin to undermine the credibility of RNAi as a powerful functional approach. This Commentary is an invitation to an open discussion started among various users of RNAi to set forth accepted standards that would insure the quality and accuracy of information in the large datasets coming out of genome-scale screens.

Nat Biotechnol. 2009 June ; 27(6): 549–555. doi:10.1038/nbt.1543.

siRNA

Transfection of small RNAs globally perturbs gene regulation by endogenous microRNAs

Aly A. Khan, Doron Betel, Martin L. Miller, Chris Sander, Christina S. Leslie[*], and **Debora S. Marks**[*]

Abstract

Transfection of small RNAs (si/miRNAs) into cells typically lowers expression of many genes. Unexpectedly, increased expression of genes also occurs. We investigated whether this upregulation results from a saturation effect, i.e. competition for intracellular small RNA processing machinery between the transfected si/miRNAs and the endogenous pool of microRNAs (miRNAs). To test this hypothesis, we analyzed genome-wide transcript responses from more than 150 published transfection experiments in 7 different cell types. We show that endogenous miRNA targets have significantly higher expression levels following transfection, consistent with an impaired effectiveness of endogenous miRNA repression. Further confirmation comes from concentration and temporal dependence. Strikingly, the profile of endogenous miRNAs can largely be inferred by correlating miRNA sites with gene expression changes after transfections. The saturation and competition effects present practical implications for miRNA target prediction, the design of si/shRNA genomic screens and siRNA therapeutics.

The NOBEL Prize in Chemistry 2020

"for the development of a method for genome editing"

was awarded jointly to

Emmanuelle Charpentier

born 1968 in Juvisy-sur-Orge, France. Ph.D. 1995 from Institut Pasteur, Paris, France. Director of the Max Planck Unit for the Science of Pathogens, Berlin, Germany.

and **Jennifer A. Doudna**

born 1964 in Washington, D.C, USA. Ph.D. 1989 from Harvard Medical School, Boston, USA. Professor at the University of California, Berkeley, USA and Investigator, Howard Hughes Medical Institute.

https://www.nobelprize.org/prizes/chemistry/2020/press-release/

CRISPR-Cas9

The CRISPR-Cas9 Team

Emmanuelle Charpentier
Jennifer Doudna
Martin Jinek
Krzysztof Chylinski
Ines Fonfara
(Michael Hauer)

Science. 2012 Aug 17;337(6096):816-21. doi: 10.1126/science.1225829

RESEARCH ARTICLE

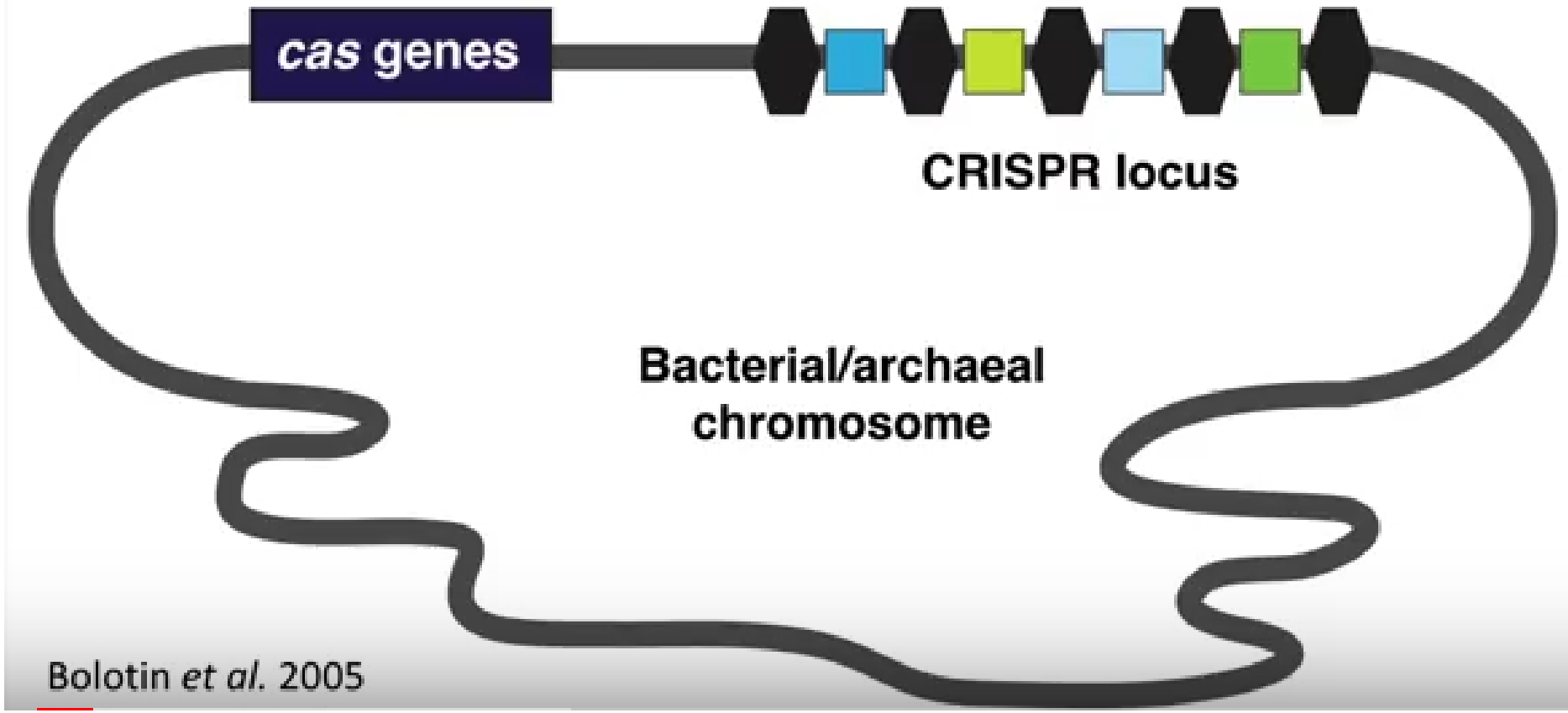
A Programmable Dual-RNA–Guided DNA Endonuclease in Adaptive Bacterial Immunity

Martin Jinek[1,2,*], Krzysztof Chylinski[3,4,*], Ines Fonfara[4], Michael Hauer[2,†], Jennifer A. Doudna[1,2,5,6,‡], Emmanuelle Charpentier[4,‡]

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated systems (Cas) provide bacteria and archaea with adaptive immunity against viruses and plasmids by using CRISPR RNAs (crRNAs) to guide the silencing of invading nucleic acids. We show here that in a subset of these systems, the mature crRNA that is base-paired to trans-activating crRNA (tracrRNA) forms a two-RNA structure that directs the CRISPR-associated protein Cas9 to introduce double-stranded (ds) breaks in target DNA. At sites complementary to the crRNA-guide sequence, the Cas9 HNH nuclease domain cleaves the complementary strand, whereas the Cas9 RuvC-like domain cleaves the non complementary strand. The dual-tracrRNA:crRNA, when engineered as a single RNA chimera, also directs sequence-specific Cas9 dsDNA cleavage. **Our study reveals a family of endonucleases that use dual-RNAs for site-specific DNA cleavage and highlights the potential to exploit the system for RNA-programmable genome editing.**

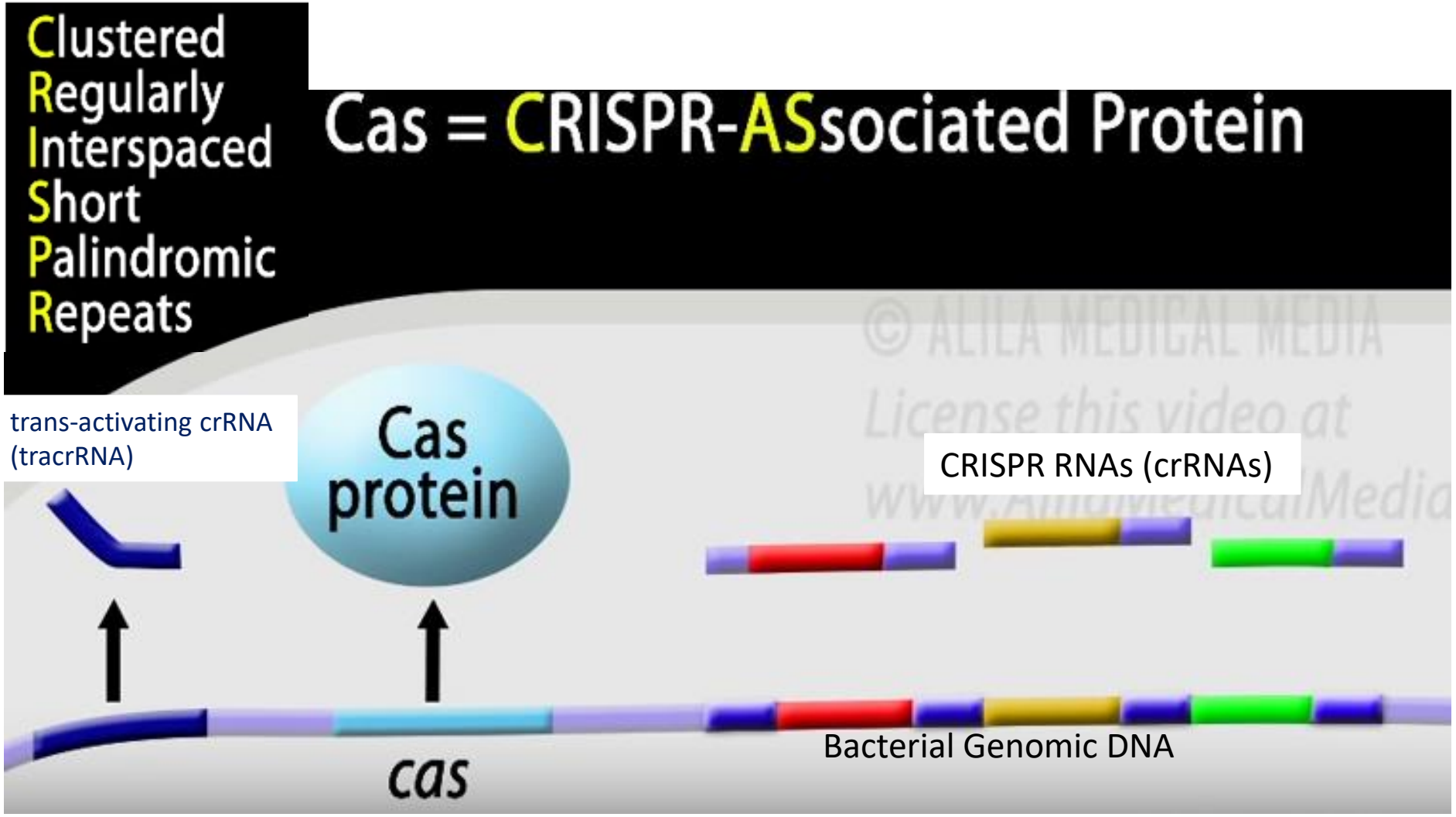
CRISPRs: Hallmarks of acquired immunity in bacteria

Clusters of Regularly Interspaced Short Palindromic Repeats (CRISPRs)

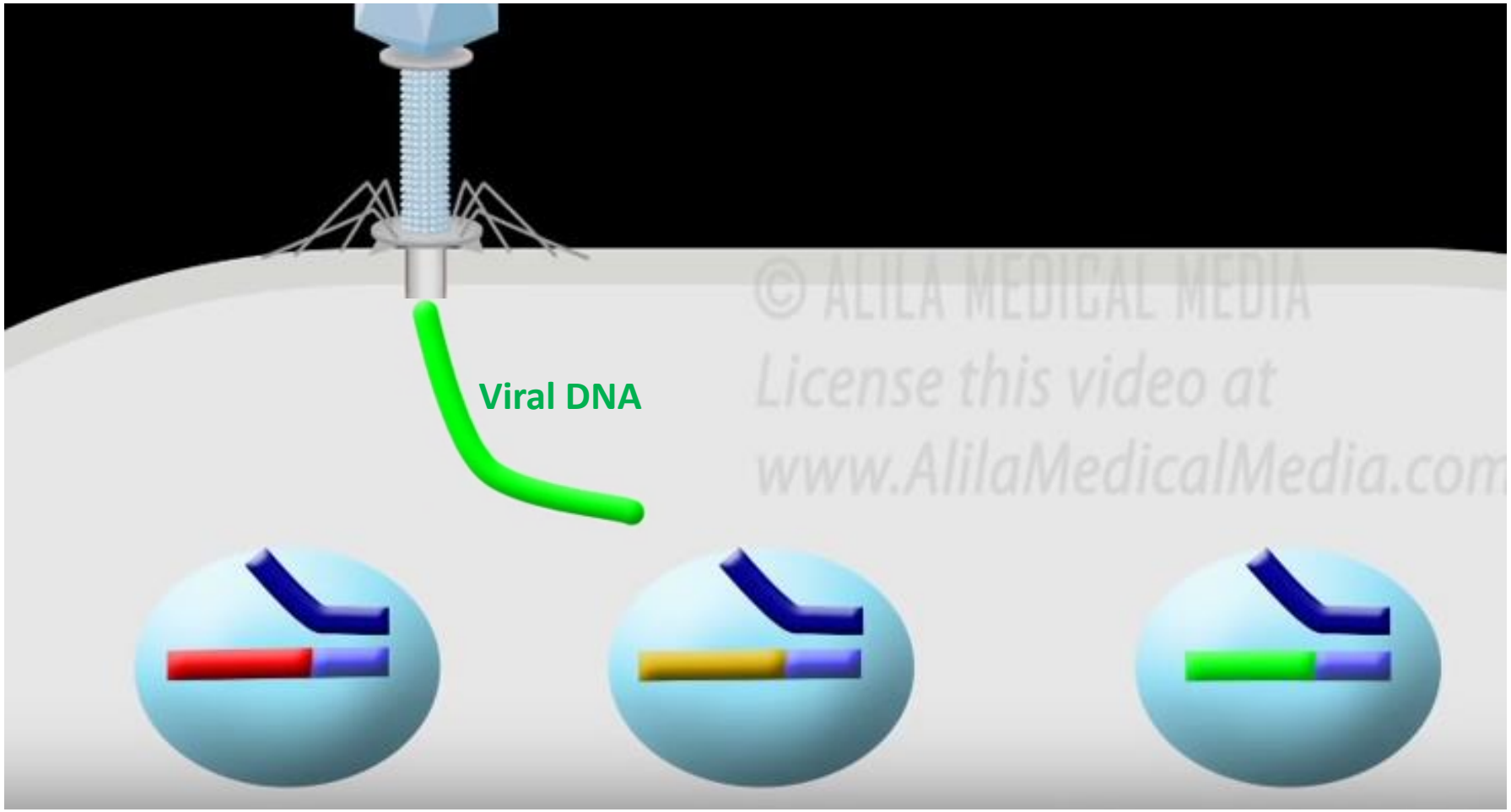


Bolotin *et al.* 2005

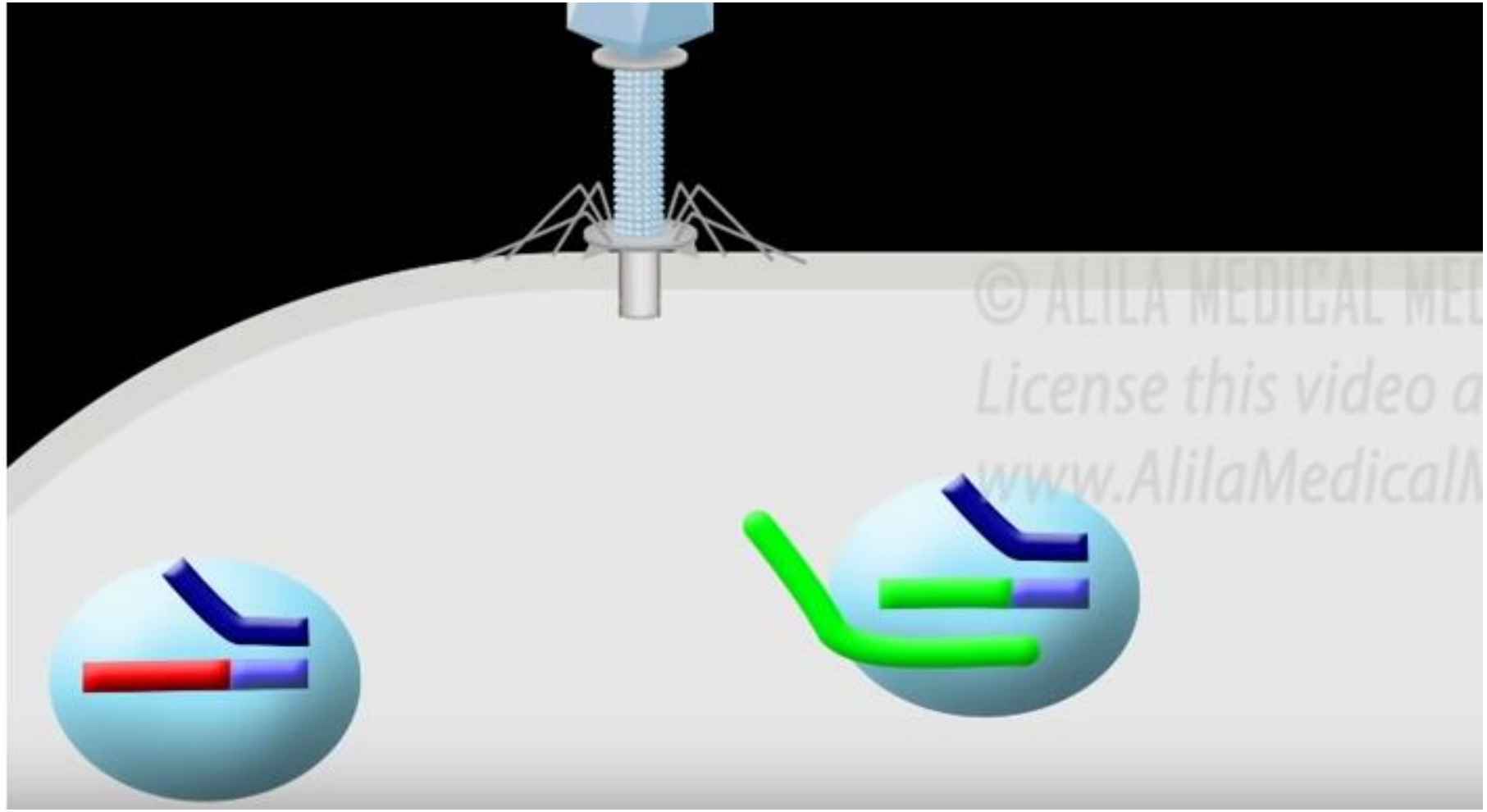
https://www.youtube.com/watch?v=O3e2_Ctty_M



The CRISPR region is a DNA library of possible bacteria enemies that need to be recognized and distroyed.



