LOSCHMIDT LABORATORIES

Synthetic protein nanomachines

Engineered large macromolecular assemblies and virus-inspired vehicles

Creating synthetic protein complexes and supramolecular assemblies for biotechnology, biomedicine and basic research

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Short recapitulation of previous lesson

- What is directed evolution?
- Directed evolution: pros and cons
- How to generate gene diversification and library creation?
- How to screen your gene library?
- What is continuous directed evolution? Examples…
- What is semi-rational protein engineering approach?

What will we talk about

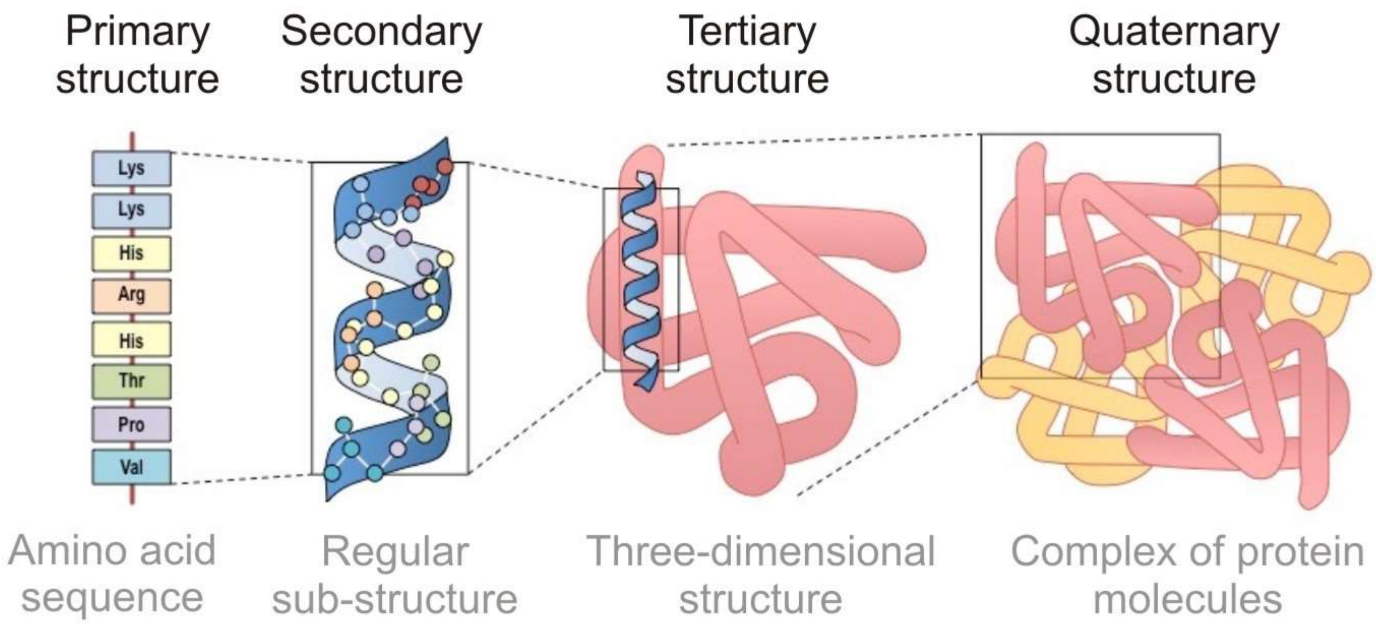
- Design of large protein nanomachines
    - Macromolecular protein assemblies
- Synergy between synthetic biology and structural biology
    - Resolution revolution, mutual benefits
- Multi-expression technologies
    - Strategies, methodology, success stories
- Synthetic virology
    - Construction of synthetic virus-based vehicles, examples
- Immunotherapy and gene therapy
    - Concepts and applications

Design of large protein nanomachines

Concepts
Methods
Applications

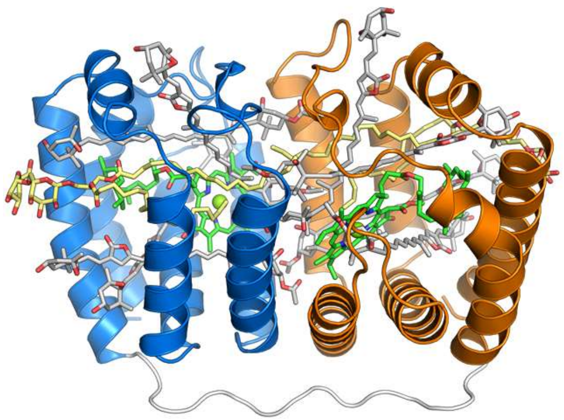
The anatomy of protein structure

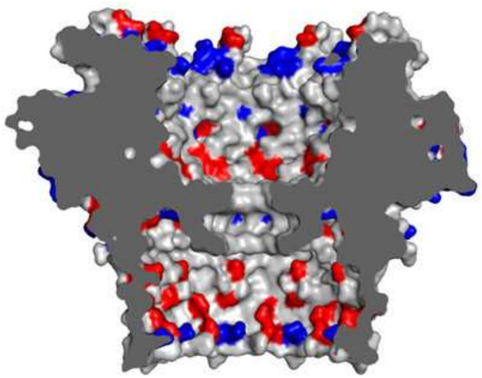
- Proteins are an important class of biological macromolecules which are polymers of amino acids
- Biochemists have distinguished several levels of structural organization of proteins

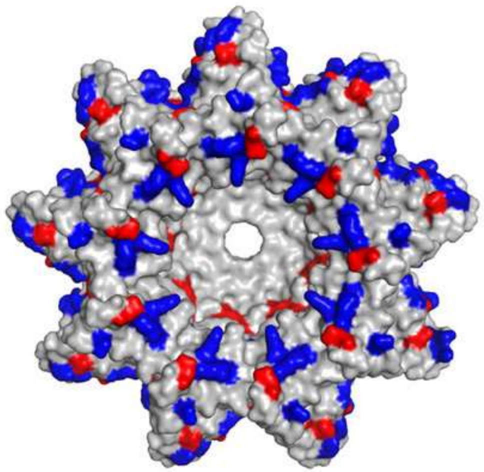


Most proteins work in multi-subunit protein complexes

- Protein function and activity are highly regulated within living cells
- To become functional, proteins commonly depend on their interactions with other molecules. These molecules are known to include other proteins, which readily interact to form **protein complexes**
- The majority of cellular processes are dependent on **protein-protein interactions** (**PPI's**)
- An additional layer of complexity is added by the interaction of different protein complexes in so called **super-complexes** or **metabolons**
- A protein complex or multi-protein complex is a group of two or more associated polypeptide chains. Different polypeptide chains may have different functions