Association with CAS, an endonuclease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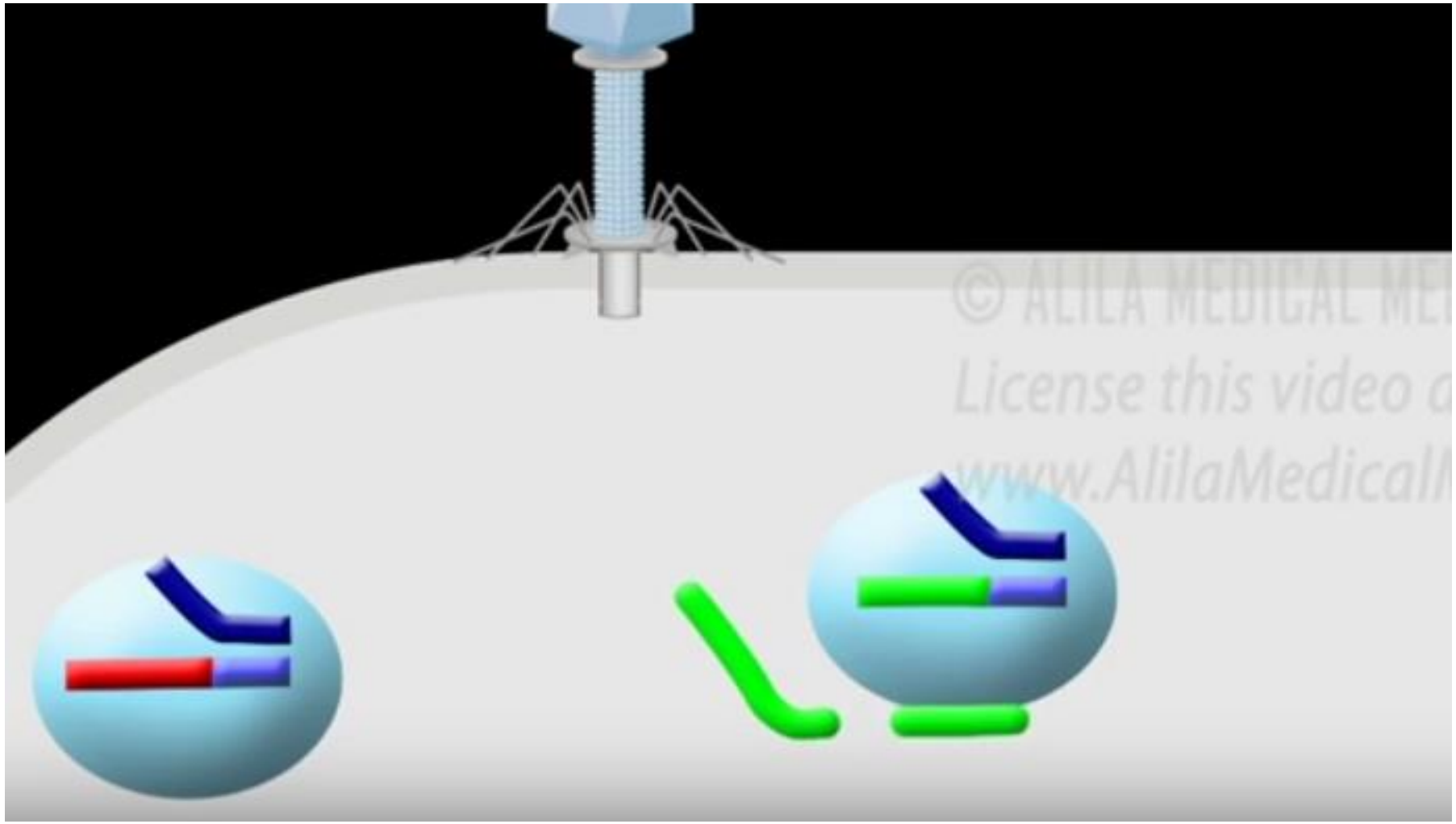
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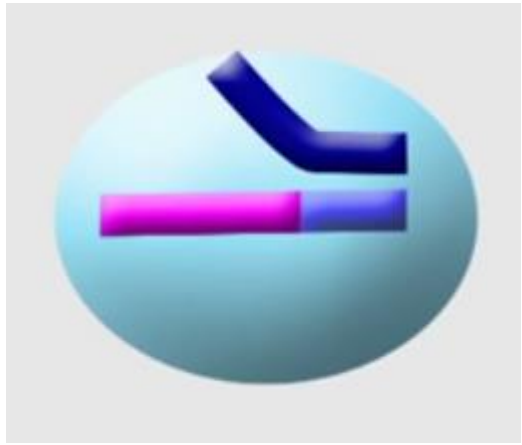
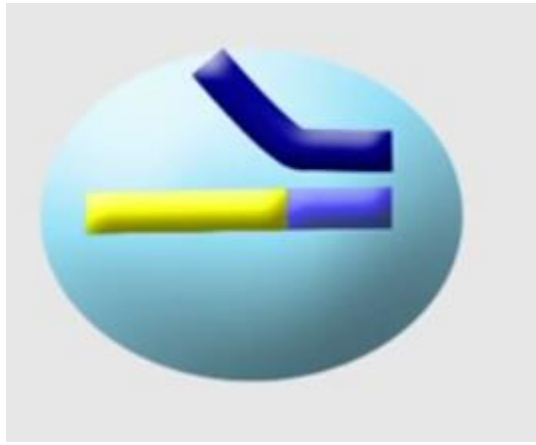
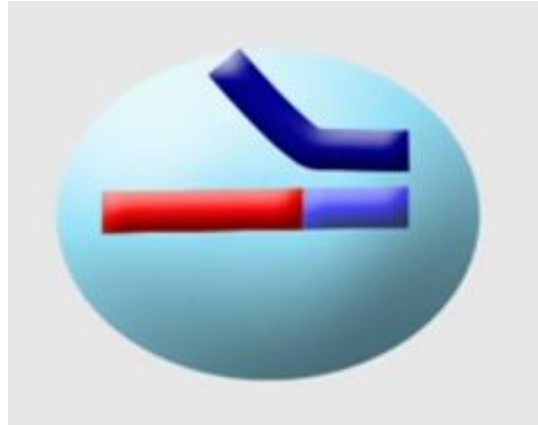
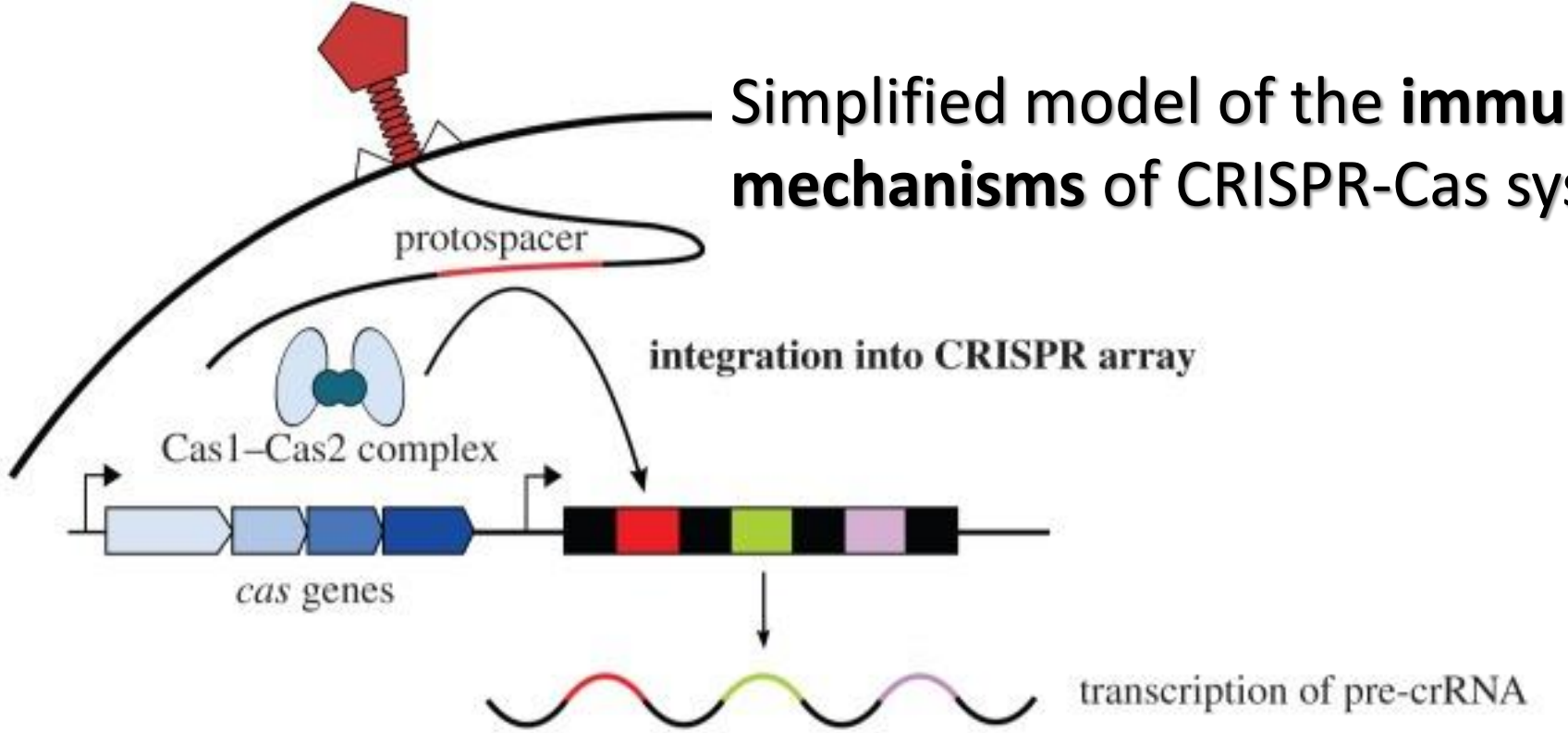






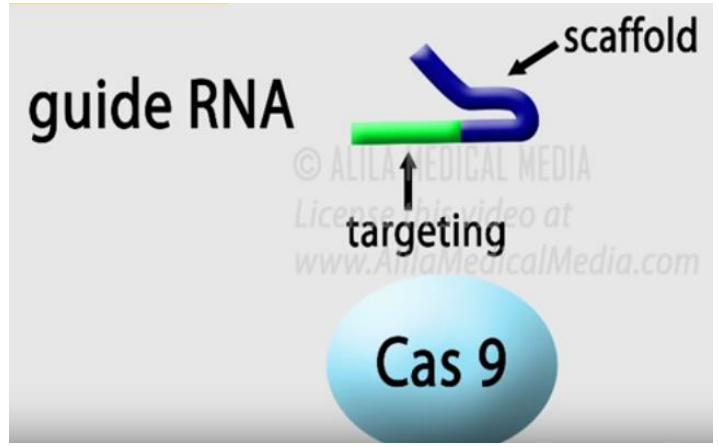
Simplified model of the **immunity mechanisms** of CRISPR-Cas systems.

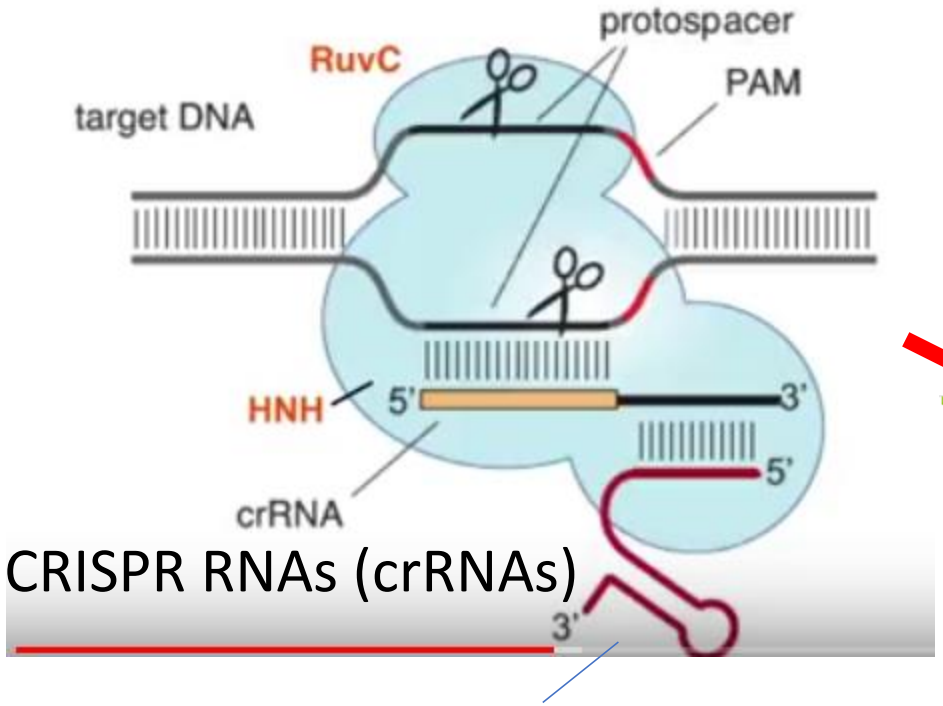


The CRISPR-Cas system is composed of a ***cas* operon** (blue arrows) and a **CRISPR array** that comprises identical repeat sequences (**black rectangles**) that are interspersed by phage-derived spacers (**coloured rectangles**). Upon **phage infection**, a sequence of the invading DNA (protospacer) is incorporated into the CRISPR array.

The CRISPR array is then transcribed into a long precursor CRISPR RNA (pre-crRNA), which is further processed. A ribonucleoprotein complex consisting of Cas9 and a tracrRNA : crRNA duplex targets and cleaves invading DNA.

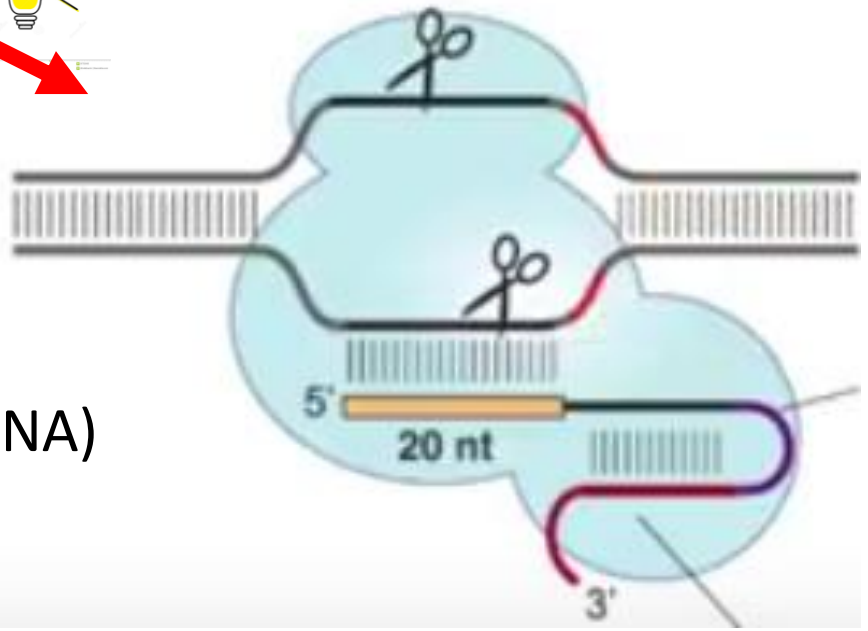
Cas9 is a dual-RNA-guided dsDNA endonuclease





CRISPR RNAs (crRNAs)

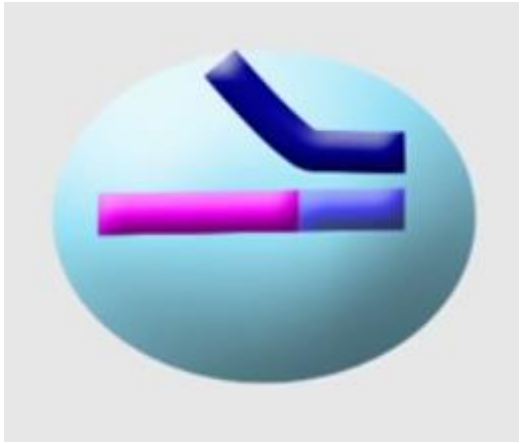
trans-activating crRNA (tracrRNA)

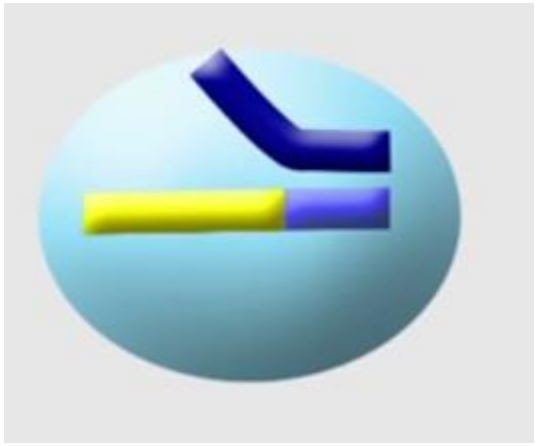


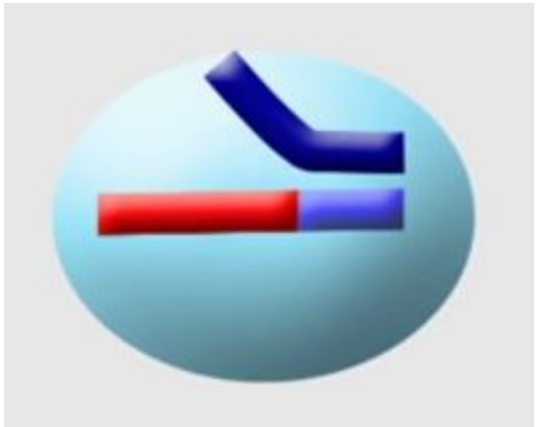
https://www.youtube.com/watch?v=2pp17E4E-O8

crRNA-tracrRNA chimera

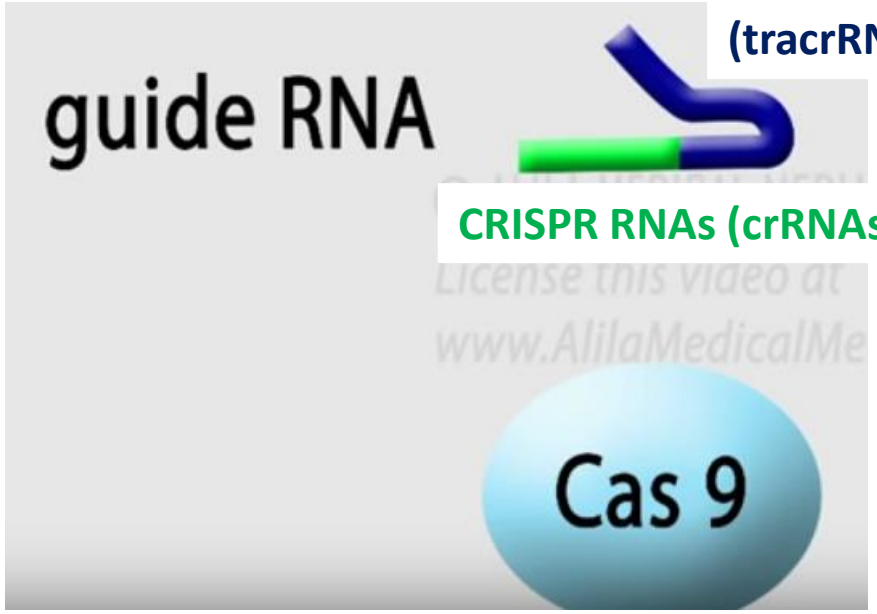
Jennifer Doudna: Genome Engineering with CRISPR-Cas9

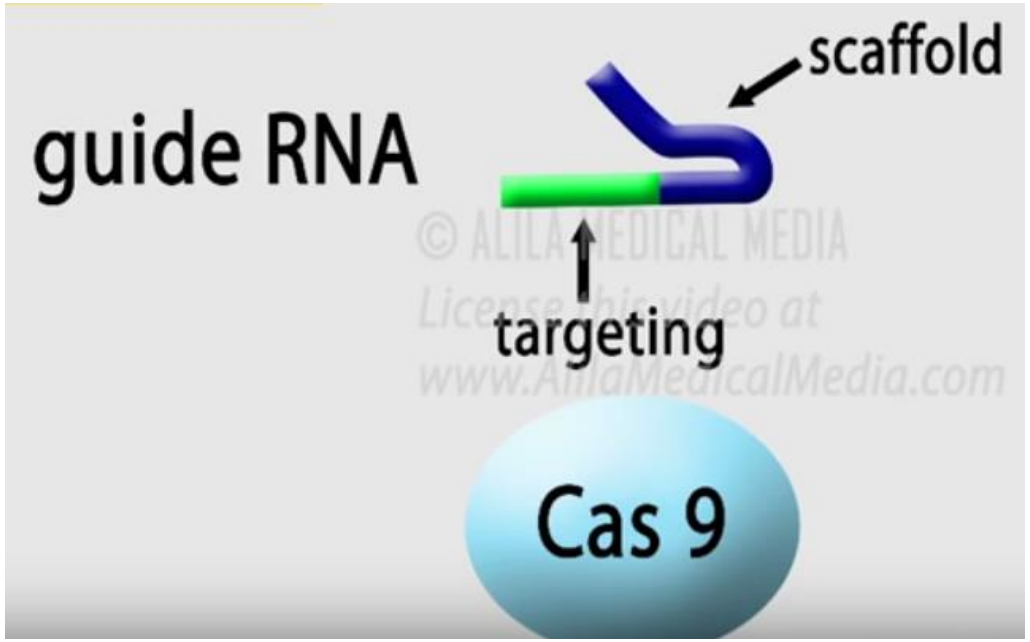


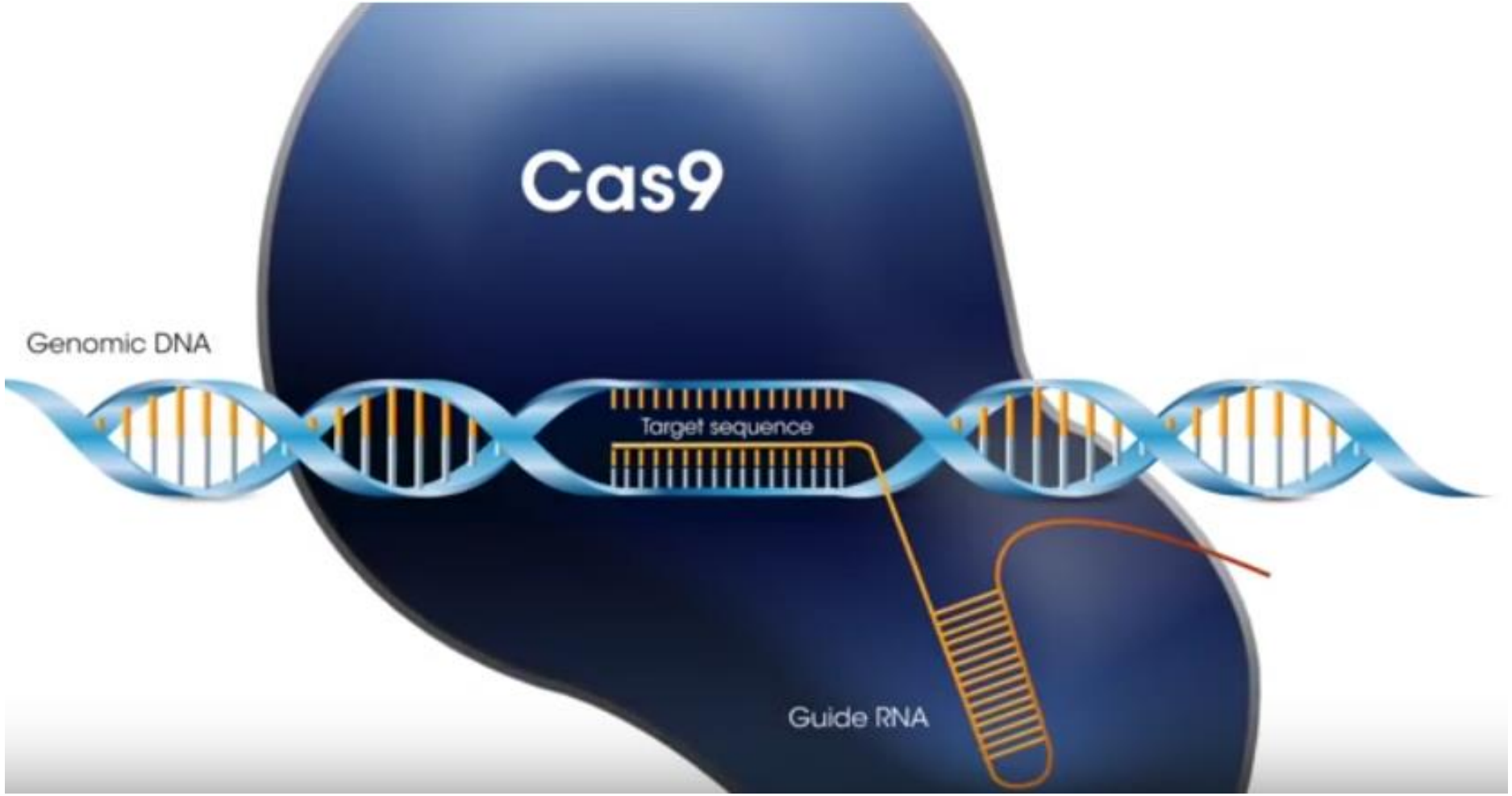




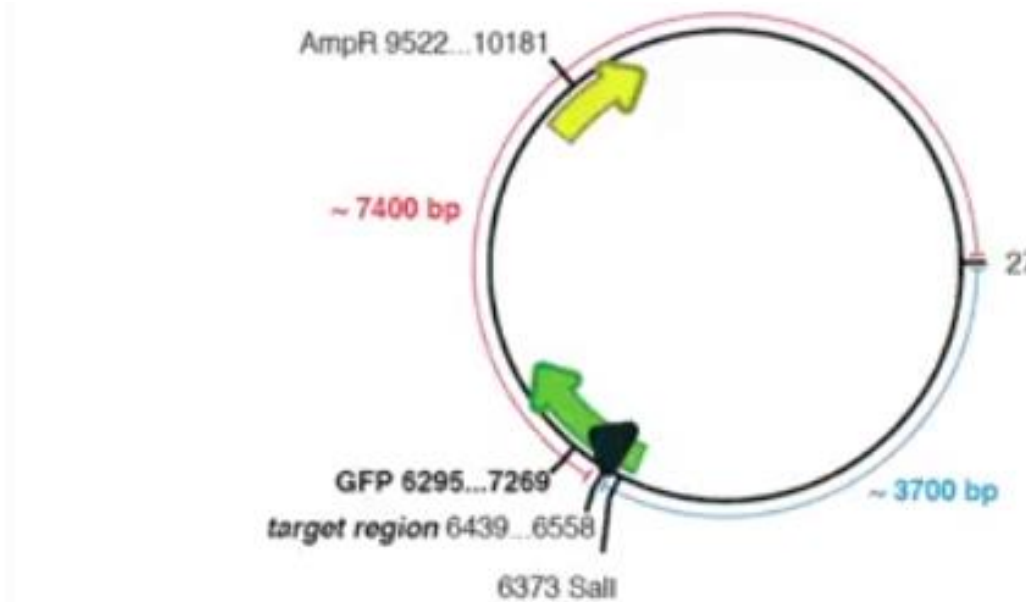
trans-activating crRNA (tracrRNA)

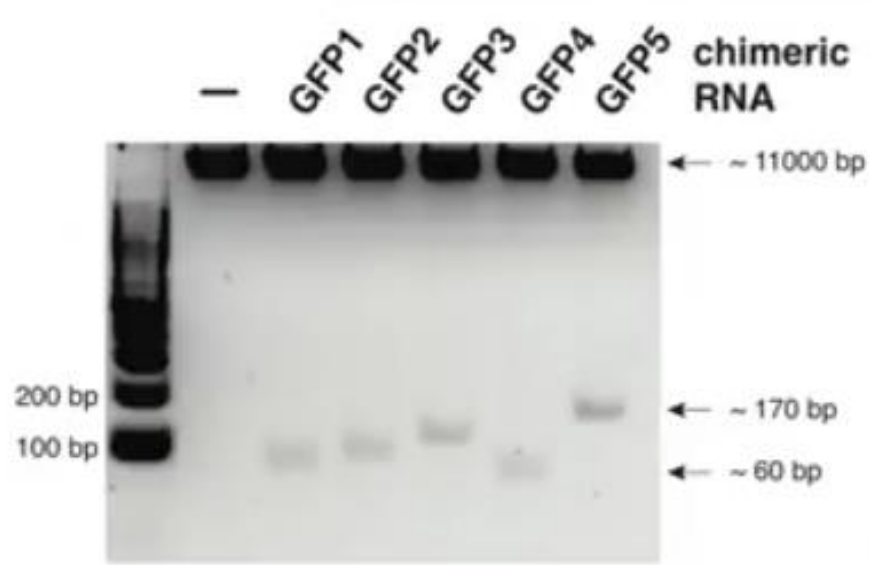


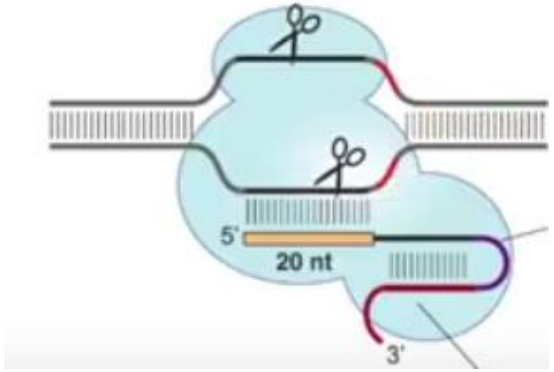



Cas9
Genomic DNA
Target sequence
Guide RNA

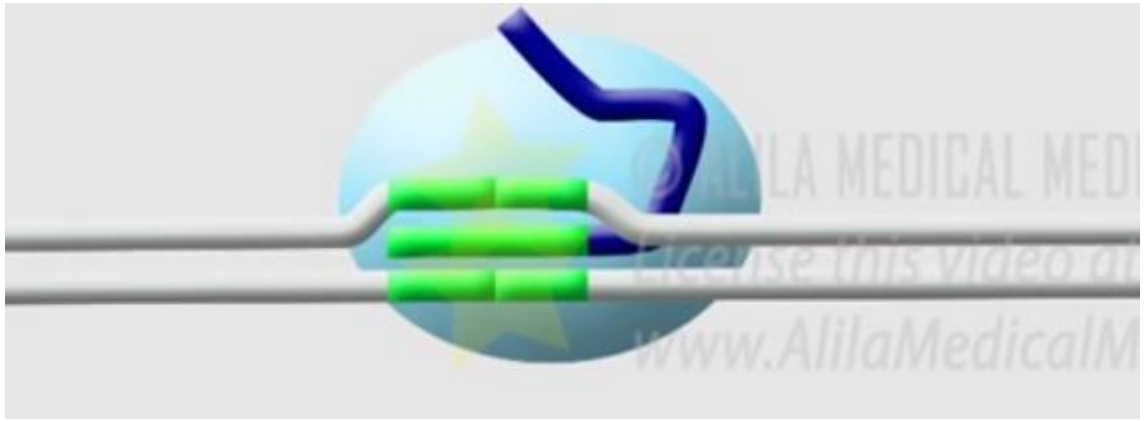
Programmed Cas9 cleaves DNA at specified sites

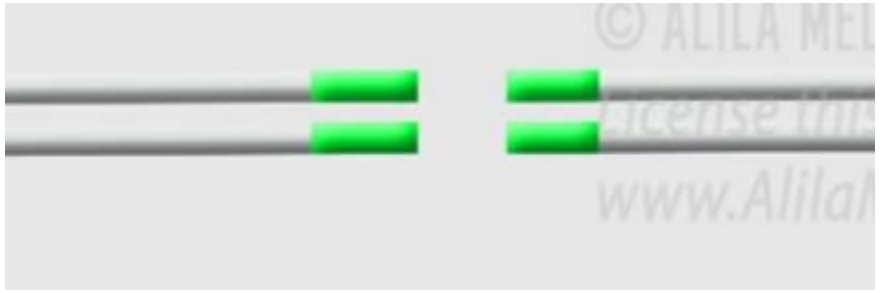




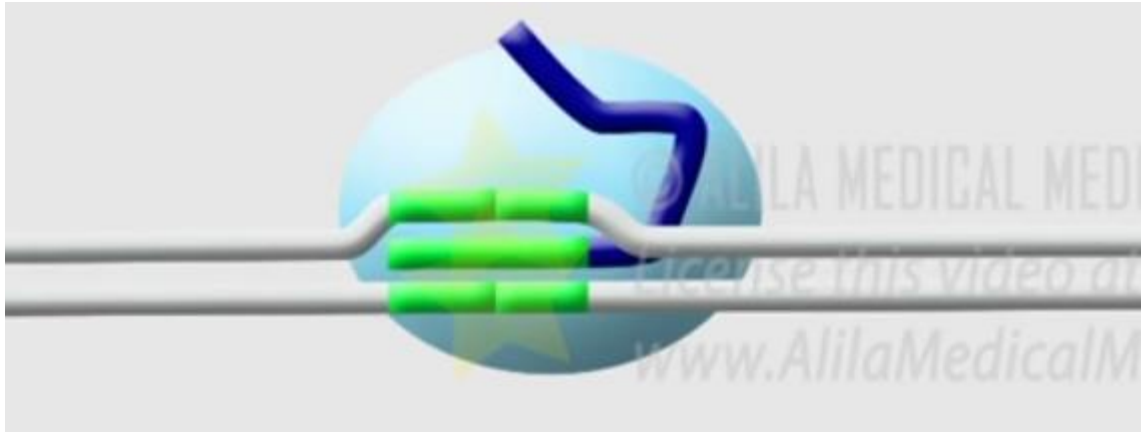


Generating a knock-out

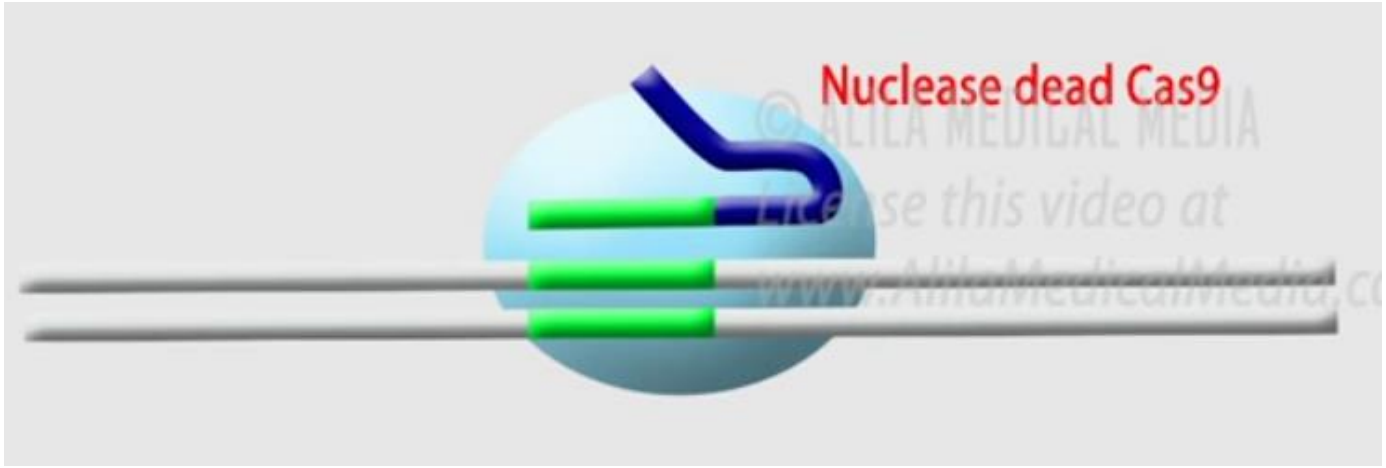


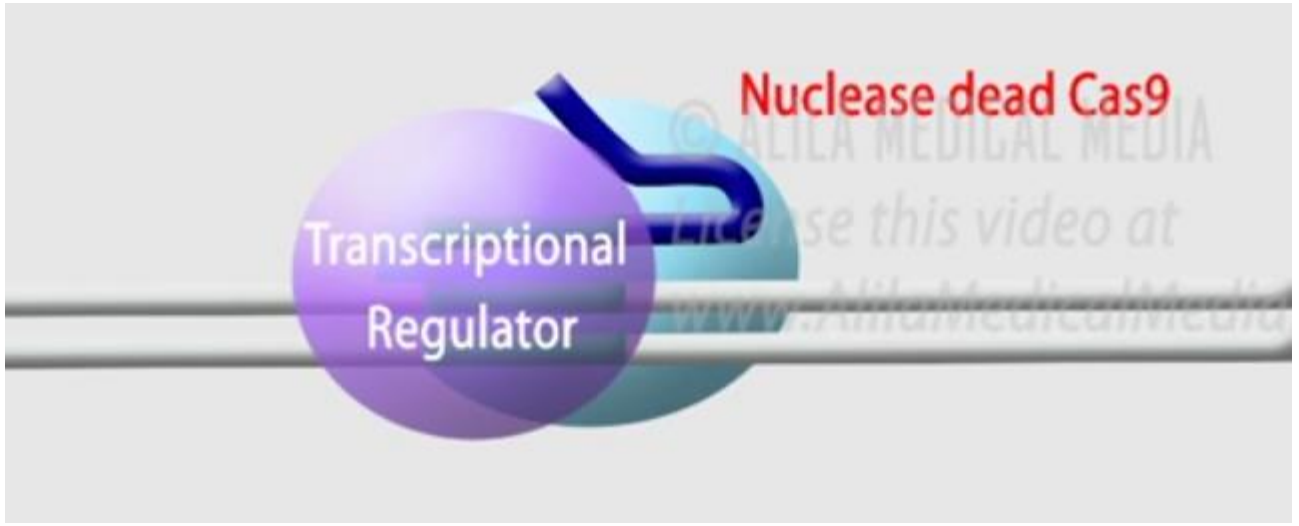


Making precise modifications (DNA editing)