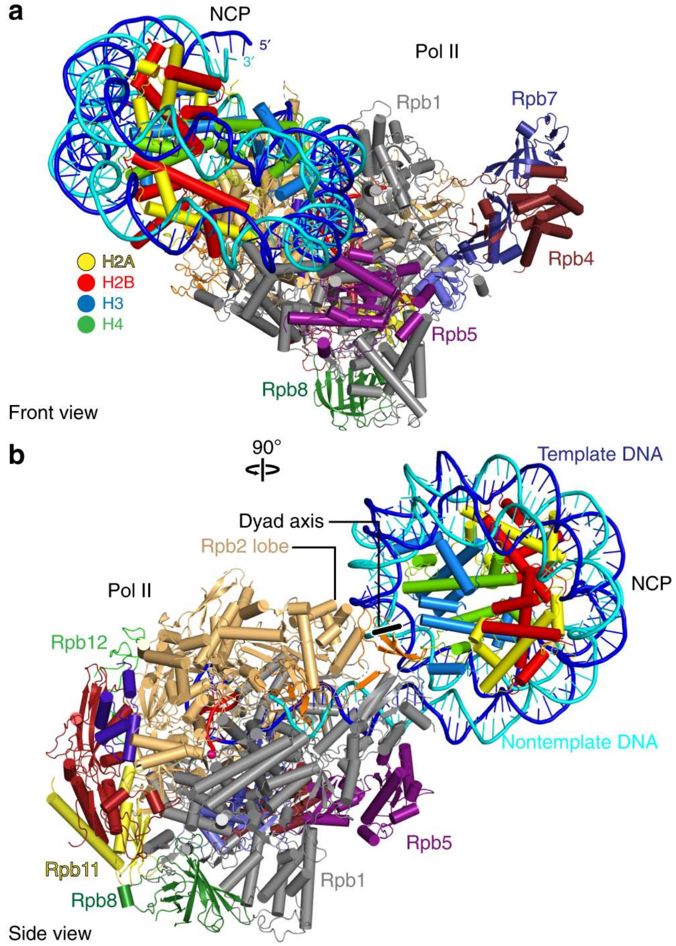


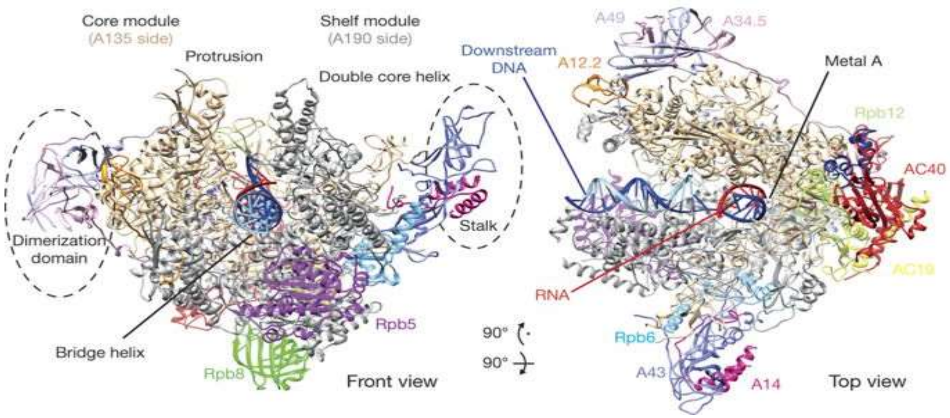




A movie of RNA polymerase II transcription

Prof. Patrick Cramer
Director at the Max Planck Institute for Biophysical Chemistry, Gottingen, DE





https://www.youtube.com/watch?v=WIMV_I88Lus

The ribosome, a large multi-subunit protein-RNA complex

- The ribosomes are protein synthesizers of the cell
- Made up of rRNAs and distinct ribosomal proteins
- Arranged into two pieces:
  - Small ribosomal subunit
  - Large ribosomal subunit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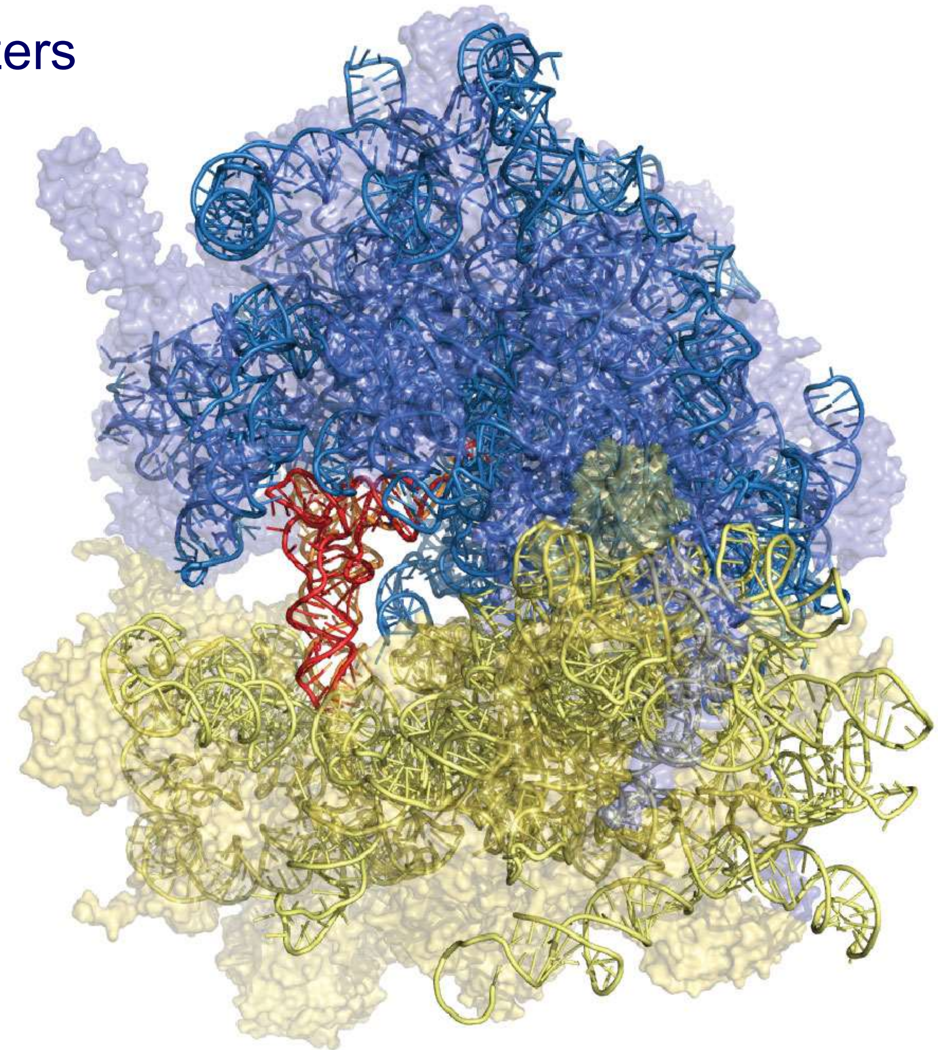
RESEARCH ARTICLE

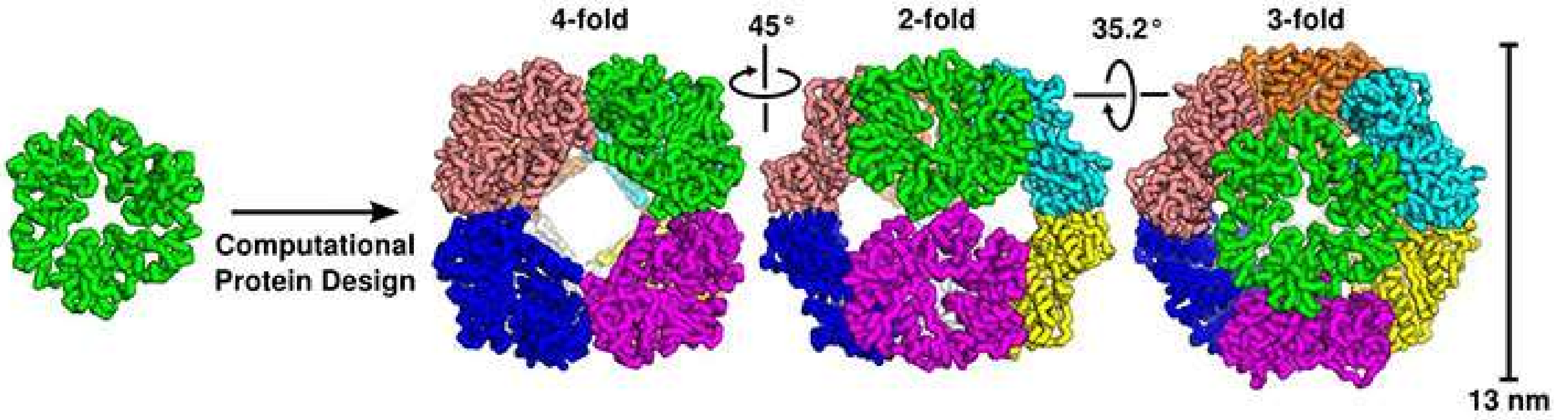
The Structure of the Eukaryotic Ribosome at 3.0 Å Resolution

Adam Ben-Shem*,†, Nicolas Garreau de Loubresse*, Sergey Melnikov*, Lasse Jenner, Gulnara Yusupova, Marat Yusupov†

+ See all authors and affiliations



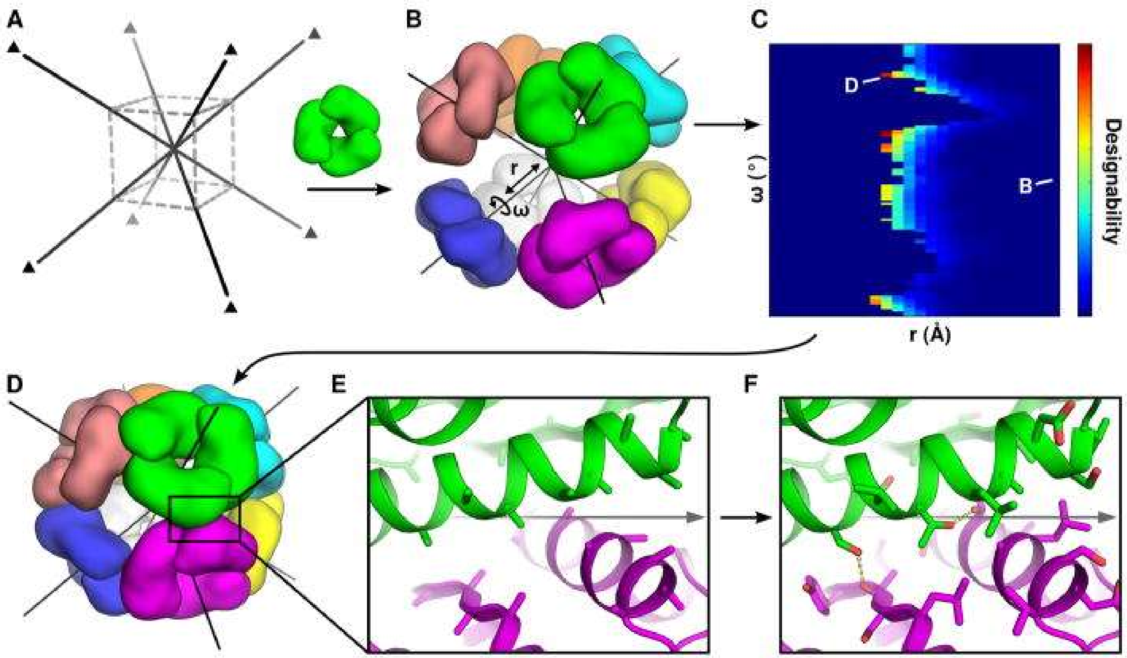
Computational design of large protein assemblies