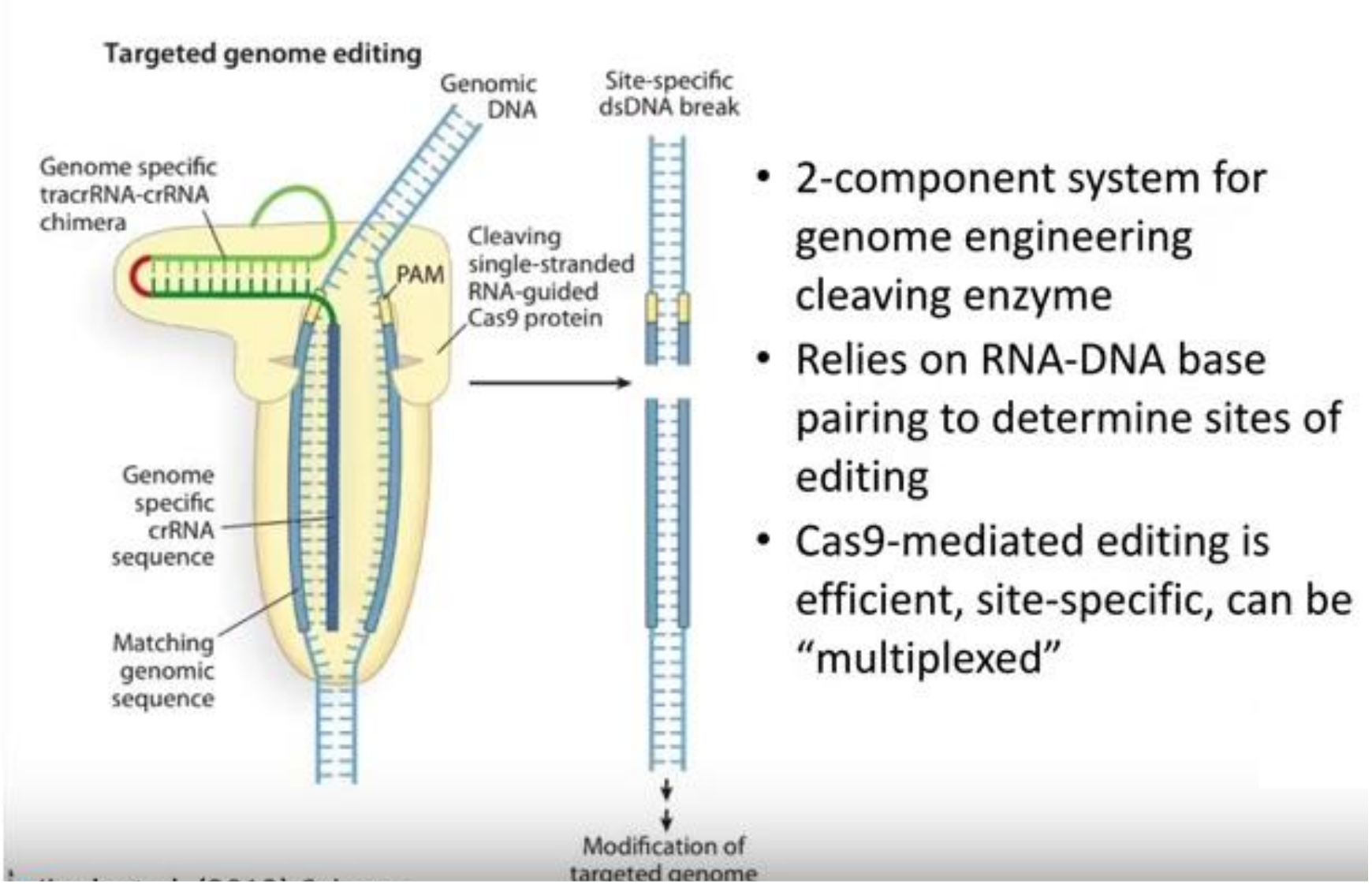


Activation/repression of target genes

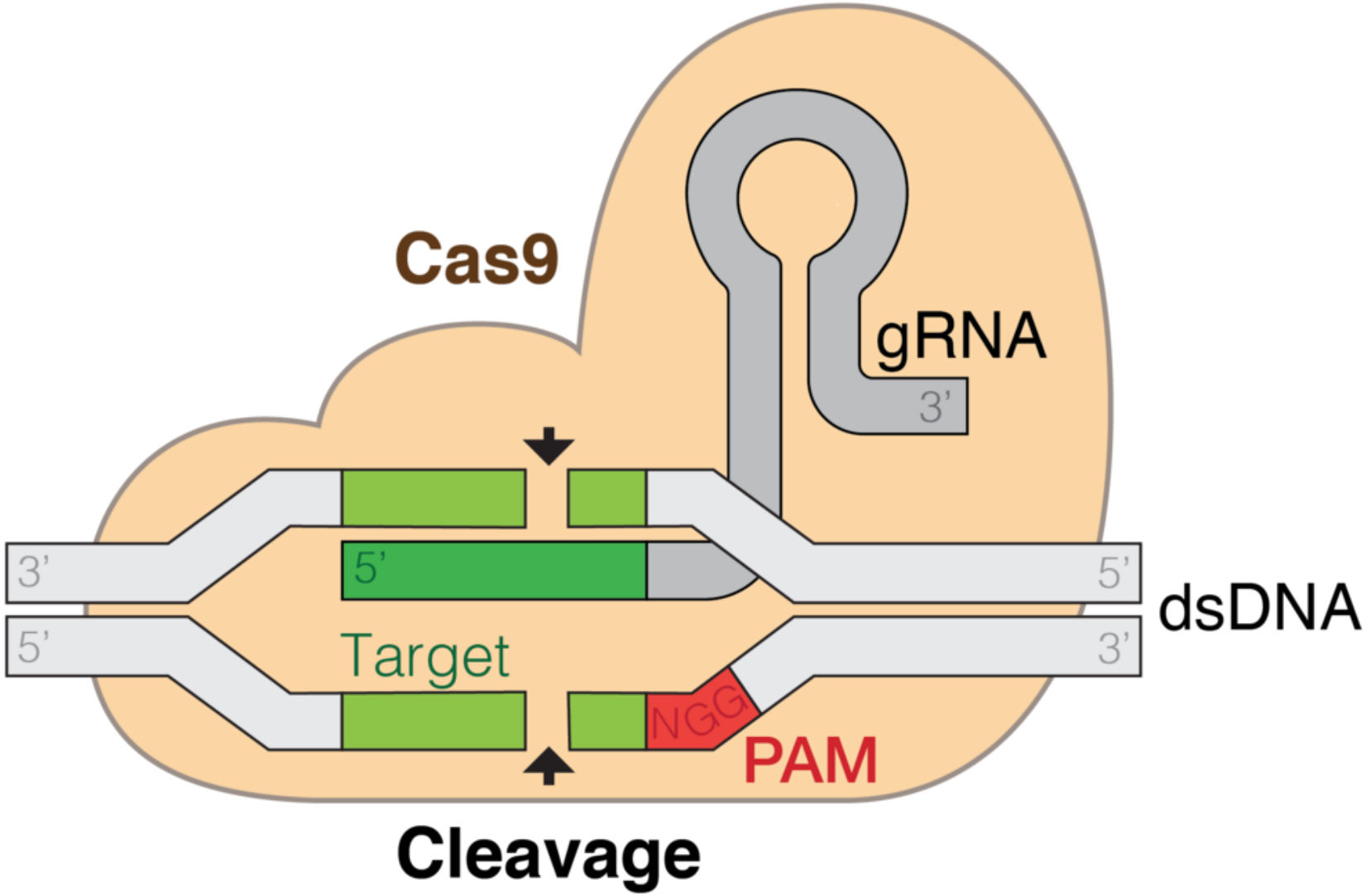


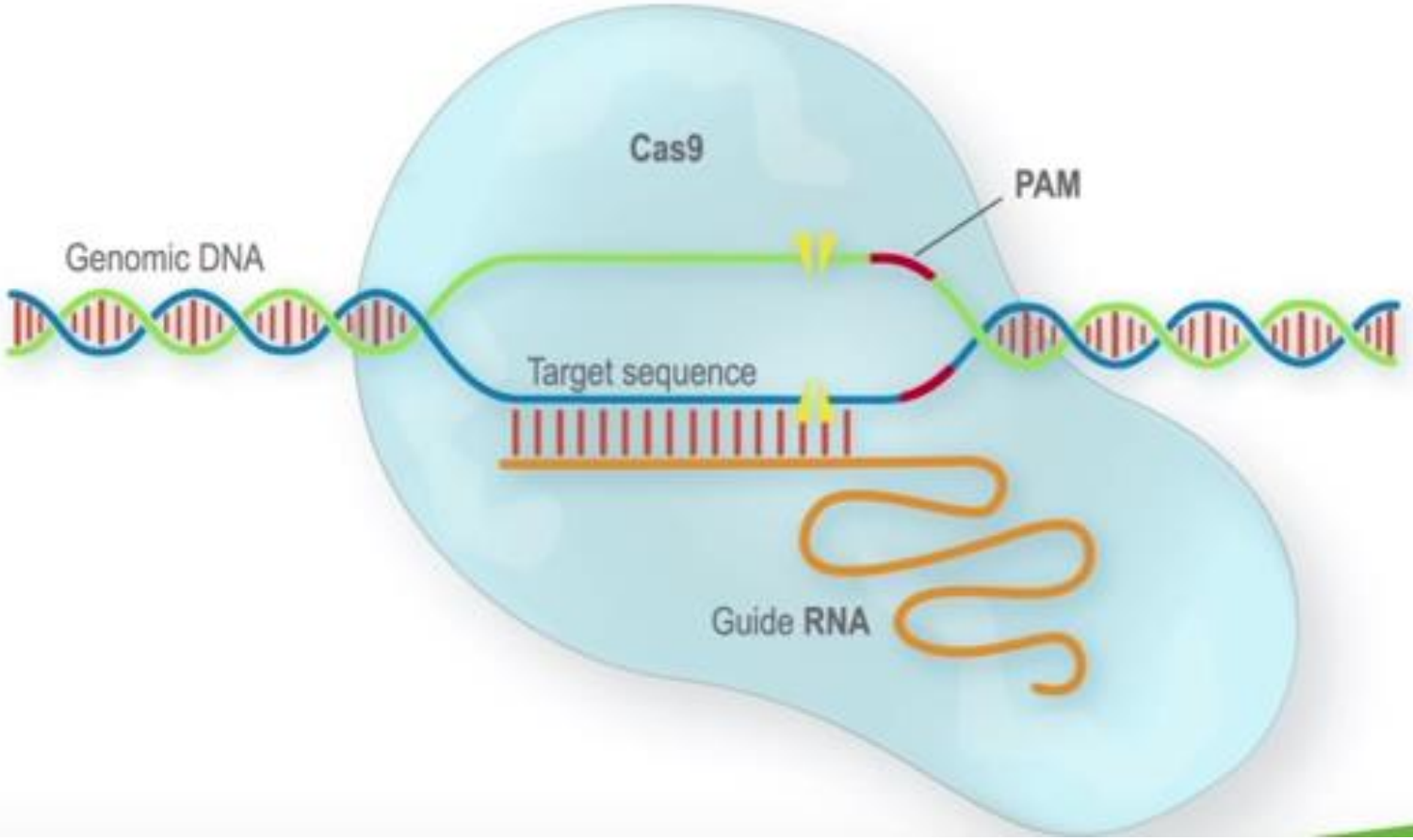


CRISPR-Cas9 technology



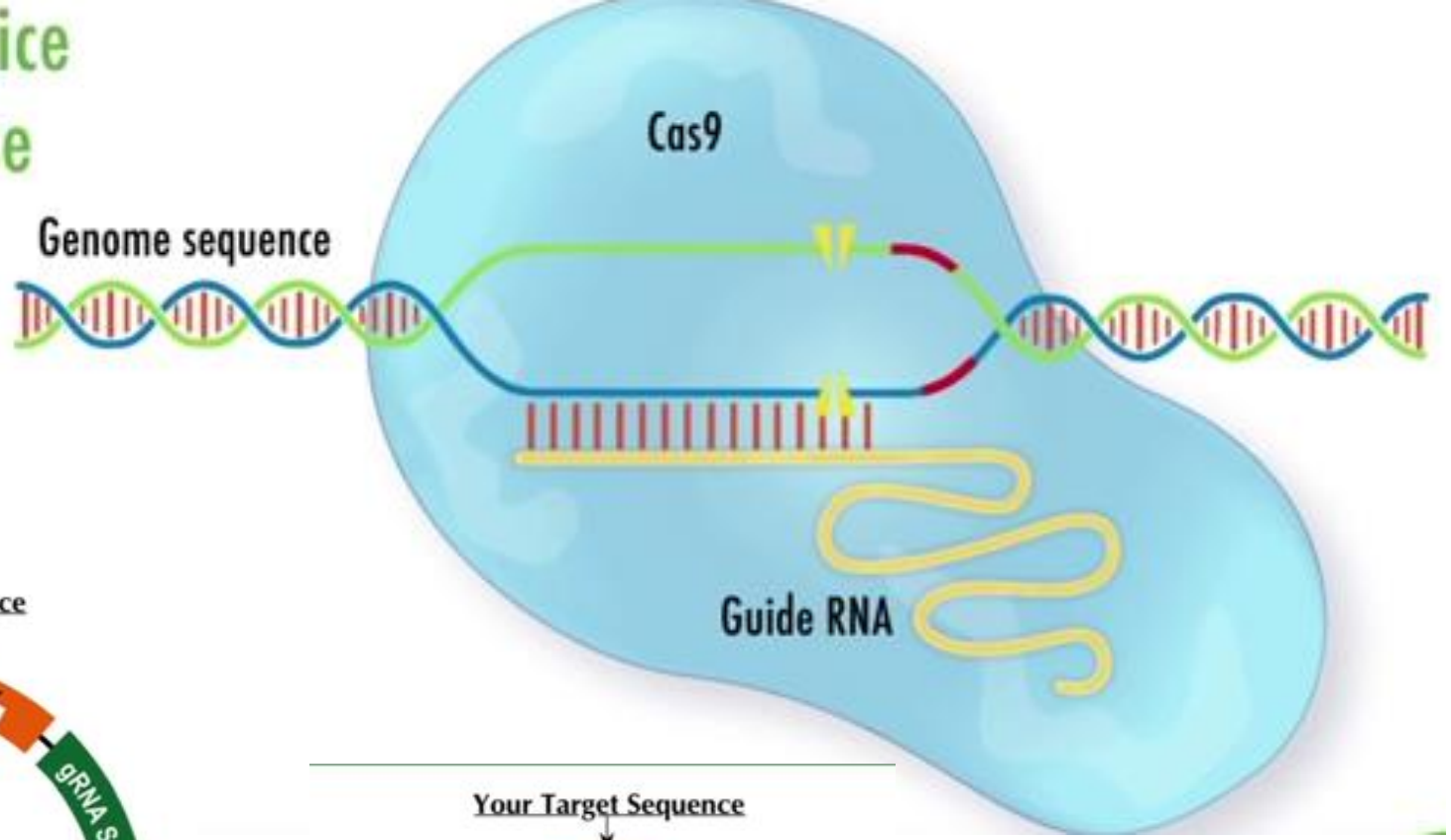
- 2-component system for genome engineering cleaving enzyme
- Relies on RNA-DNA base pairing to determine sites of editing
- Cas9-mediated editing is efficient, site-specific, can be “multiplexed”

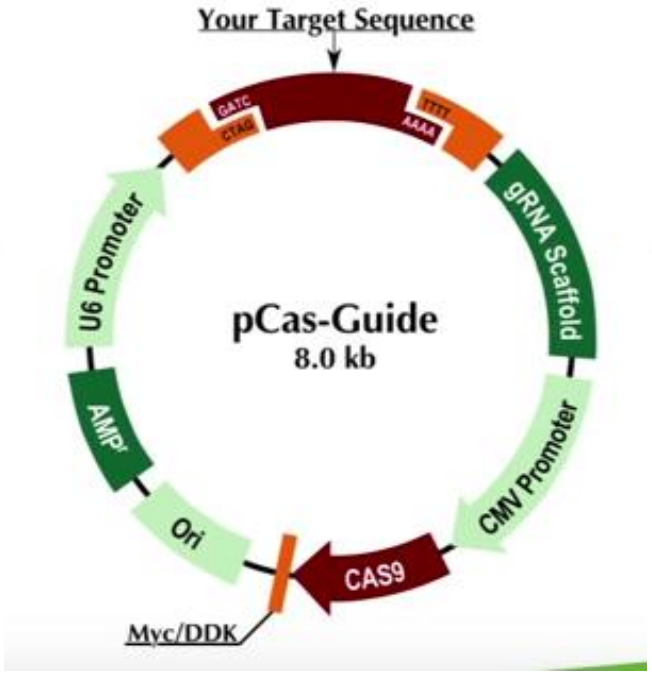

Cas9
gRNA
3'
3'
5'
5'
dsDNA
5'
Target
3'
NGG
PAM
Cleavage

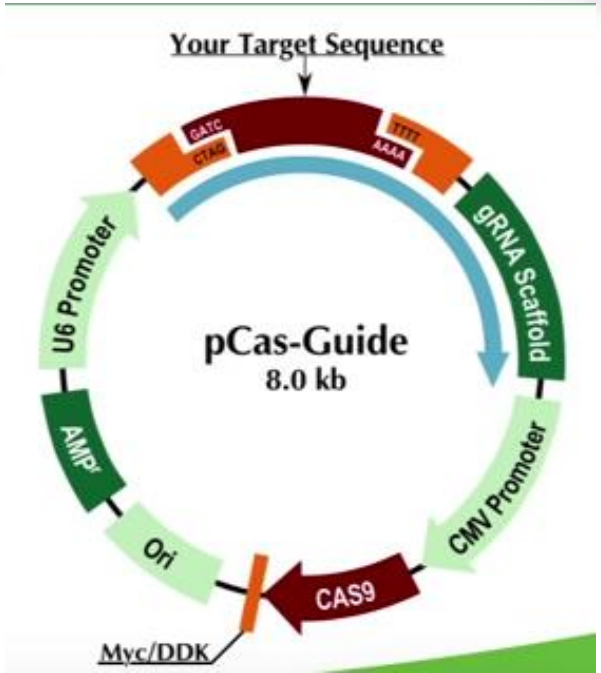


https://www.youtube.com/watch?v=0dRT7slyGhs

+ Homing Device
+ Endonuclease





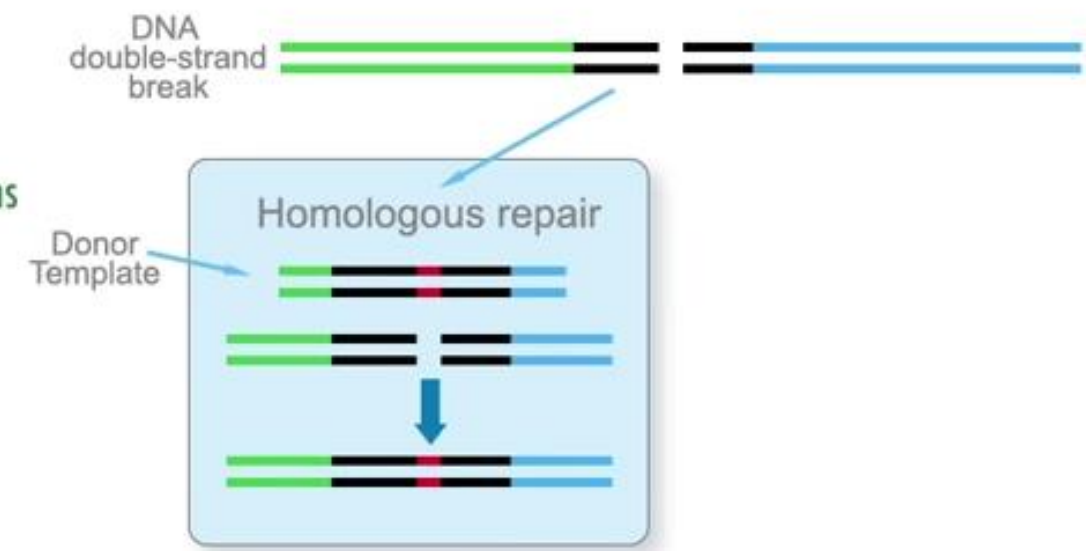


CRISPR | Cas9

Two Main Mechanisms to Repair DSB

Desired modifications

+ Gene knock-out
+ Gene tagging
+ Specific mutations/insertion/deletions
+ Knock-in (report genes)
+ Promoter study



CRISPR | Cas9

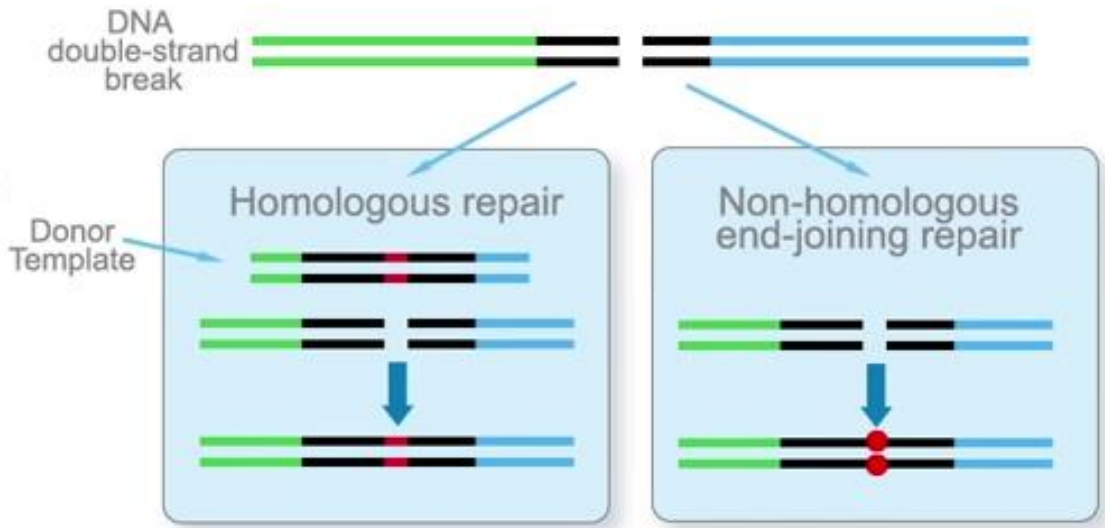
Two Main Mechanisms to Repair DSB

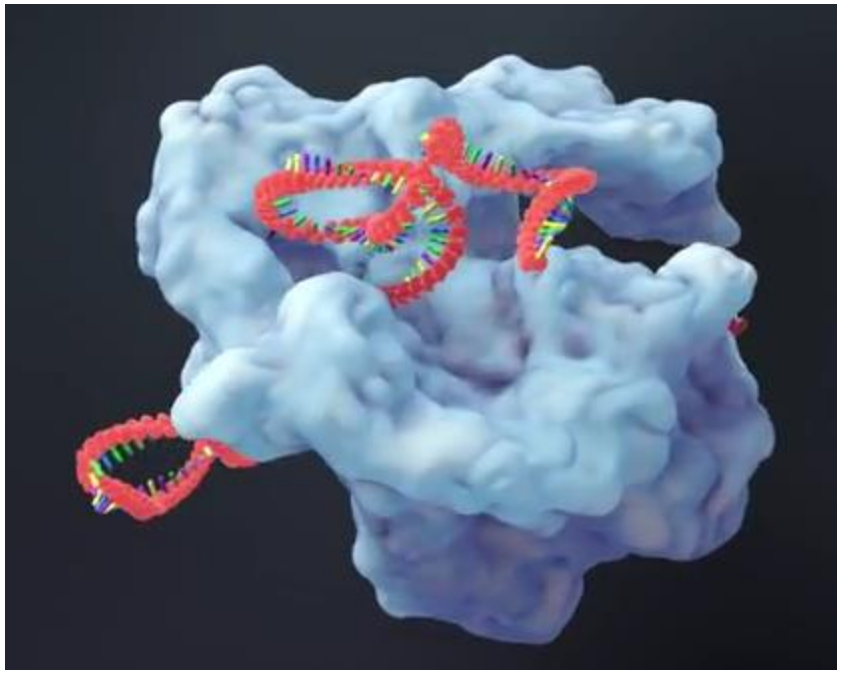
Desired modifications

+ Gene knock-out
+ Gene tagging
+ Specific mutations/insertion/deletions
+ Knock-in (report genes)
+ Promoter study

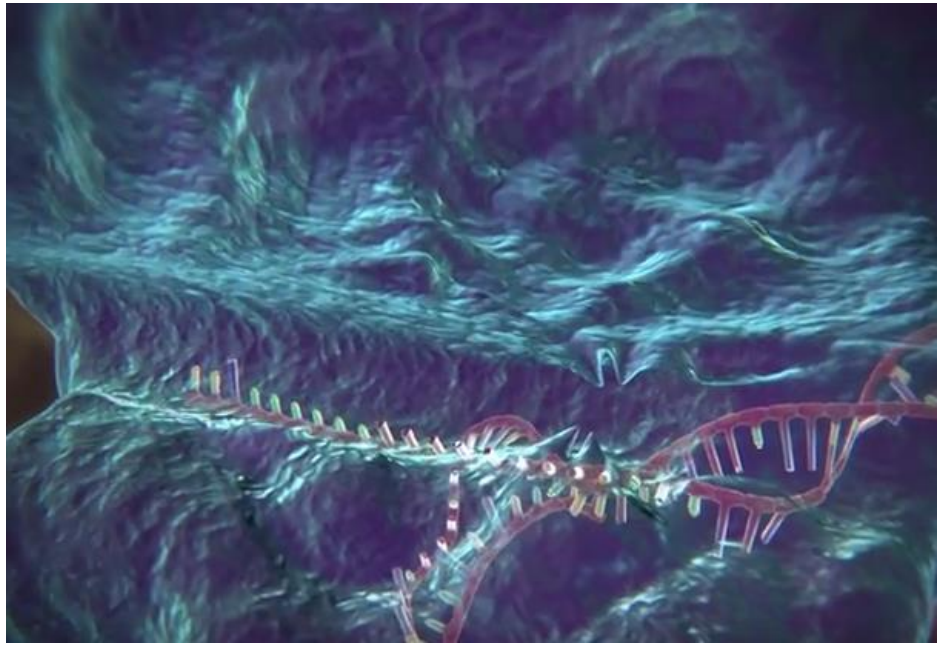
Potential

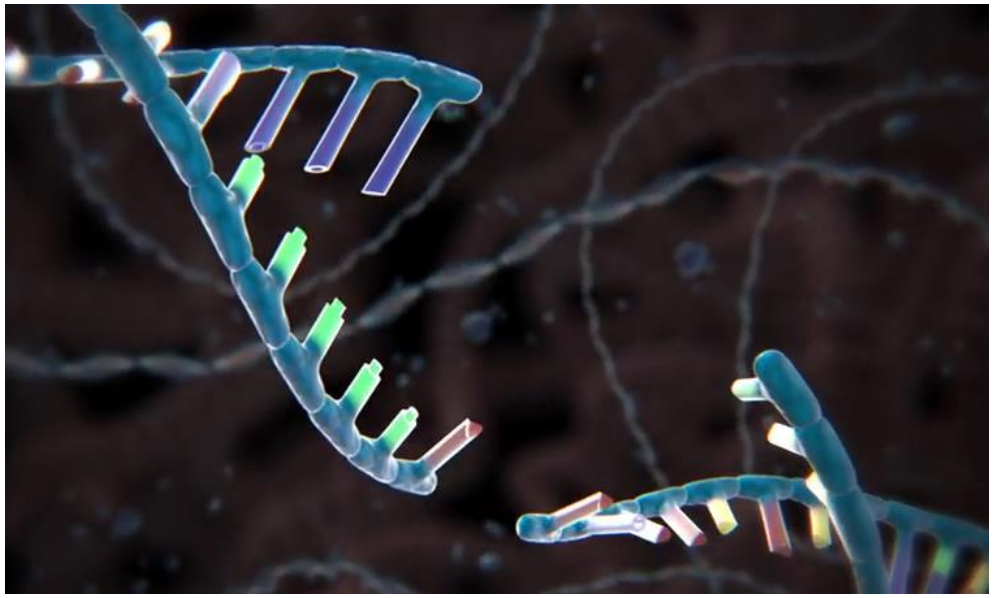
+ Mutations
+ Insertions | deletions
+ Gene knockout