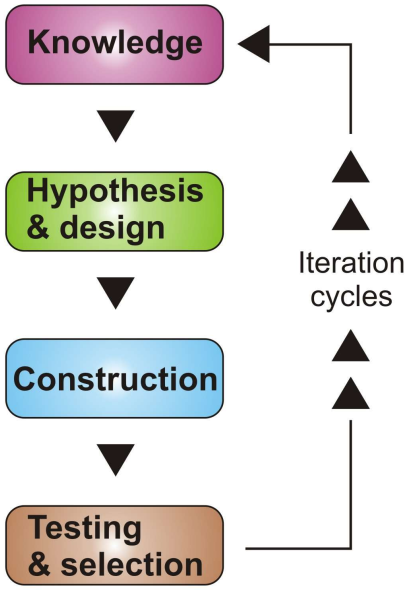


- Self assembling protein materials play critical roles in biology.
- Proteins have many features that make them attractive as building blocks for the development of completely new classes of advanced functional materials.
- There are efforts to develop general approaches for creating new self assembling nanostructures, and using these approaches to develop a next generation of synthetic biology solutions (nanoreactors, nanocages, vaccines and drug delivery vehicles).

Science 2012 336 (6085):1171-4. doi: 10.1126/science.1219364

General approach to designing self-assembling protein nanomaterials

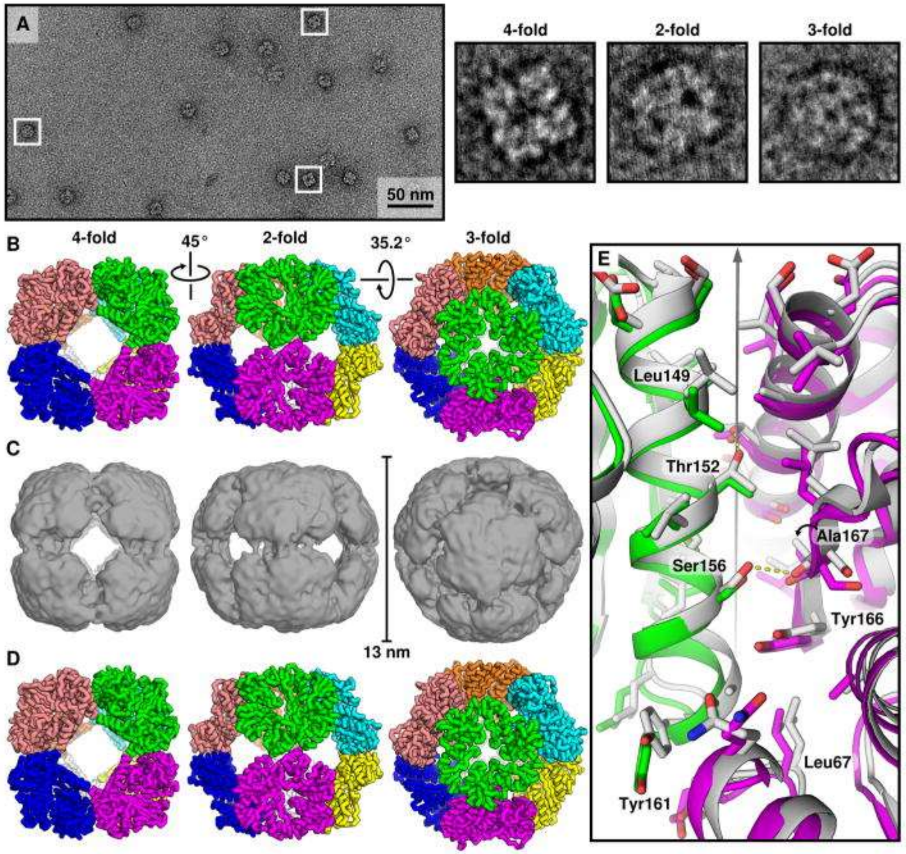




(**A**) First, a target symmetric architecture is chosen. Octahedral point group symmetry is used in this example; the threefold rotational axes are marked here by triangles and shown as black lines throughout. The dashed cube is shown to orient the viewer. (**B**) Multiple copies of the building block are symmetrically arranged in the target architecture by aligning their shared symmetry axes. The pre-existing organization of the oligomeric building block fixes several (in this case four) rigid body degrees of freedom (DOFs). (**C**) Symmetrical docking is performed by systematically varying the two DOFs (moves are applied symmetrically to all subunits) and computing the suitability of each configuration for interface design (red: more suitable; blue: less suitable). Points corresponding to the docked configurations in panels (B), in which the building blocks are not in contact, and (**D**), a highly complementary interface, are indicated (**E**, **F**).

Science 2012 336 (6085):1171-4. doi: 10.1126/science.1219364

Computational design of self-assembling protein nanomaterials: experimental validation



(**A**) A representative negative stain electron micrograph of O3-33. Selected particles (boxed in white) that resemble views of the design model along its 4-fold, 2-fold, and 3-fold rotational axes, shown in (B), are enlarged at right. (**B**) The O3-33 design model, depicted in ribbon format. Each trimeric building block is shown in a different color. (**C**) The density map from a 20 Å resolution cryo-EM reconstruction of O3-33 clearly recapitulates the architecture of the design model. (**D**) The crystal structure of O3-33 (R32 crystal form). Images in (B) to (D) are shown to scale along the three types of symmetry axes present in point group O. (**E**) The designed interface in O3-33, highlighting the close agreement between the crystal structure (green and magenta) and the design model (white). Oxygen atoms are red; nitrogens, blue. Hydrogen bonds between the building blocks are shown as yellow dashes, and an octahedral 2-fold rotational axis that passes through the interface is shown as a gray line. Residues in which substitution disrupted self-assembly are labeled.

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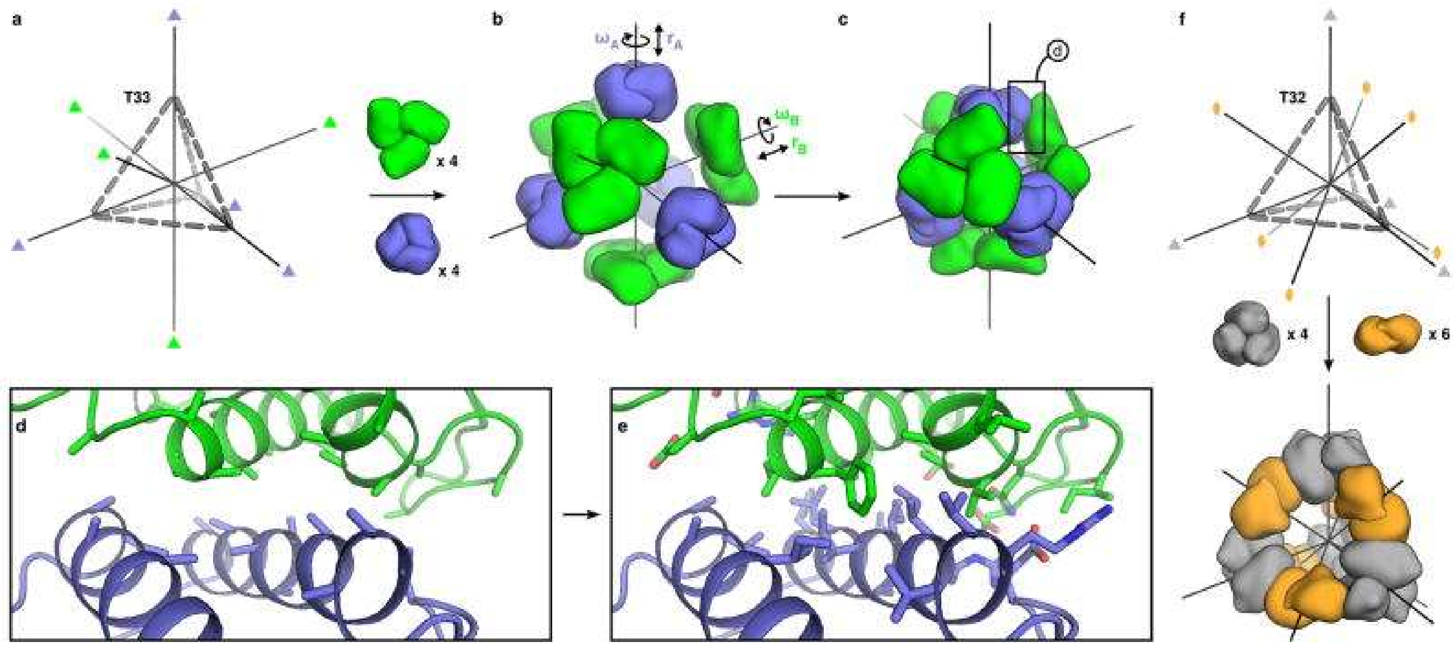
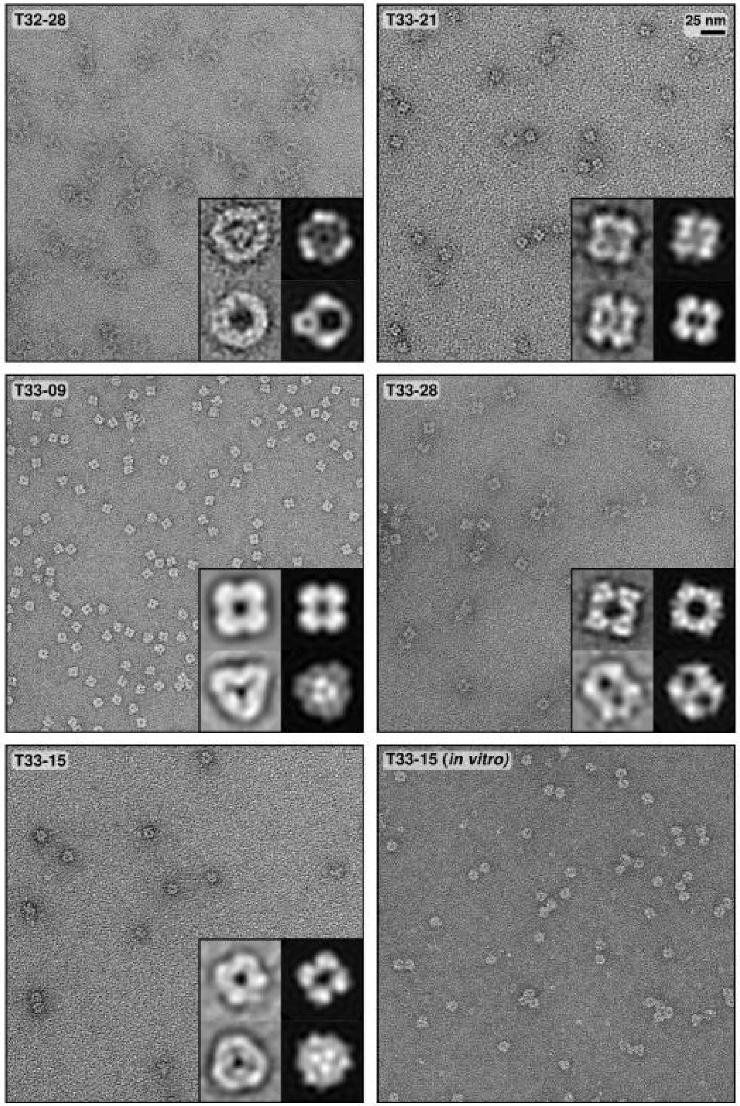
Design of multi-component protein materials: overview