https://www.youtube.com/watch?v=4YKFw2KZA5o





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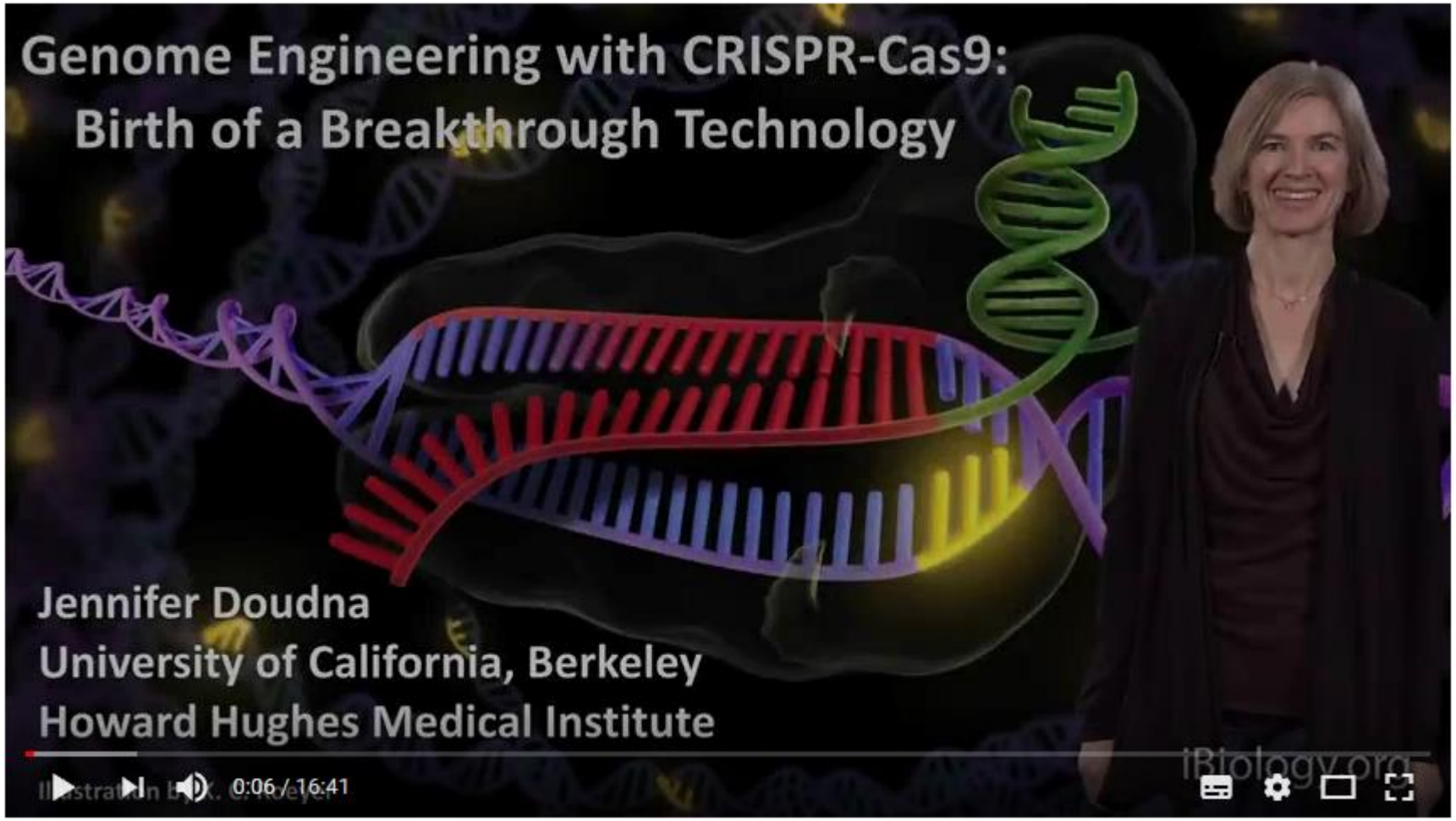
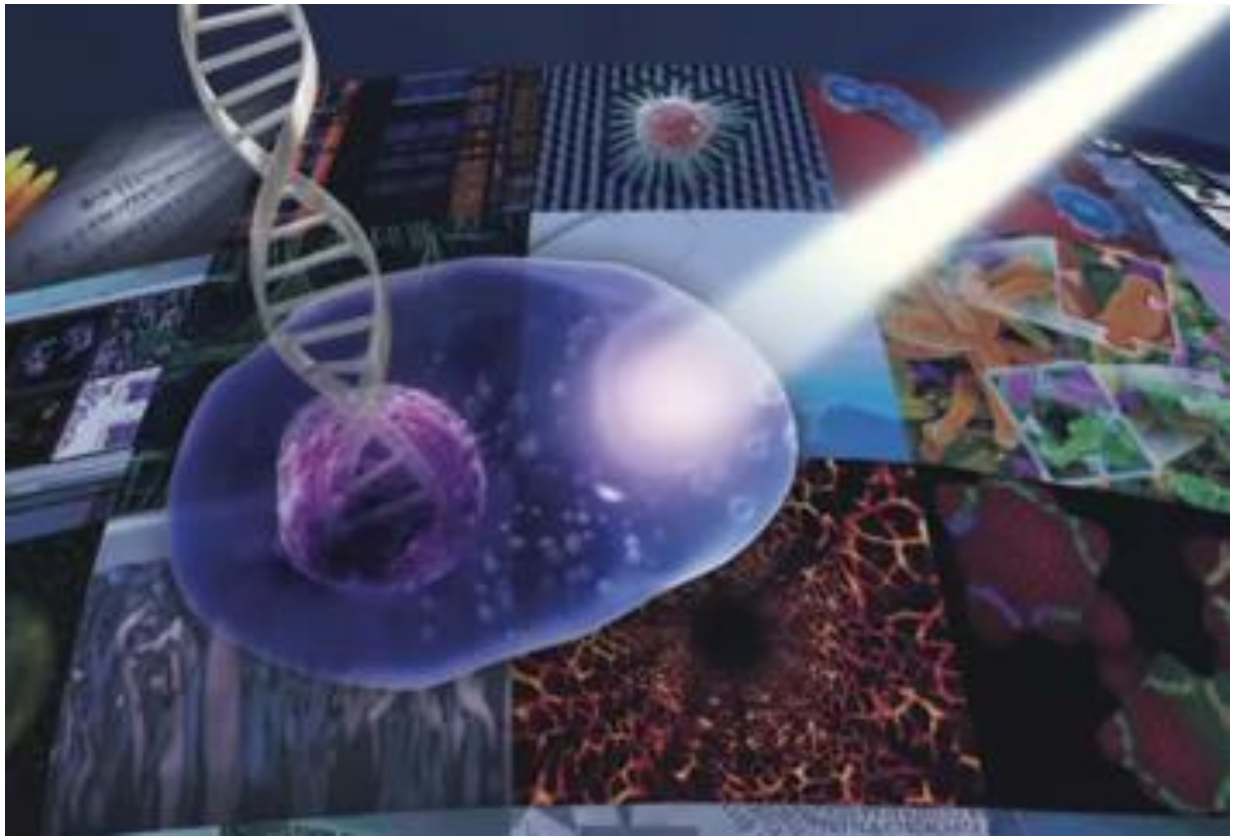


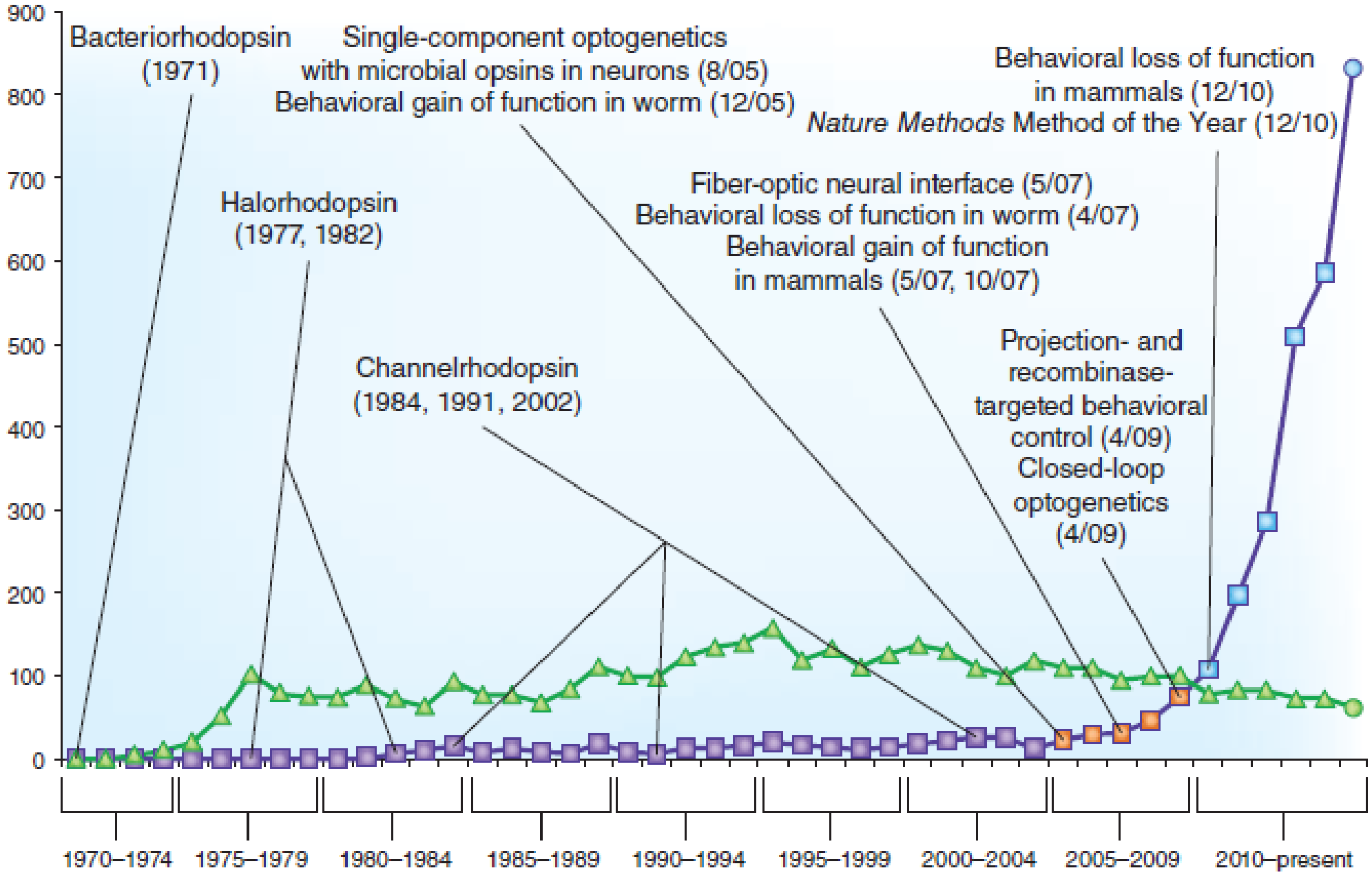
https://www.youtube.com/watch?v=SuAxDVBt7kQ

Optogenetic: method of the year 2010



https://www.youtube.com/watch?v=I64X7vHSHOE

Optogenetics is the combination of genetic and optical methods to cause or inhibit well defined events in specific cells of living tissue and behaving animals


900
800
700
600
500
400
300
200
100
0
Bacteriorhodopsin (1971)
Halorhodopsin (1977, 1982)
Channelrhodopsin (1984, 1991, 2002)
Single-component optogenetics with microbial opsins in neurons (8/05)
Behavioral gain of function in worm (12/05)
Fiber-optic neural interface (5/07)
Behavioral loss of function in worm (4/07)
Behavioral gain of function in mammals (5/07, 10/07)
Projection- and recombinase-targeted behavioral control (4/09)
Closed-loop optogenetics (4/09)
Behavioral loss of function in mammals (12/10)
Nature Methods Method of the Year (12/10)
1970–1974
1975–1979
1980–1984
1985–1989
1990–1994
1995–1999
2000–2004
2005–2009
2010–present

Optogenetics

Karl Deisseroth

Optogenetics is a technology that allows targeted, fast control of precisely defined events in biological systems as complex as freely moving mammals. By delivering optical control at the speed (millisecond-scale) and with the precision (cell type–specific) required for biological processing, optogenetic approaches have opened new landscapes for the study of biology, both in health and disease.

Optogenetics: 10 years of microbial opsins in neuroscience

Karl Deisseroth

Over the past 10 years, the development and convergence of microbial opsin engineering, modular genetic methods for cell-type targeting and optical strategies for guiding light through tissue have enabled versatile optical control of defined cells in living systems, defining modern optogenetics. Despite widespread recognition of the importance of spatiotemporally precise causal control over cellular signaling, for nearly the first half (2005–2009) of this 10-year period, as optogenetics was being created, there were difficulties in implementation, few publications and limited biological findings. In contrast, the ensuing years have witnessed a substantial acceleration in the application domain, with the publication of thousands of discoveries and insights into the function of nervous systems and beyond. This Historical Commentary reflects on the scientific landscape of this decade-long transition.

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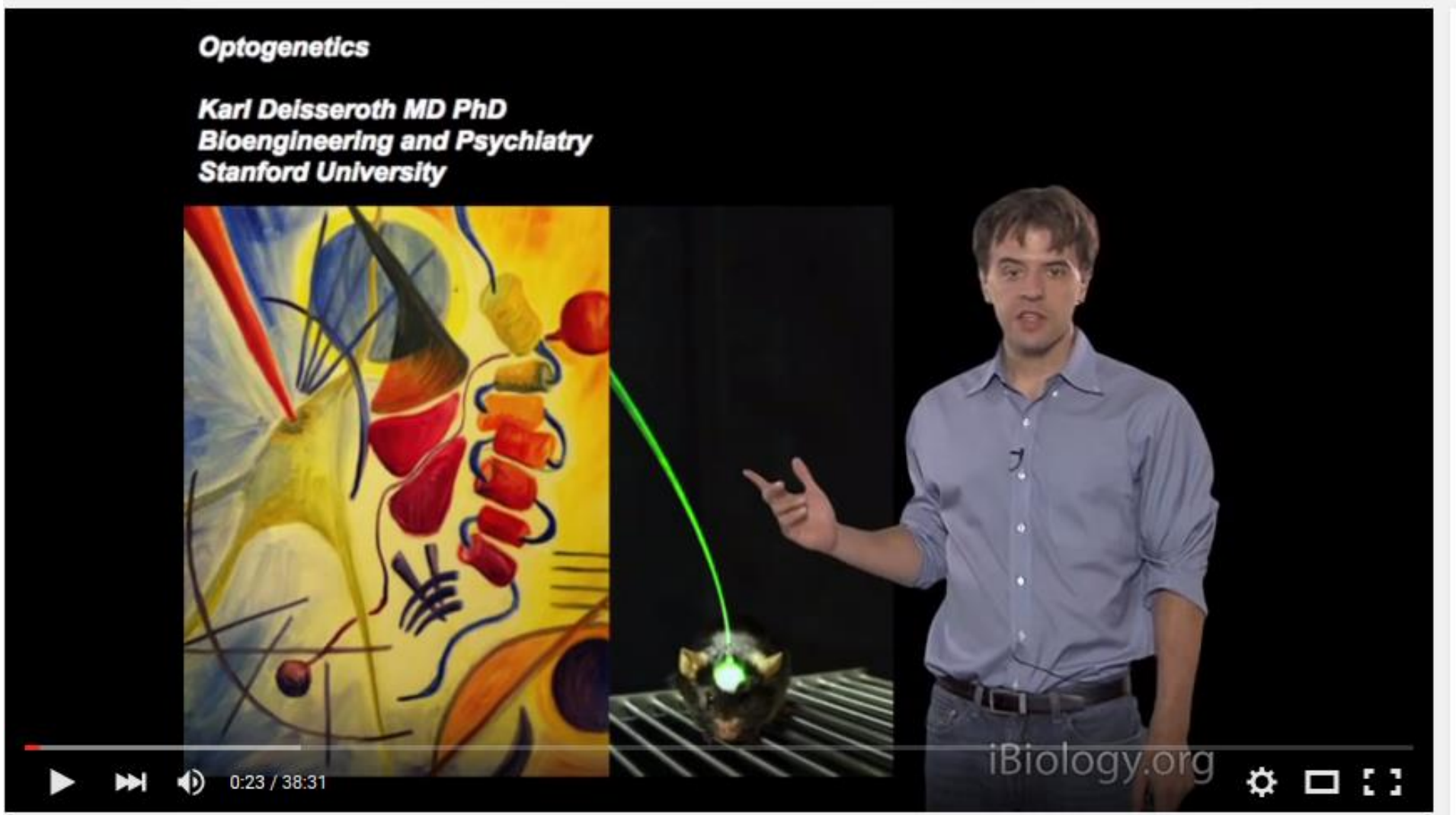
Tools and reagents developed are freely available
http://www.optogenetics.org/
http://addgene.org/

https://www.youtube.com/watch?v=PSbNCoImUTA

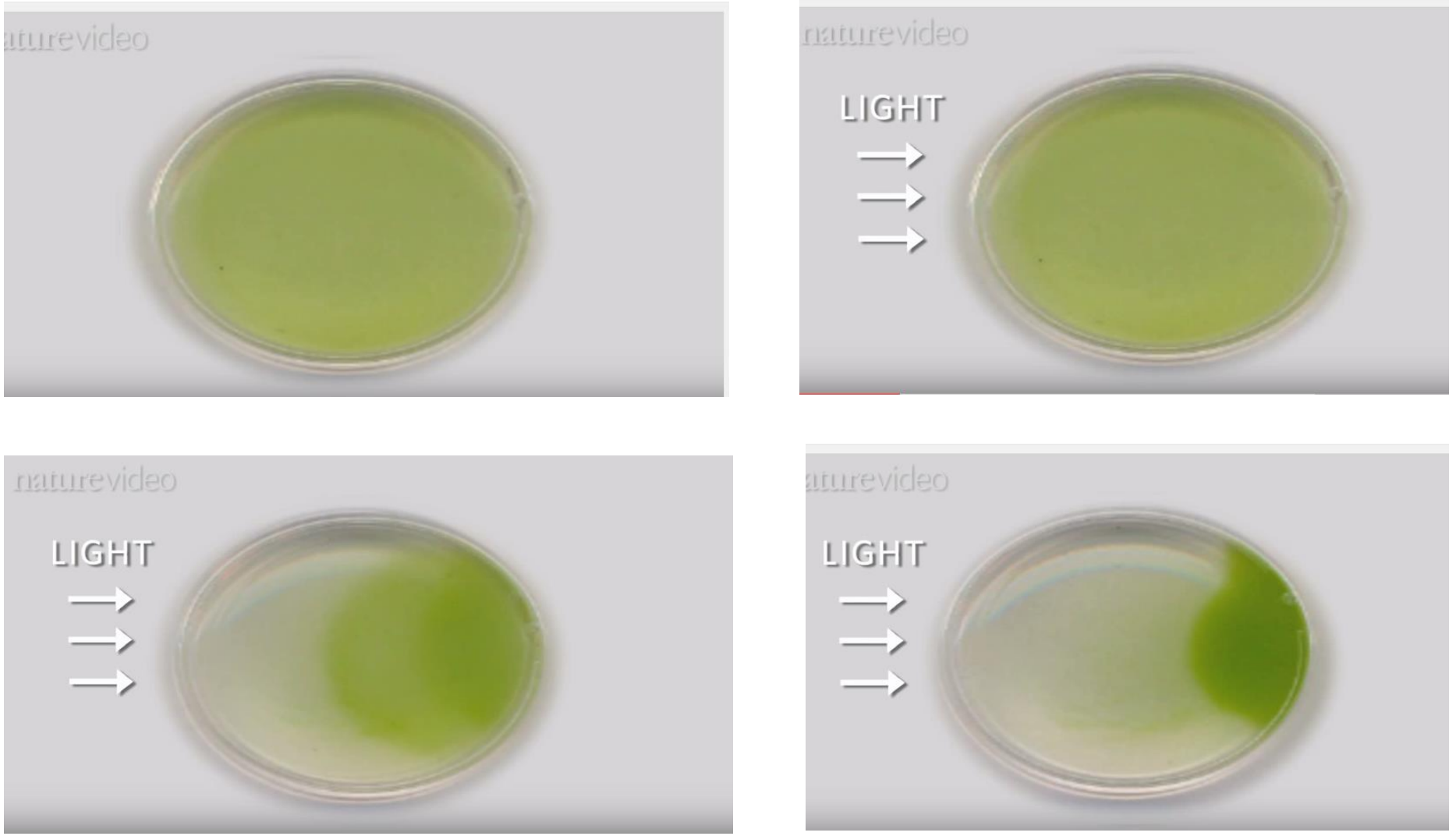


Microscopy: Optogenetics (Karl Deisseroth)

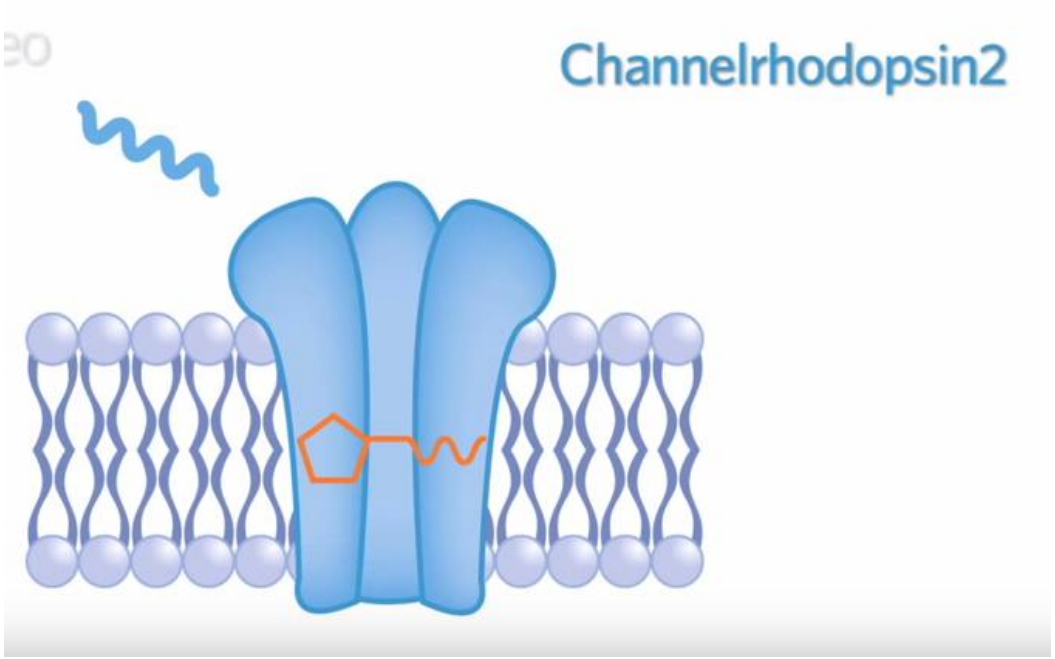
Optogenetics is the combination of genetic and optical methods to cause or inhibit well defined events in specific cells of living tissue and behaving animals