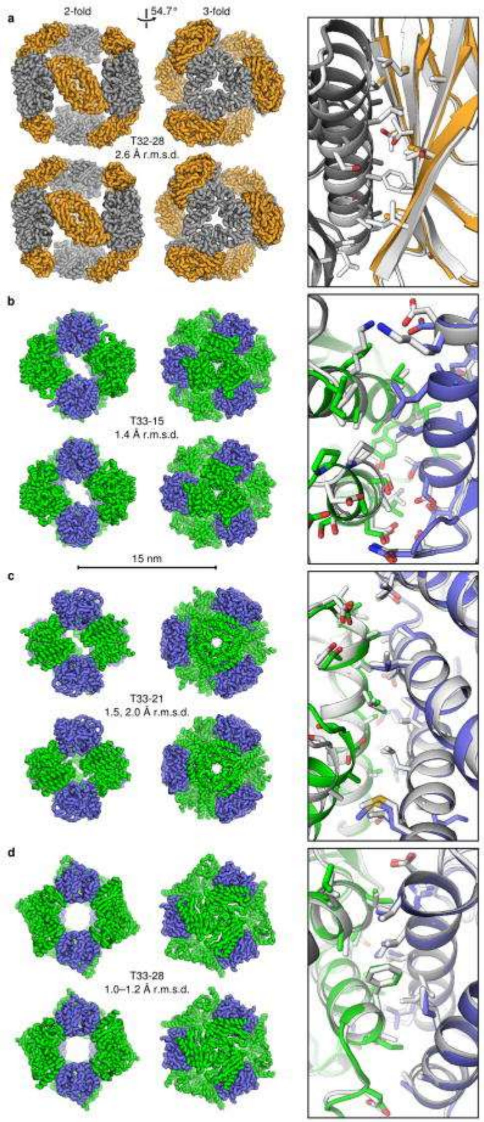


a, The T33 architecture comprises four copies each of two distinct trimeric building blocks (green and blue) arranged with tetrahedral point group symmetry (24 total subunits; triangles indicate three-fold symmetry axes). **b,** Each building block has two rigid body degrees of freedom, one translational (r) and one rotational (ω), that are systematically explored during docking. **c–d,** The docking procedure, which is independent of the amino acid sequence of the building blocks, identifies large interfaces with high densities of contacting residues formed by well-anchored regions of the protein structure. **e,** Amino acid sequences are designed at the new interface to stabilize the modeled configuration and drive coassembly of the two components. **f,** In the T32 architecture, four trimeric (grey) and six dimeric (orange) building blocks are aligned along the three-fold and two-fold symmetry axes passing through the vertices and edges of a tetrahedron, respectively.

Nature. 2014 Jun 5; 510(7503): 103–108

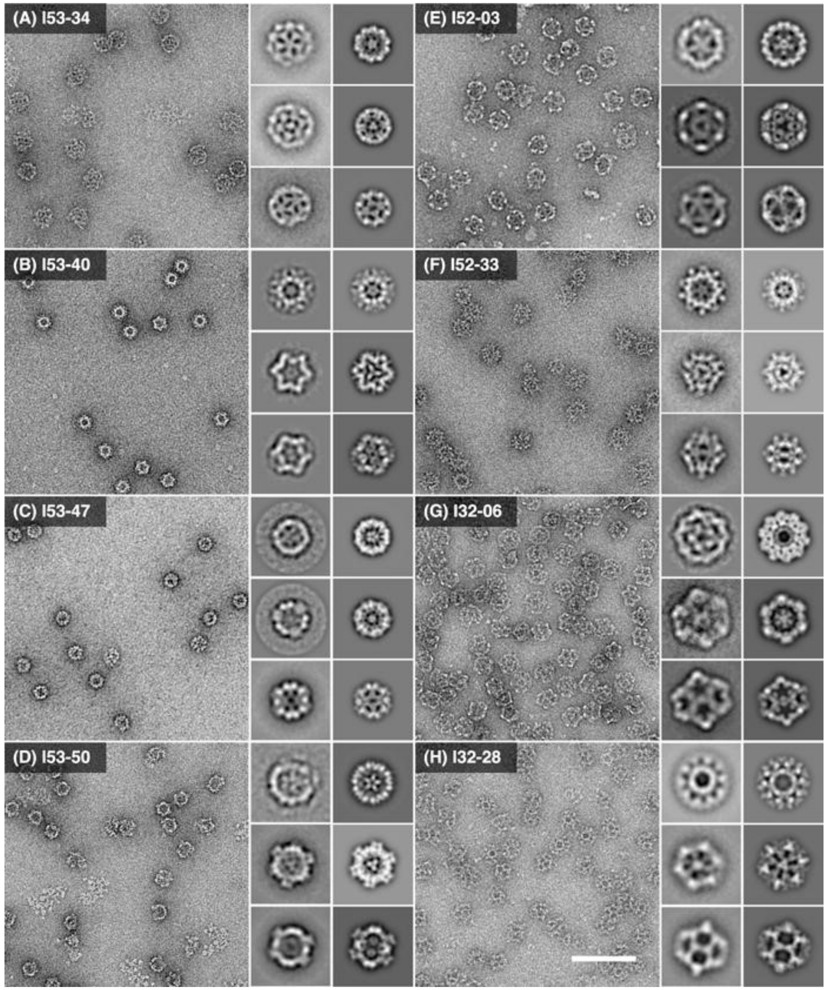
Structures of designed two-component protein nanomaterials

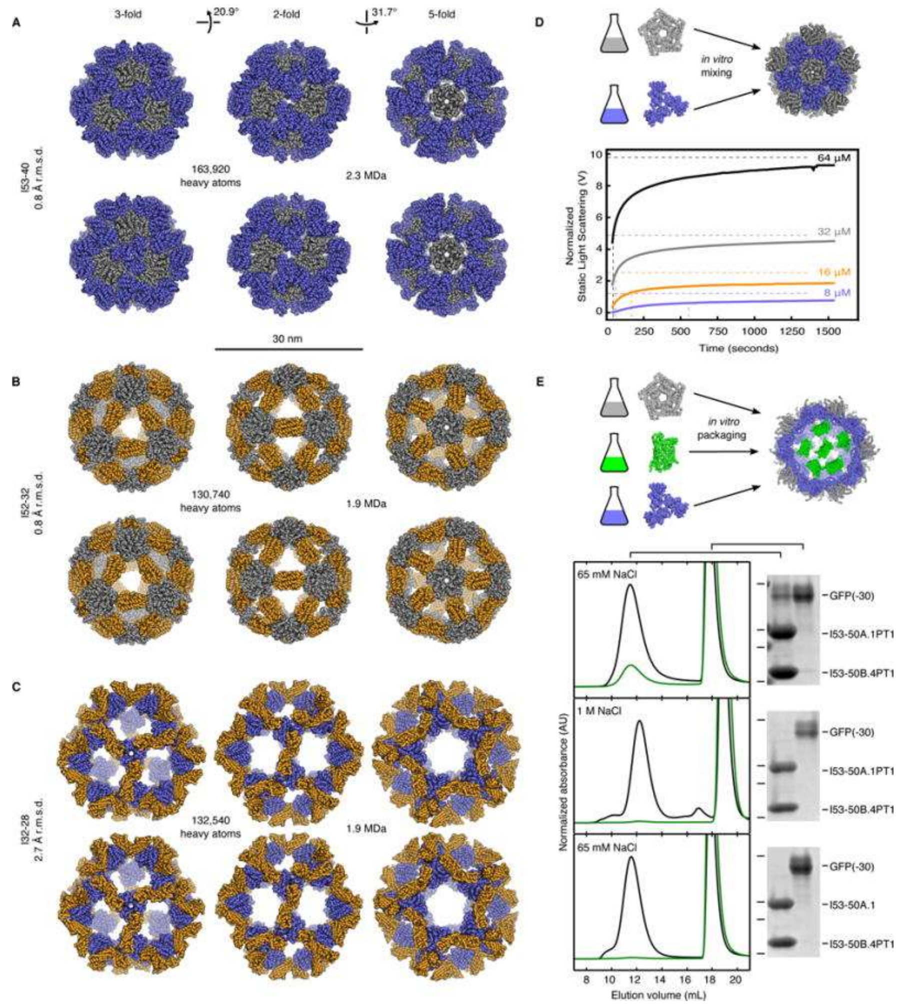




Nature. 2014 Jun 5; 510(7503): 103–108

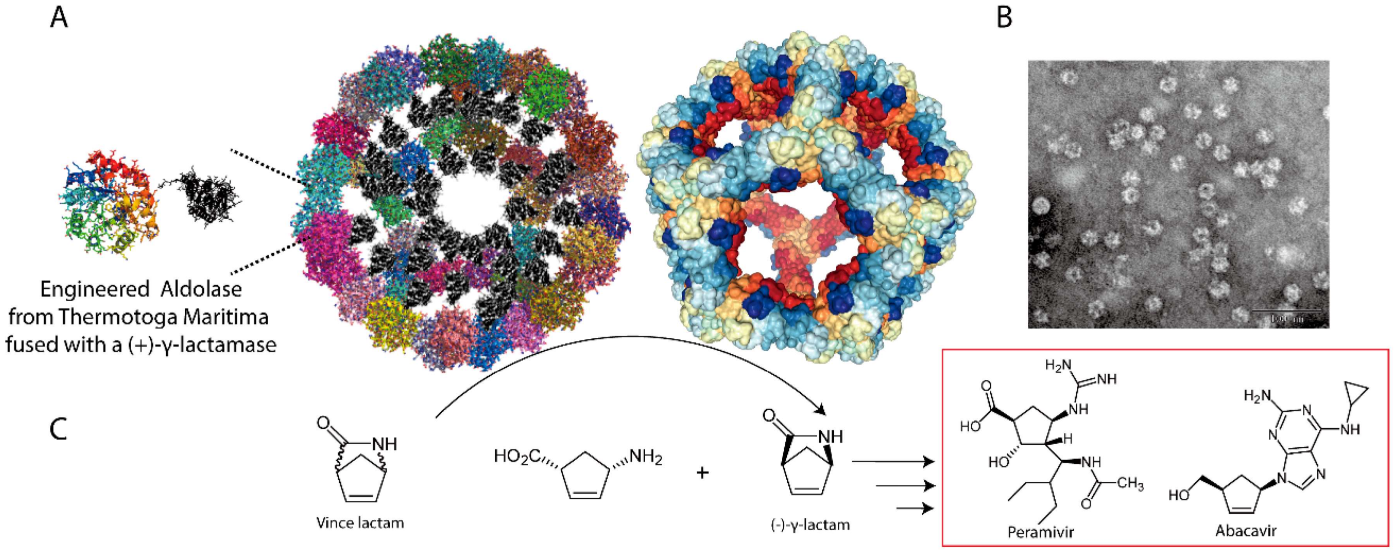
Accurate design of megadalton-scale two-component icosahedral protein complexes