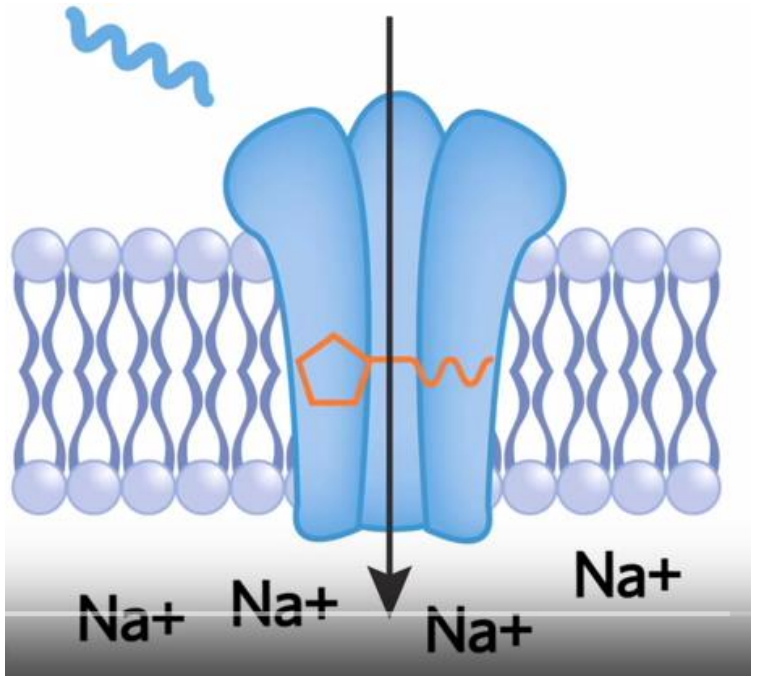
This technology, involves 3 core features:

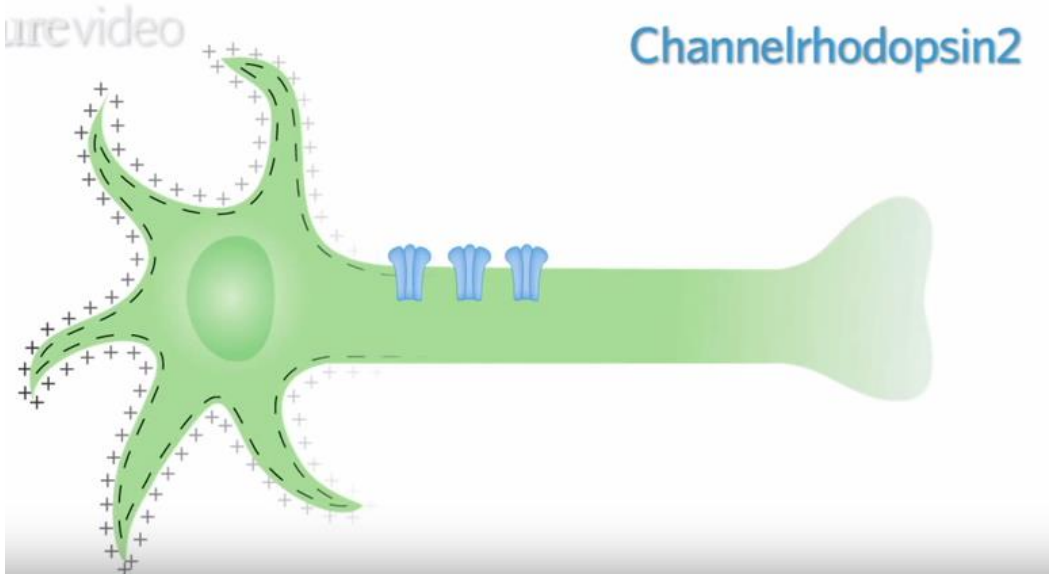
1- microbial opsins
2- methods to obtain cell specific opsin expression
3. methods to guide strong and precisely timed light

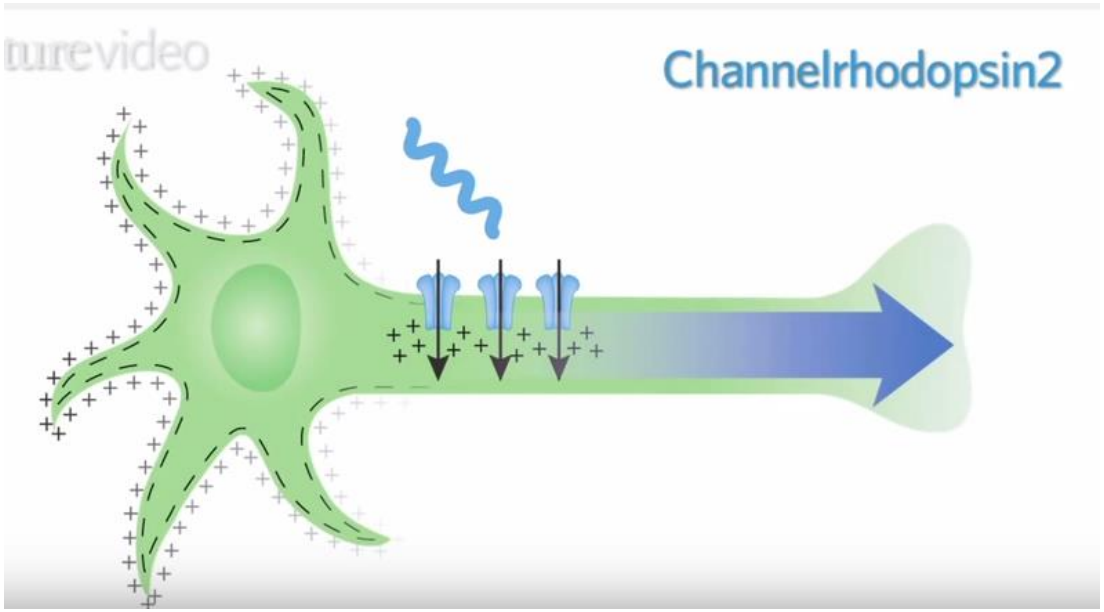


1- microbial **opsins**, members of an ancient gene family adapted from evolutionarily distant organisms such as *algae* and *archaebacteria*, with each gene encoding a distinct protein that directly elicits electrical current across cellular membranes in response to light

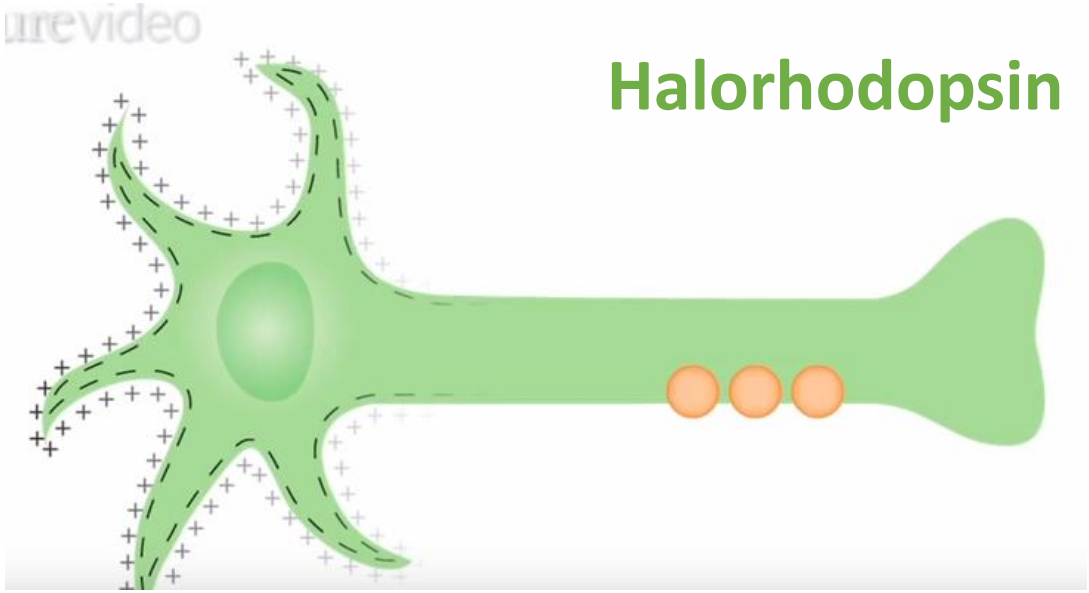

eo
Channelrhodopsin2


Na+
Na+
Na+
Na+

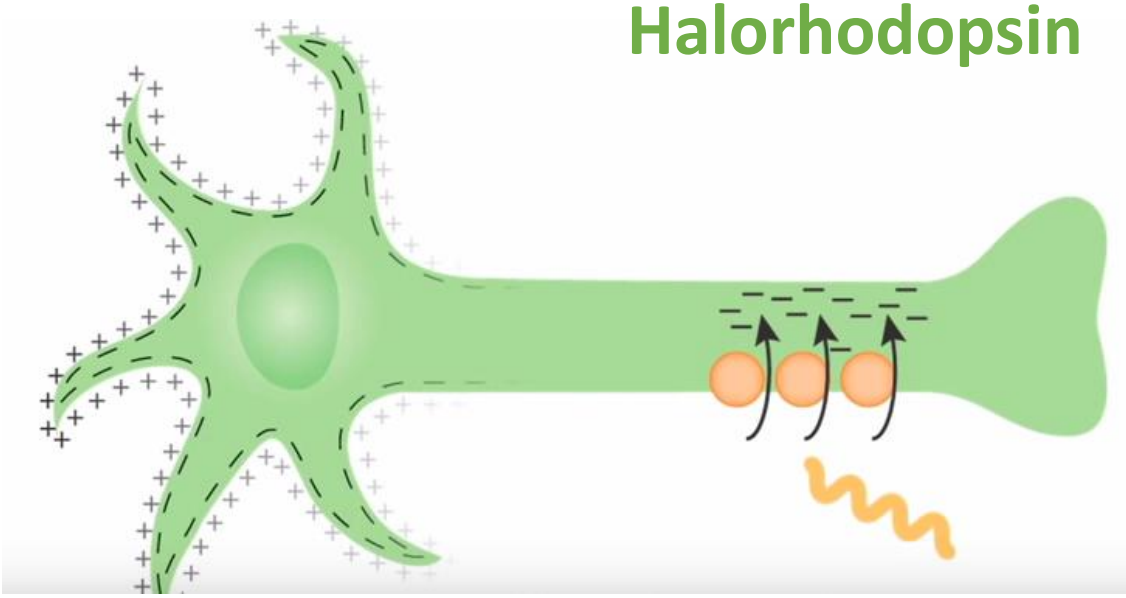

Channelrhodopsin2


Channelrhodopsin2

Halorhodopsin



Halorhodopsin



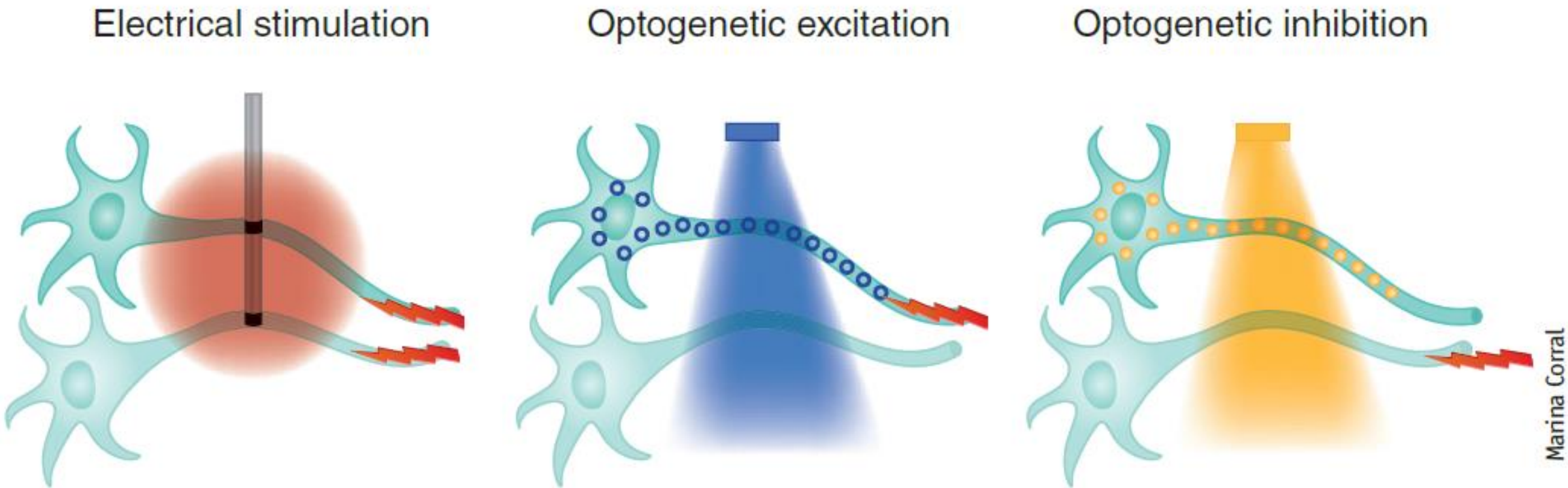
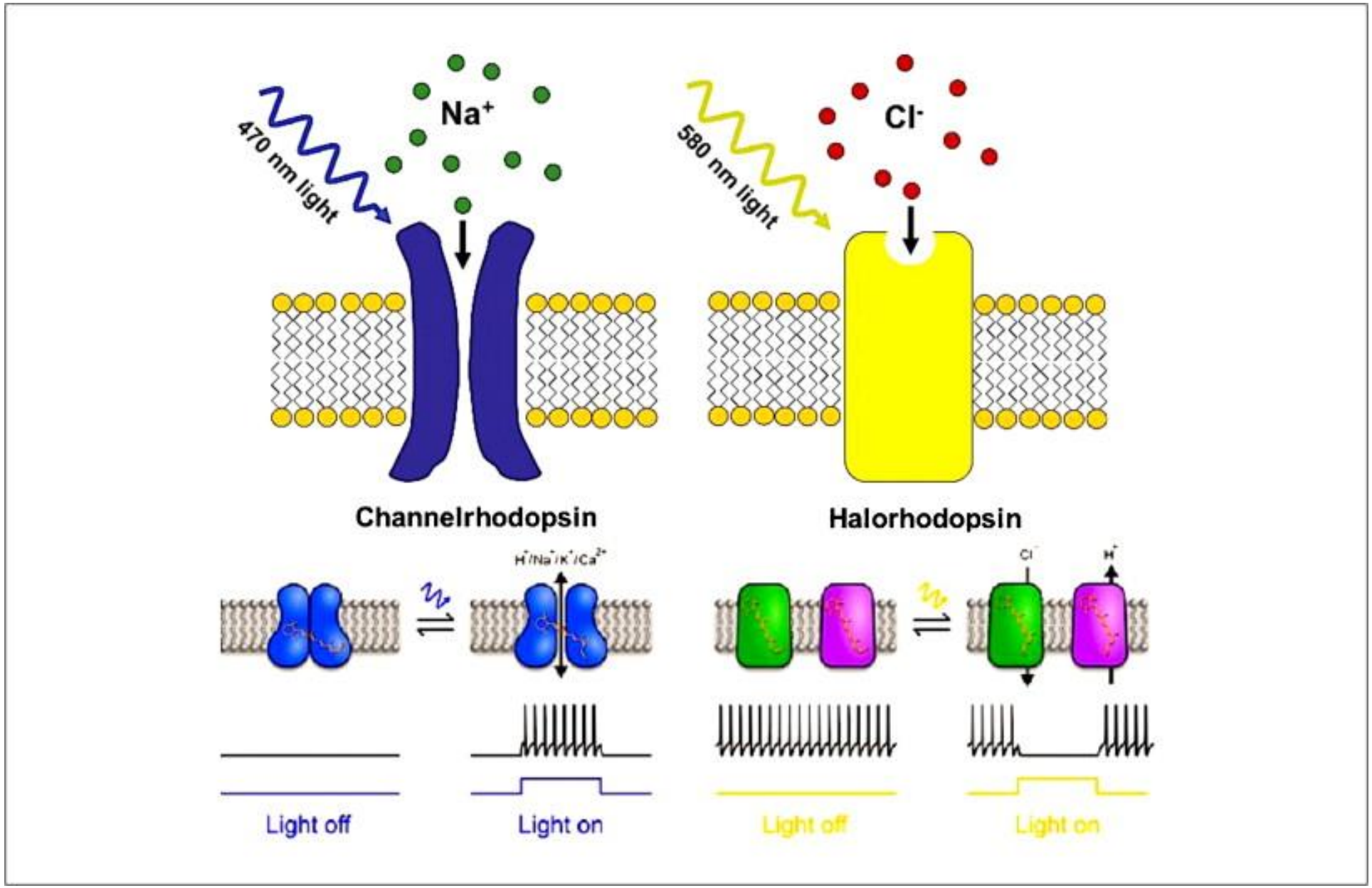
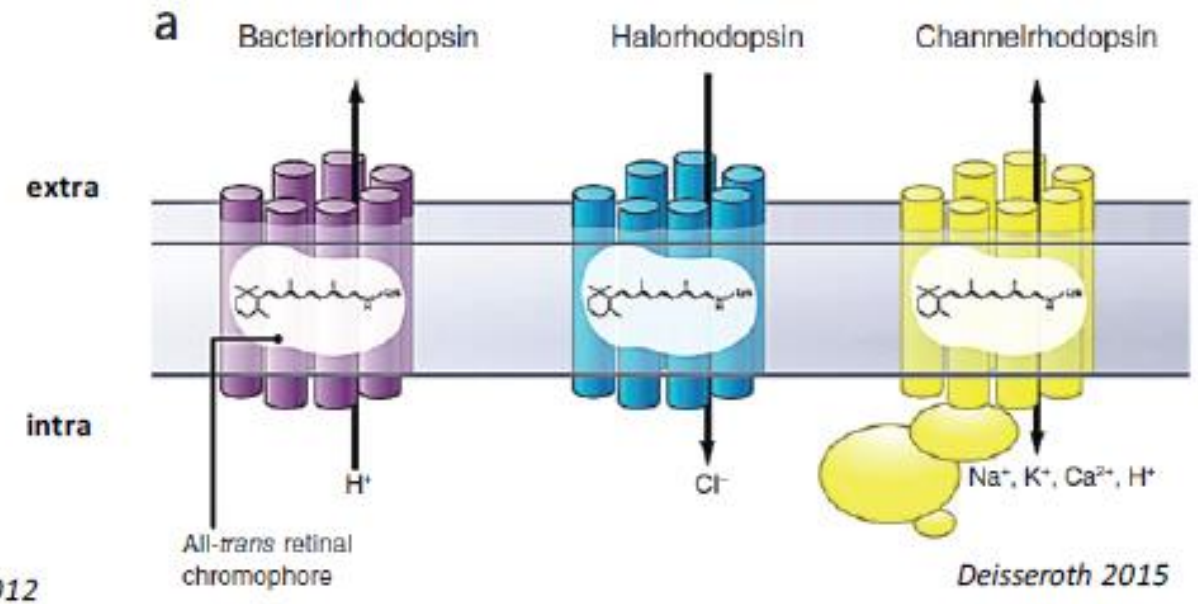


Figure 2 | Principle of optogenetics in neuroscience. Targeted excitation (as with a blue light–activated channelrhodopsin) or inhibition (as with a yellow light–activated halorhodopsin), conferring cellular specificity and even projection specificity not feasible with electrodes while maintaining high temporal (action-potential scale) precision.

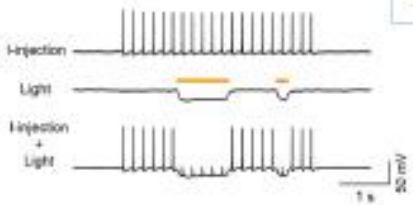


Microbial opsins (type I): transduce photons into electrical current



Deisseroth 2015

Chow et al. 2012



Hyperpolarizating current

Arch, eArch, eArch3.0, ArchT

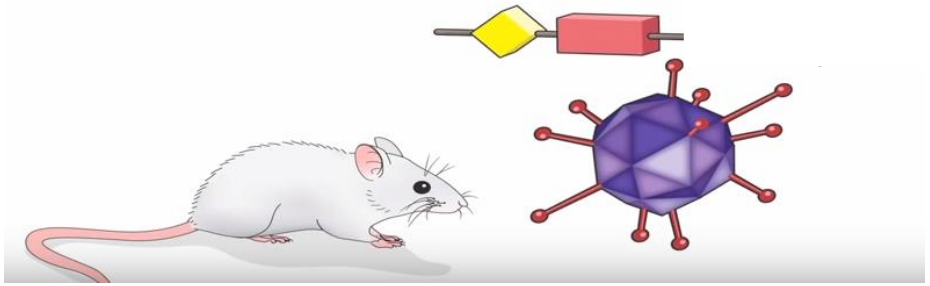
NphR, NphR3.0

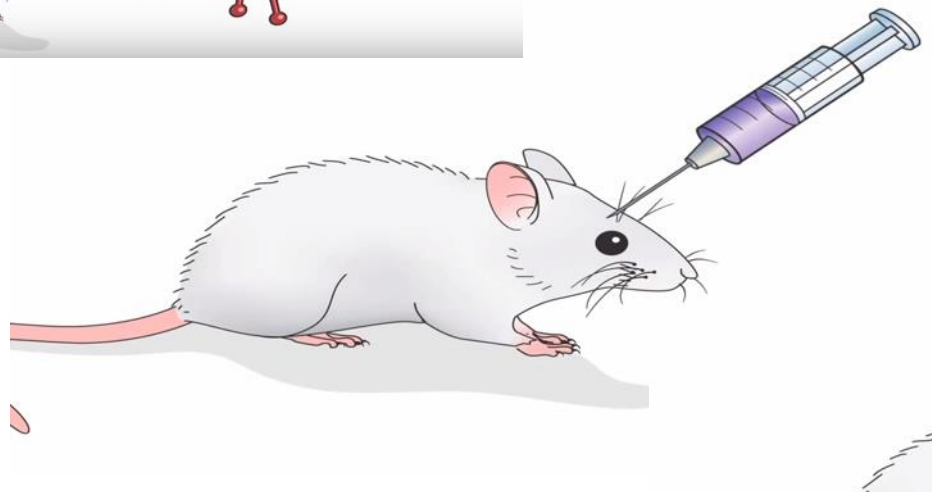
Depolarizating current

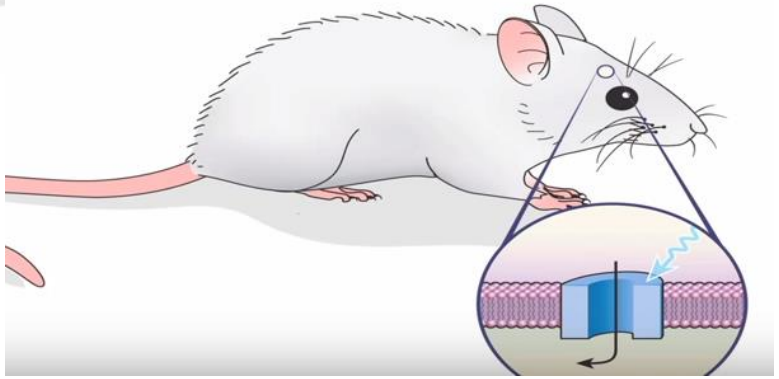
ChR2, ChETa, C1V1

2-general methods for targeting sufficiently strong and specific opsin gene expression to well-defined cellular elements in the brain

you can use a specific promoter!

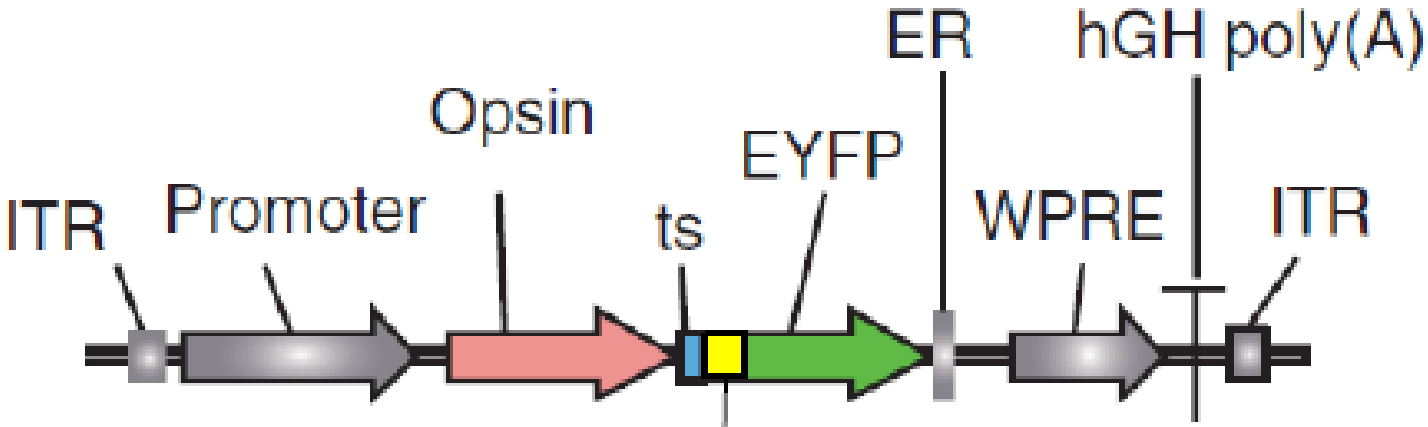


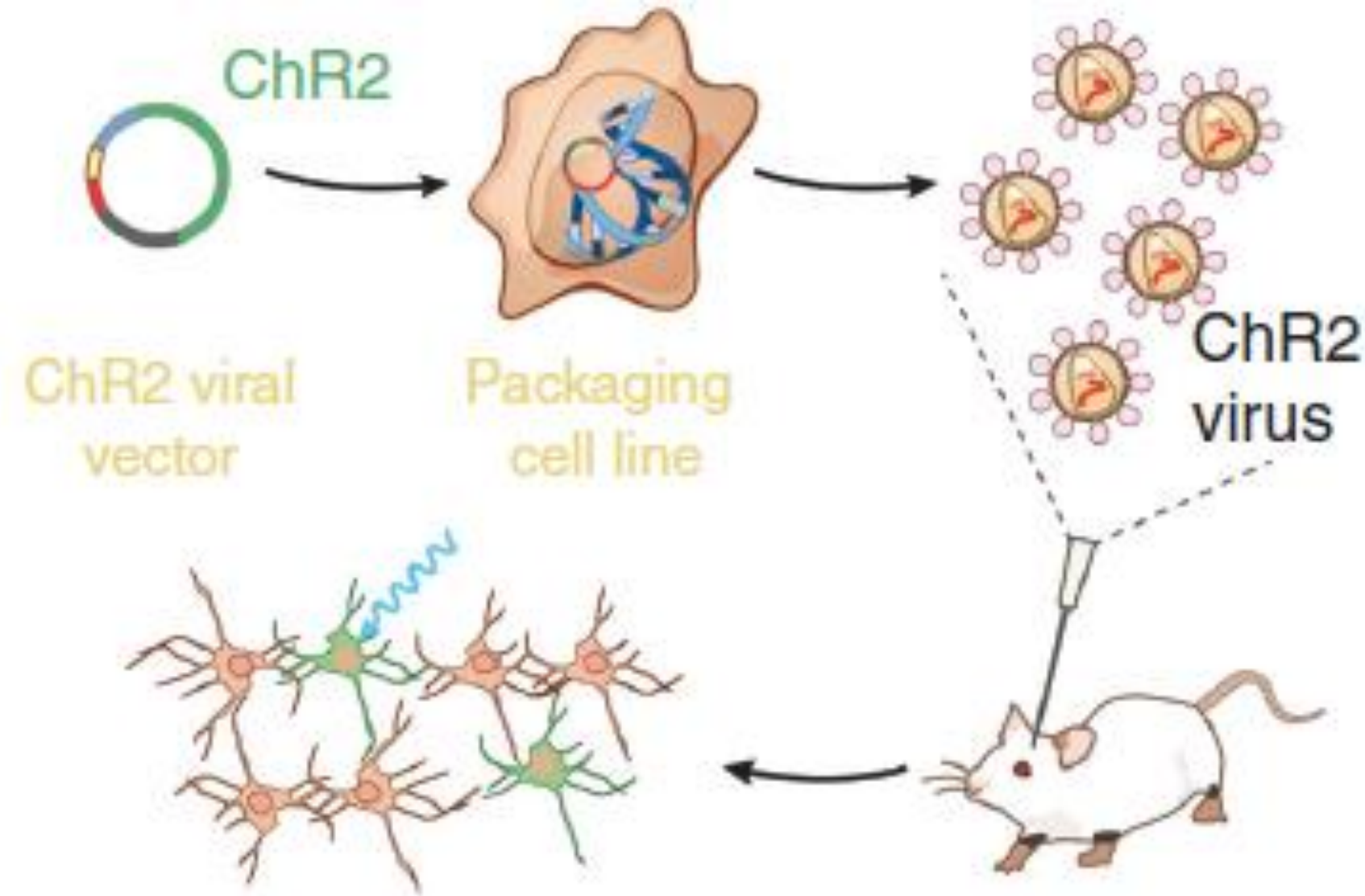




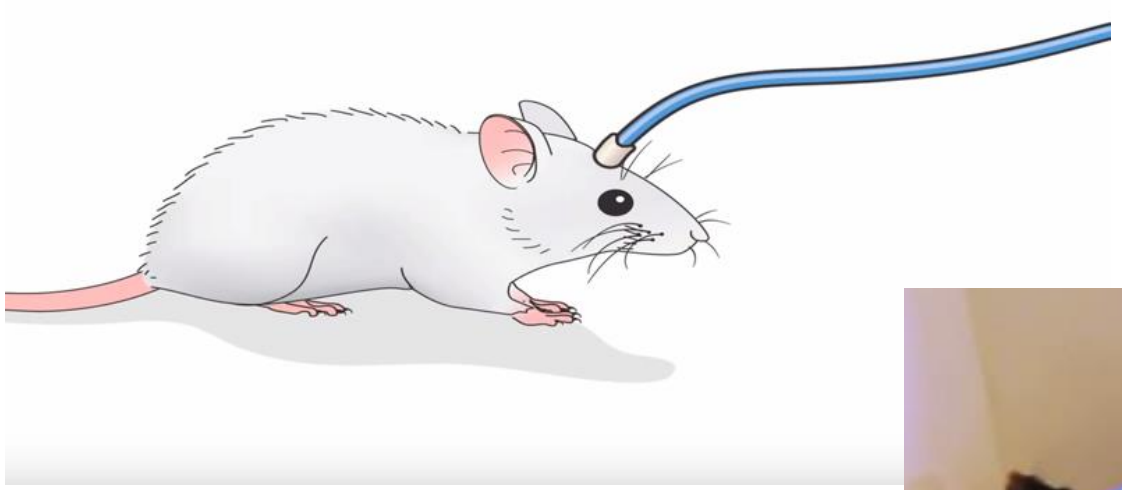
Vector:

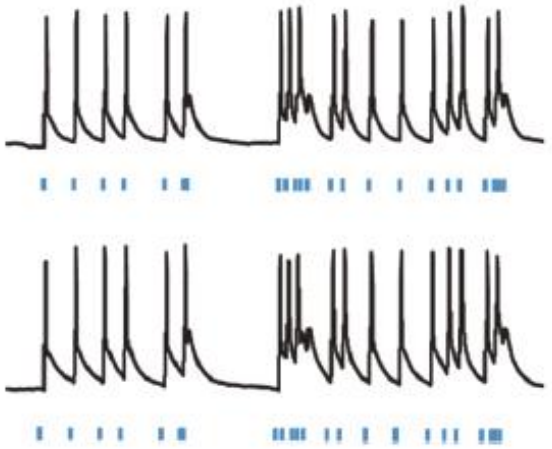
AAV: different serotypes
Lentivirus
Transgenic mouse lines



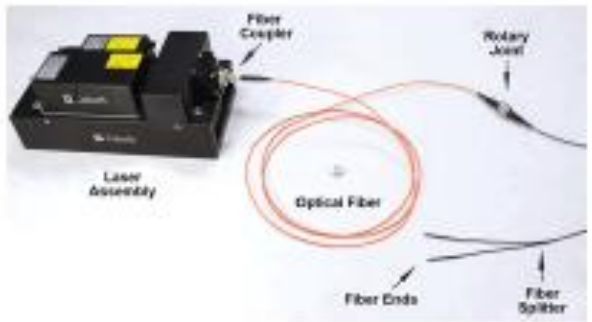

ChR2
ChR2 viral vector
Packaging cell line
ChR2 virus

3. general methods for guiding sufficiently strong and precisely timed light to specific brain regions, cells or parts of cells while the experimental subject carries out behaviours of interest.



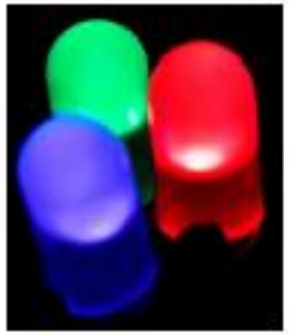


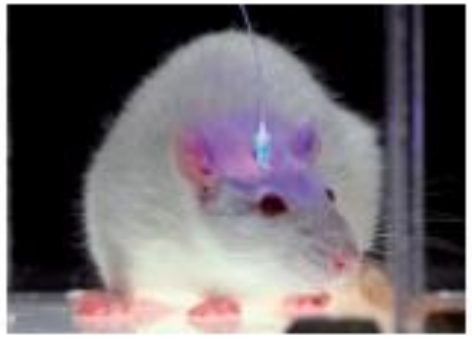
Fiber from laser source



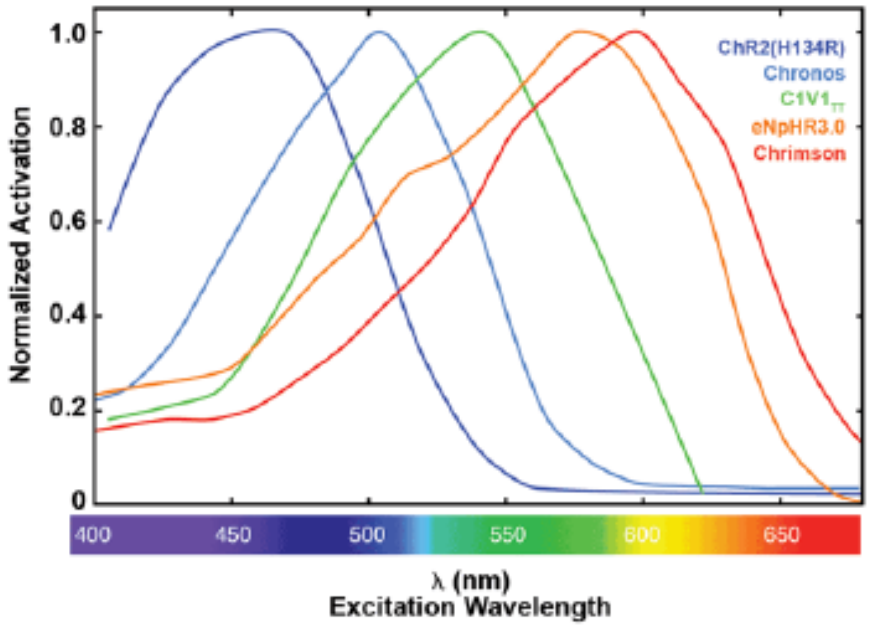
Expensive but stable, higher power, spatially precise, can collect emitted fluorescence

LEDs



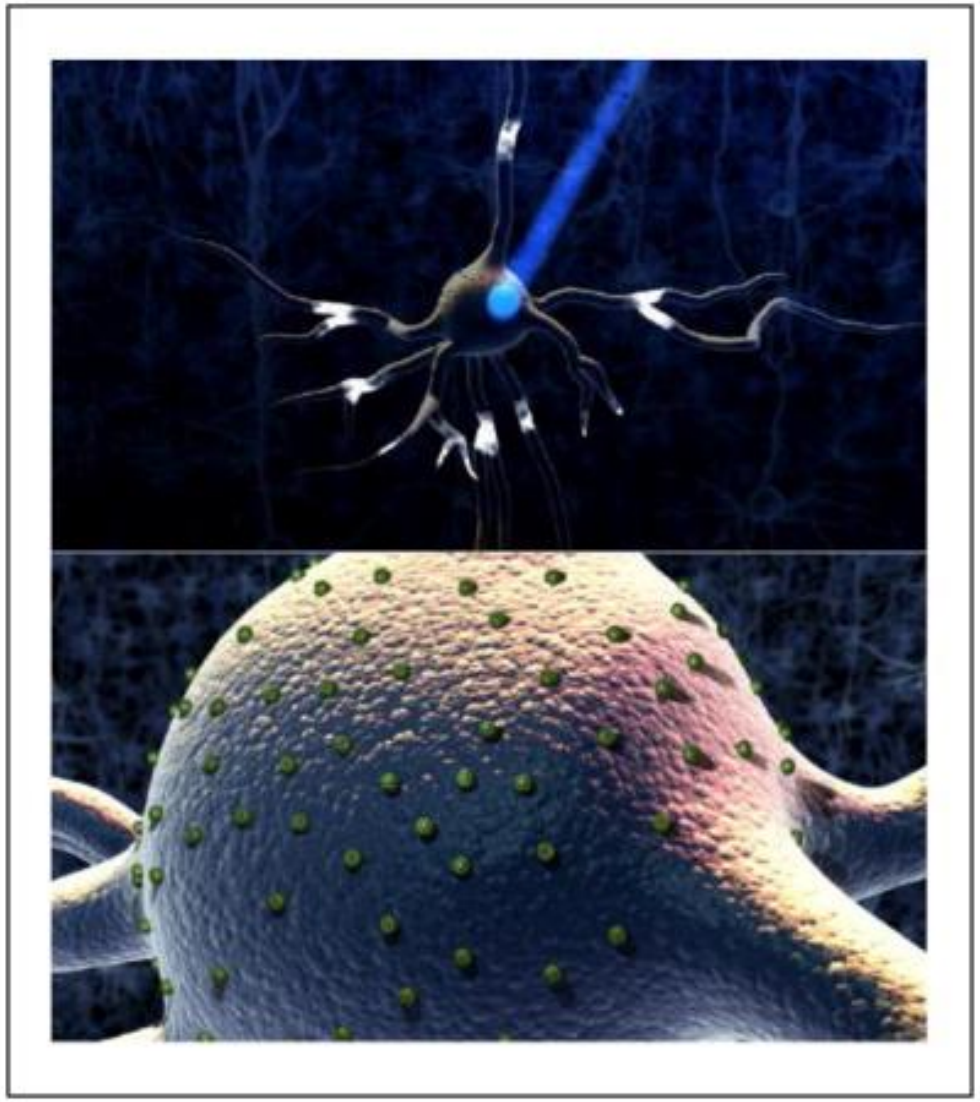


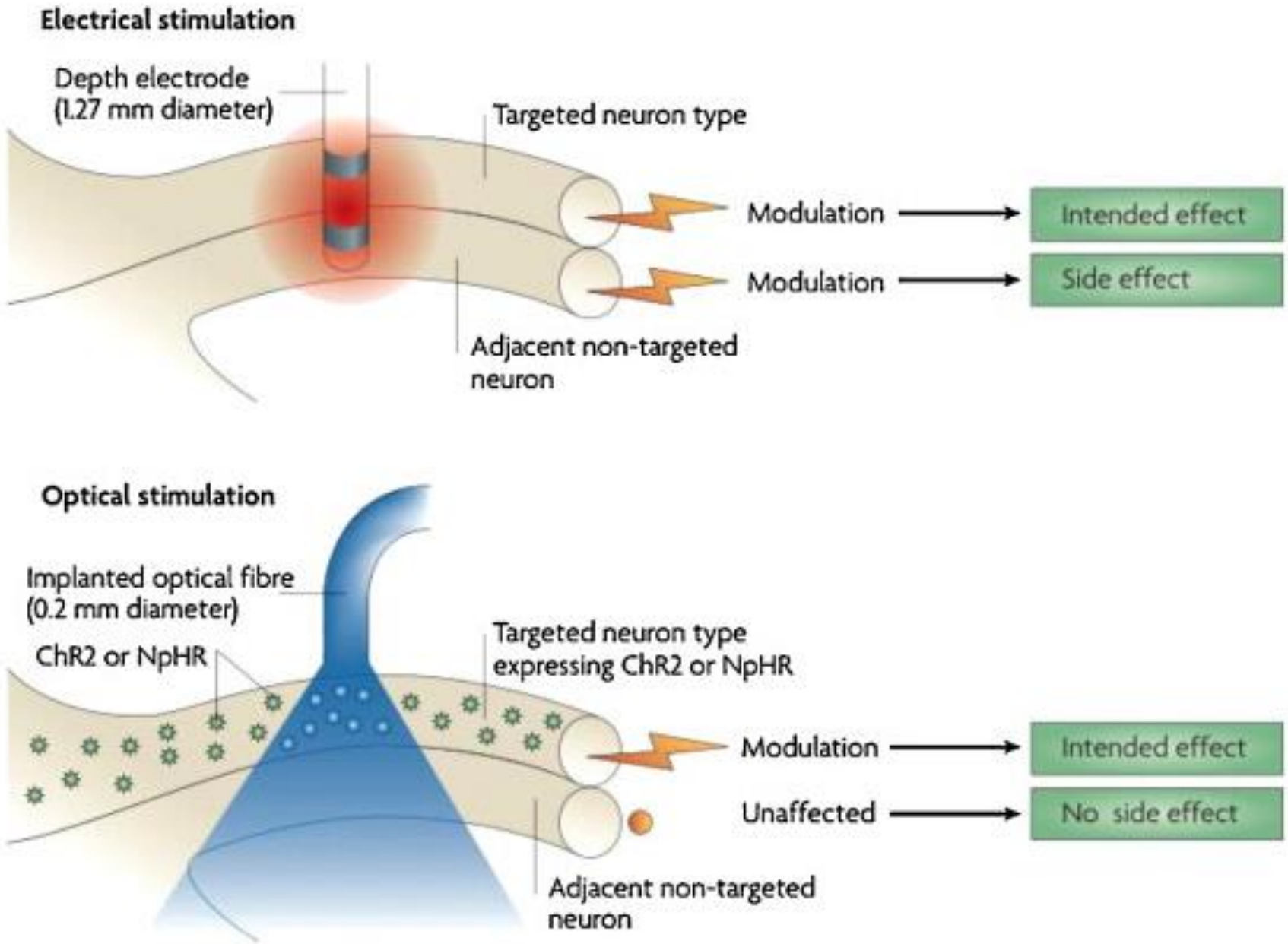
Cheap but warm up the tissue, often not enough power is delivered, less precise in space, no back collection



Neural activation through illumination

The control over the neuron lies in with the light switch. This precise control is achieved by altering the illumination light temporally or spatially. Other factors include the physical size of the light source, intensity and illumination volume.




Electrical stimulation
Depth electrode
(1.27 mm diameter)
Targeted neuron type
Modulation
Intended effect
Modulation
Side effect
Adjacent non-targeted
neuron
Optical stimulation
Implanted optical fibre
(0.2 mm diameter)
ChR2 or NpHR
Targeted neuron type
expressing ChR2 or NpHR
Modulation
Intended effect
Unaffected
No side effect
Adjacent non-targeted
neuron

Reflecting on the past 10 years brings up a final point, always worth making in the context of optogenetics, regarding a concept that could be conveyed more widely to the public: **the essential value of exploratory basic science research**, even for investigators, institutions and funding agencies primarily interested in health and translational research.

It seems unlikely that the initial experiments described here would have been fundable, as such, by typical grant programs focusing on a disease state, on a translational question, or even on solidly justified basic science.
Though clinical and commercial applications are not addressed here, the advances brought by microbial opsin-based optogenetics may inform the pathophysiology and treatment of neurological and psychiatric disease states, as well as other clinical conditions, in addition to the broad basic science discoveries described above that have from the beginning constituted the core motivation of optogenetics.

Karl Deisseroth

With optogenetics, investigators see the subject's behavior and physiology change in real time while specific cells and projections are controlled—a connection still thrilling after all these years.

The connection with light links experimenter and subject, but also in some sense spans the tree of life: microbial DNA has yet again returned to eukaryotic cells, a recurrent and curious theme of life on earth over billions of years, to provide another symbiosis, this one scientific.

Such relationships coevolve and persist once formed, and though biology as a whole in the coming years will continue to move in unexpected directions, the ancient microbial opsins now seem inextricably part of our journey.

Karl Deisseroth

http://web.stanford.edu/group/dlab/optogenetics/

Design of opto-RTKs and ways to light-control RTK downstream signalling

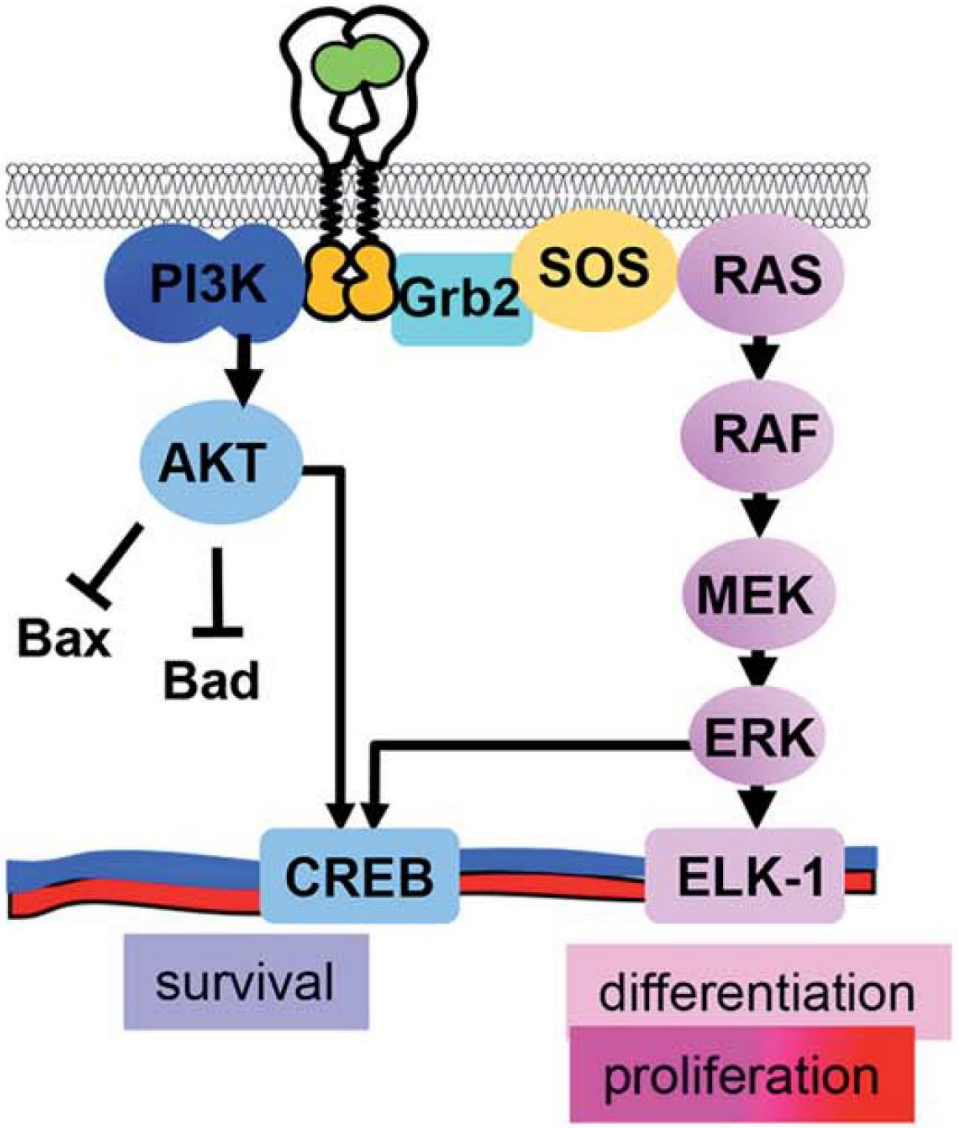
ROYAL SOCIETY OF CHEMISTRY

Light control of RTK activity: from technology development to translational research

Anna V. Leopold [a] and Vladislav V. Verkhusha *[ab]

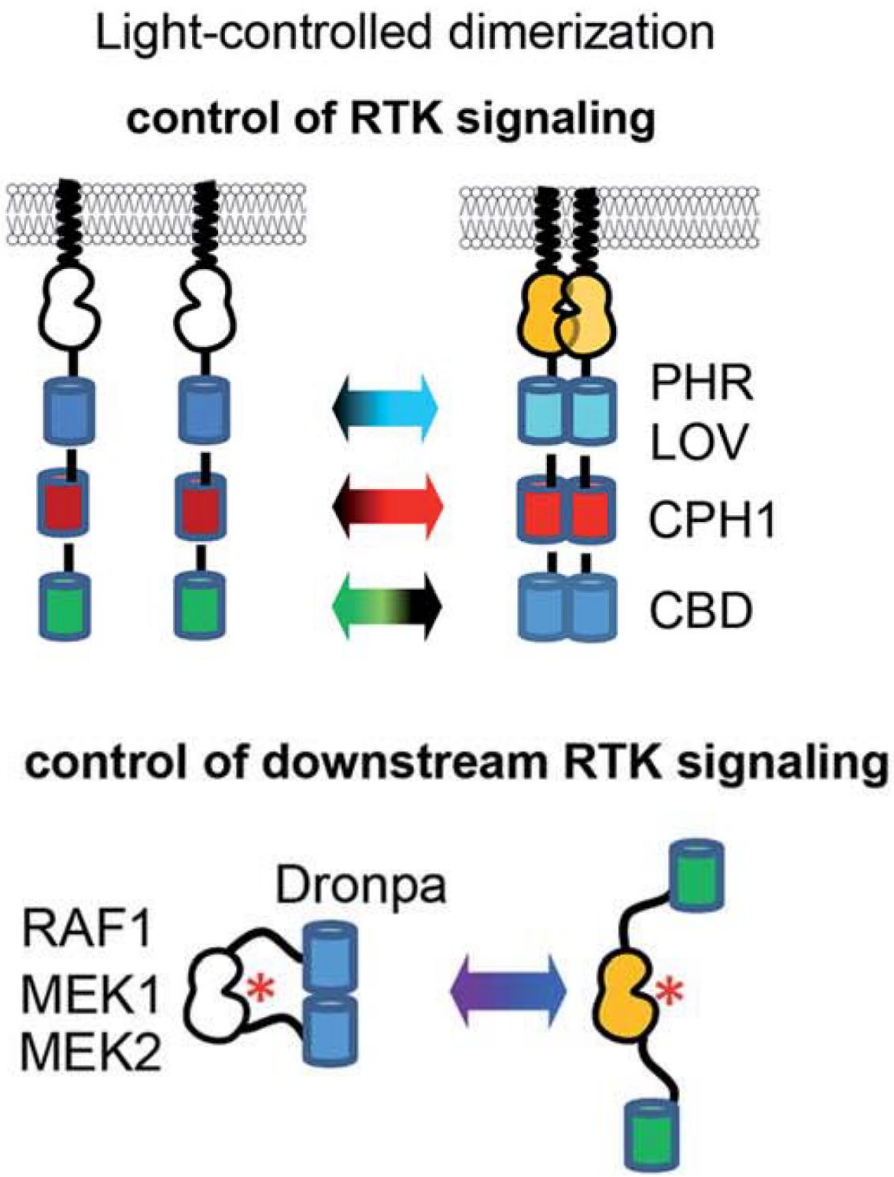
Inhibition of receptor tyrosine kinases (RTKs) by small molecule inhibitors and monoclonal antibodies is used to treat cancer. Conversely, activation of RTKs with their ligands, including growth factors and insulin, is used to treat diabetes and neurodegeneration. However, conventional therapies that rely on injection of RTK inhibitors or activators do not provide spatiotemporal control over RTK signaling, which results in diminished efficiency and side effects. Recently, a number of optogenetic and optochemical approaches have been developed that allow RTK inhibition or activation in cells and *in vivo* with light. Light irradiation can control RTK signaling non-invasively, in a dosed manner, with high spatio-temporal precision, and without the side effects of conventional treatments. Here we provide an update on the current state of the art of optogenetic and optochemical RTK technologies and the prospects of their use in translational studies and therapy.

Activation of RTK signalling pathways by growth factors



Growth factor binding leads to the dimerization of the RTK and activation of the downstream signaling.

Activation of RTK signalling pathways by light



RTK intracellular domains are fused to photoreceptors, which dimerize upon action of light. This leads to dimerization and activation of RTKs.

Dimerization is used for the photo-caging of the MEK catalytic center.

Inactive domains are shown in white, while activated domains are shown in orange.

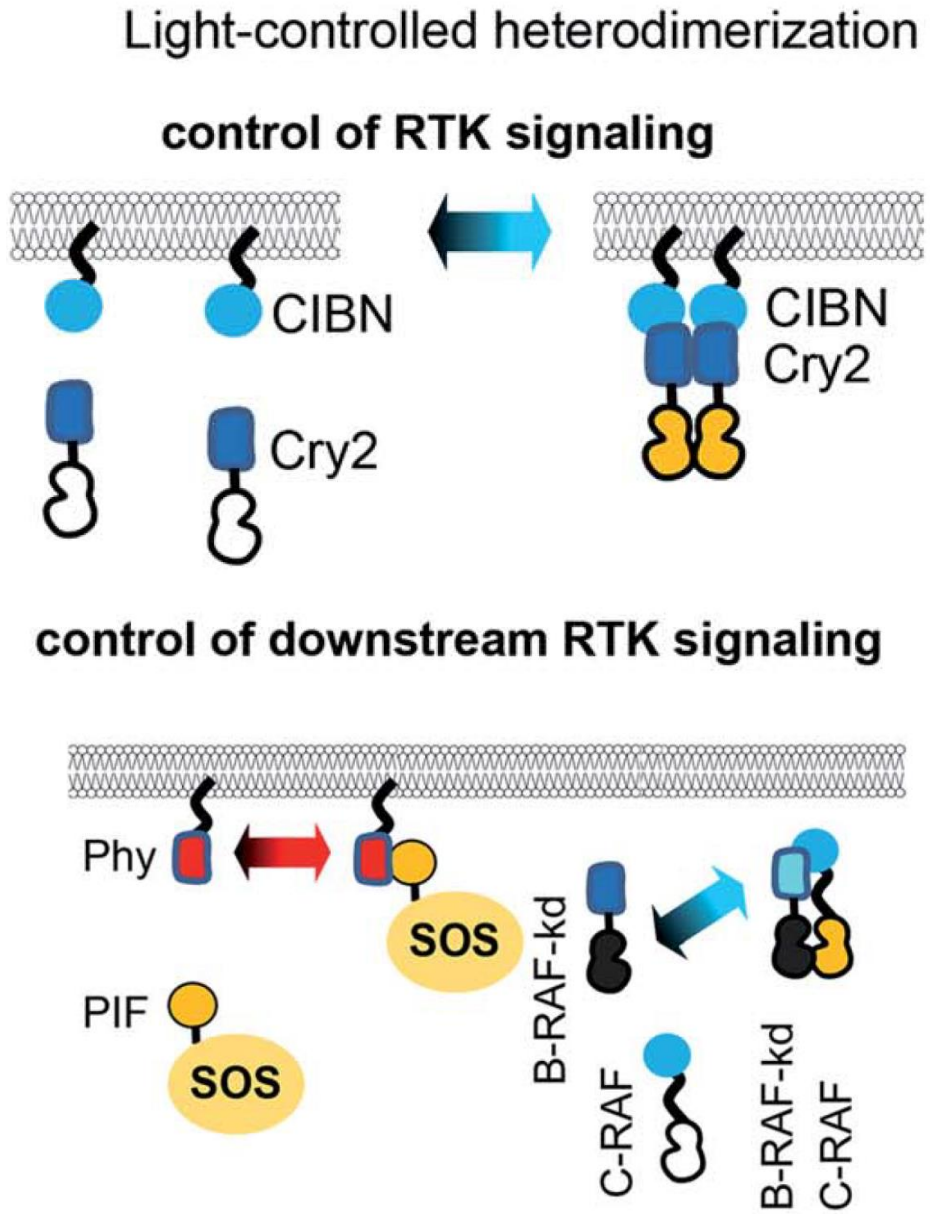
The toolbox of available opto-RTKs includes **blue**, **green**, **red**, and **far-red**/**near-infrared** light controlled RTKs.

Among **blue-light** controlled photoreceptors are light oxygen-voltage (LOV) domains and various derivatives of cryptochrome 2 (Cry2), including its photolyase homology domain (PHR). They dimerize upon action of blue light and use a chromophore (favin mononucleotide) available in mammalian tissues.

Opto-RTKs controllable with **green** and **red** light need addition of exogenous chromophores to cell culture medium. For example, a cobalamin binding domain (CBD)-based opto-FGFR1 requires B12 vitamin as the chromophore.

Far-red/near-infrared light controlled opto-RTKs were developed based on cyanobacterial phytochromes and bacterial phytochromes, for example cyanobacterial phytochrome 1 (CPH1) from Synechocystis. CPH1 dimerizes upon exposure to far-red (630 nm) light and dissociates under near-infrared (780 nm) light.
Far-red and near-infrared light exhibit lower phototoxicity and deeper penetrance into mammalian tissues, but CPH1-based opto-RTKs also need an external chromophore, such as phycocyanobilin.

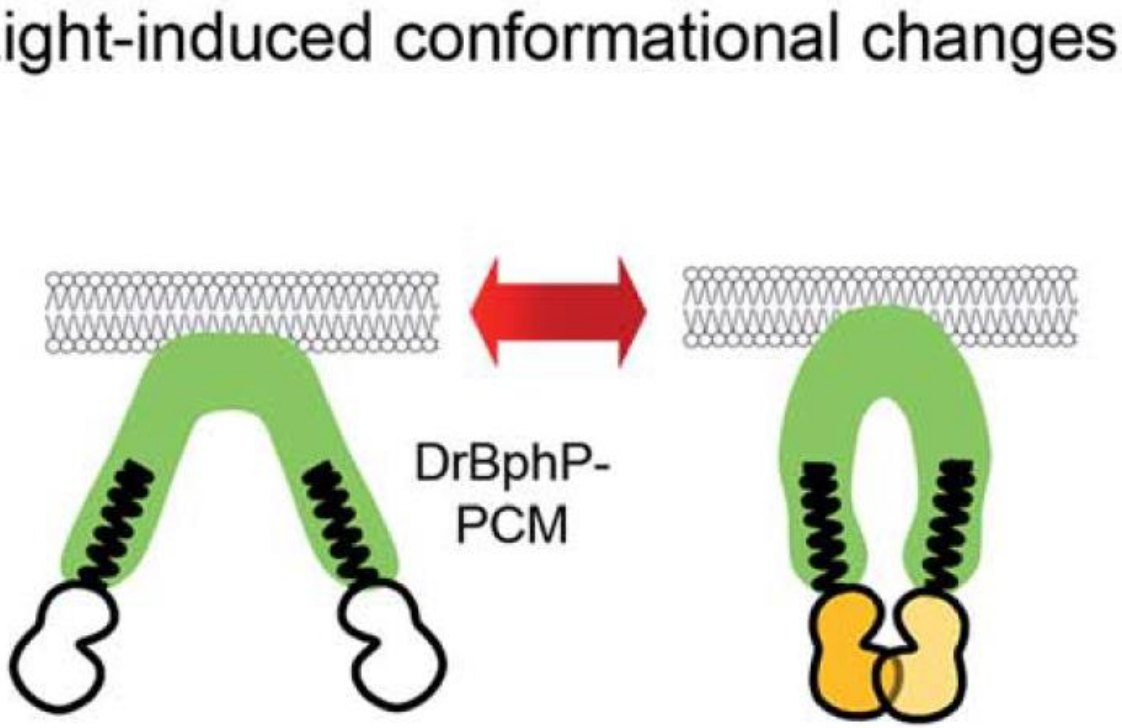
Activation of RTK signalling pathways by light



Intracellular RTK domains are fused to cryptochrome 2 (Cry2). Illumination with blue light leads to the simultaneous translocation of Cry2-RTK to the PM and its activation.

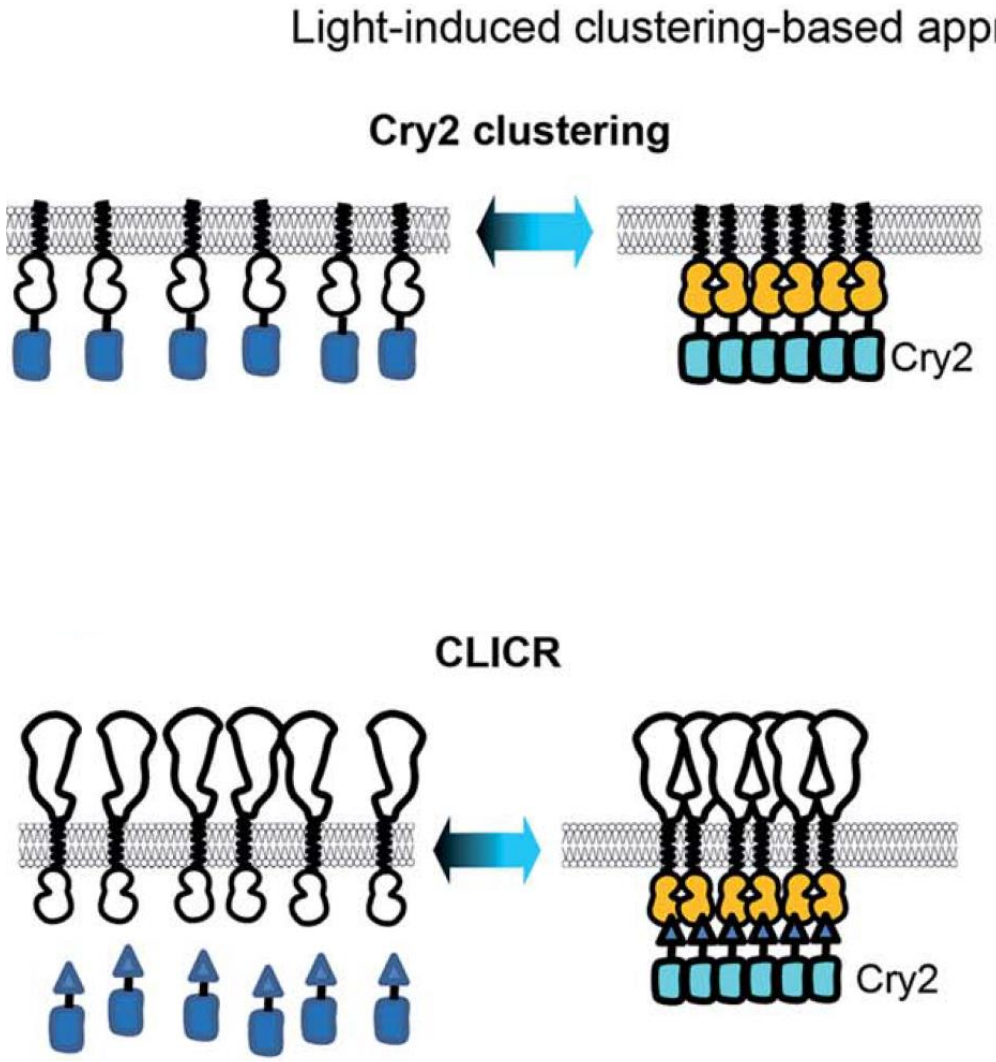
Light-controlled translocation of the SOS to the PM leads to activation of downstream ERK cascade starting from RAS. Heterodimerization of Cry2-B-RAF and CIBN–C-RAF-kd leads to the activation of the ERK cascade starting from MEK.

Activation of RTK signalling pathways by light



RTK intracellular domains are attached to the photosensory core (PCM) of bacterial phytochrome of D. radiodurans (DrBphP). Upon action of near-infrared light DrBphP-PCM undergoes conformational changes, leading to RTK activation.

Activation of RTK signalling pathways by light



RTK intracellular domains are fused to Cry2 photoreceptor. Light-induced clustering of Cry2 leads to the activation of opto-RTKs.

Endogenous RTK activation using CLICR. PLCg-SH2-motif is fused to Cry2. Upon action of light SH2-Cry2 fusions cluster and interact with endogenous RTKs. Inactive RTK domains are shown in white while activated RTK domains are shown in orange.