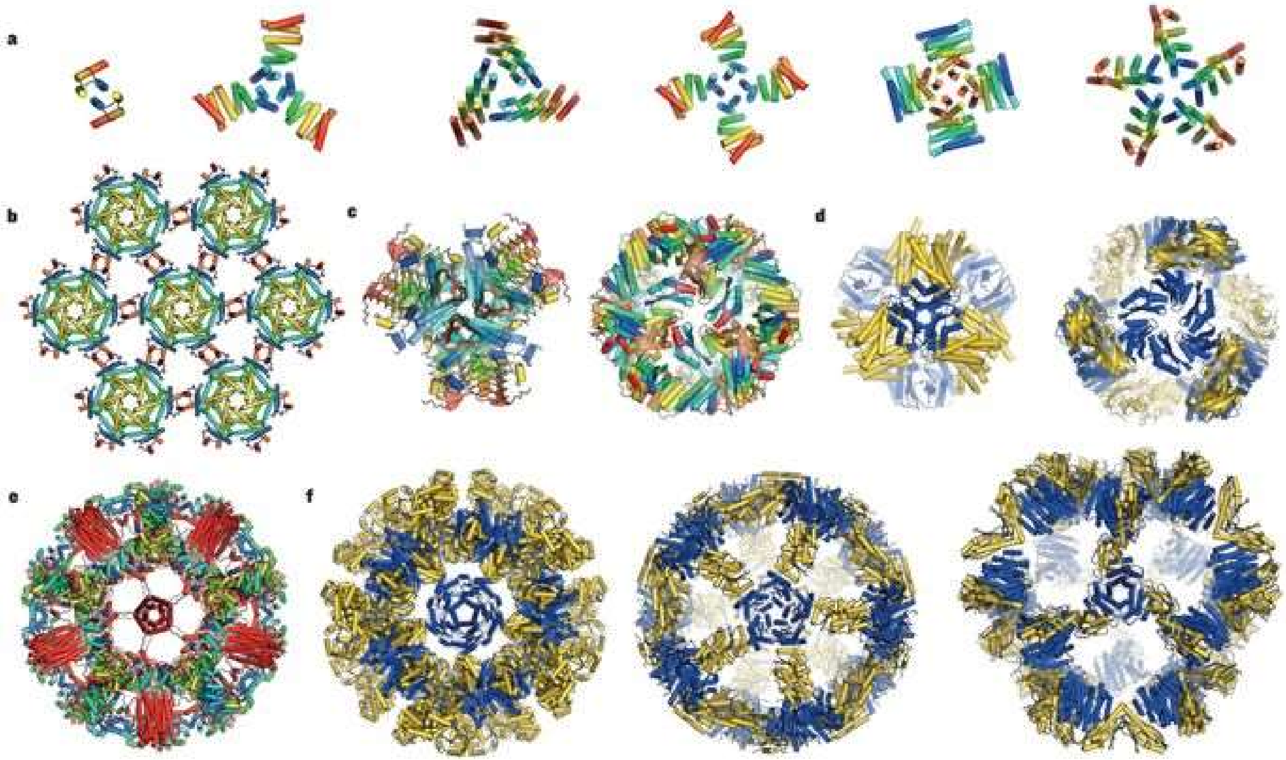
Science 2016; 353(6297): 389–394, doi: 10.1126/science.aaf8818

Artificial protein dodecahedrons



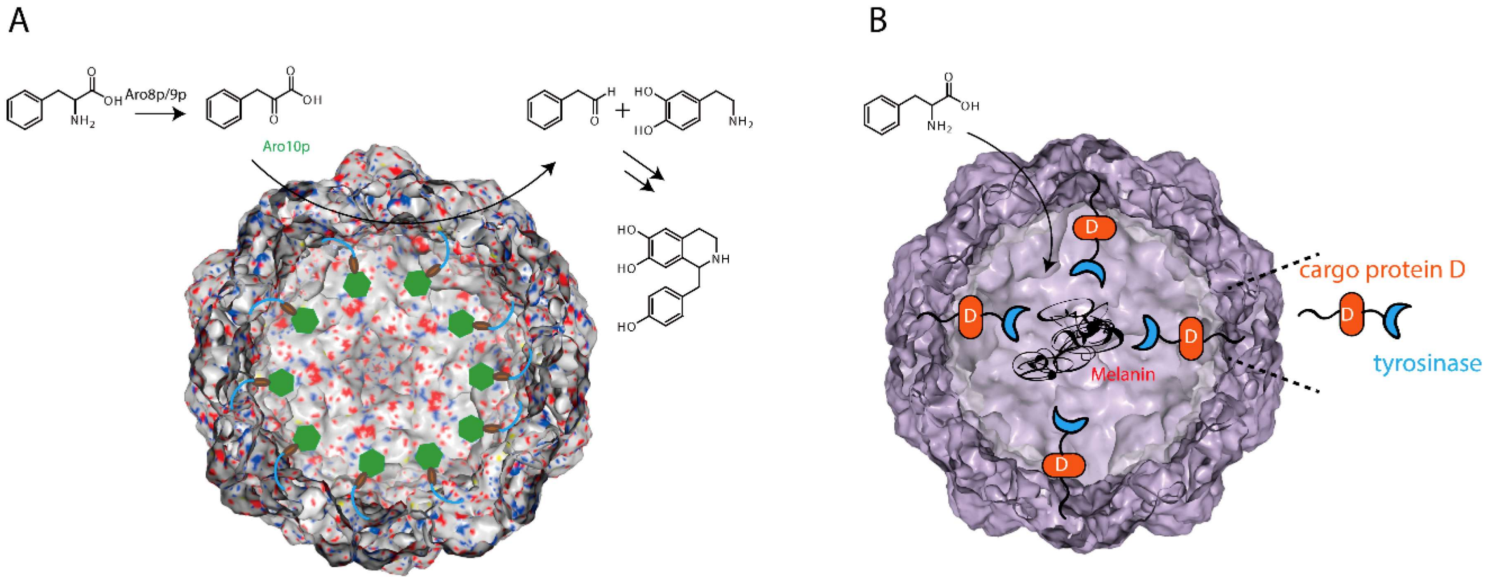
- The engineered trimeric aldolase from *Thermotoga maritima* with a modified interface was fused with an industrial biocatalyst (+)-γ-lactamase from *Microbacterium hydrocarbonoxydans* and the hybridized protein could be self-assembled into an organelle-like nanodevice.
- The constructed nanoreactor is readily used for enzymatic resolution of Vince lactam, an important intermediate for synthesis of carbocyclic nucleoside medicines. Notably, the designed nanoreactors could confer a significant benefit to the biocatalyst cargo. The encapsulated (+)-γ-lactamase exhibits significantly improved stabilities with respect to heat, organic solvent, and protease degradation. Moreover, it shows better substrate tolerance than the free enzyme.
- This research demonstrates that bio-designed artificial protein nanocages are an effective way to improve the stability and strength of biocatalysts and might have broader applications in sustainable catalysis and synthetic biology.

Synthetic large protein nanocages to improve drug delivery



Computational models of the 10 successful designs are shown via molecular surface representations (design names are shown above each model). Each design comprises a pairwise combination of pentameric (grey), trimeric (blue), or dimeric (orange) building blocks aligned along icosahedral fivefold, threefold, and twofold symmetry axes, respectively. All models are shown to scale relative to the 30 nanometer scale bar.

Encapsulins as nanocontainers for nanoreactor design



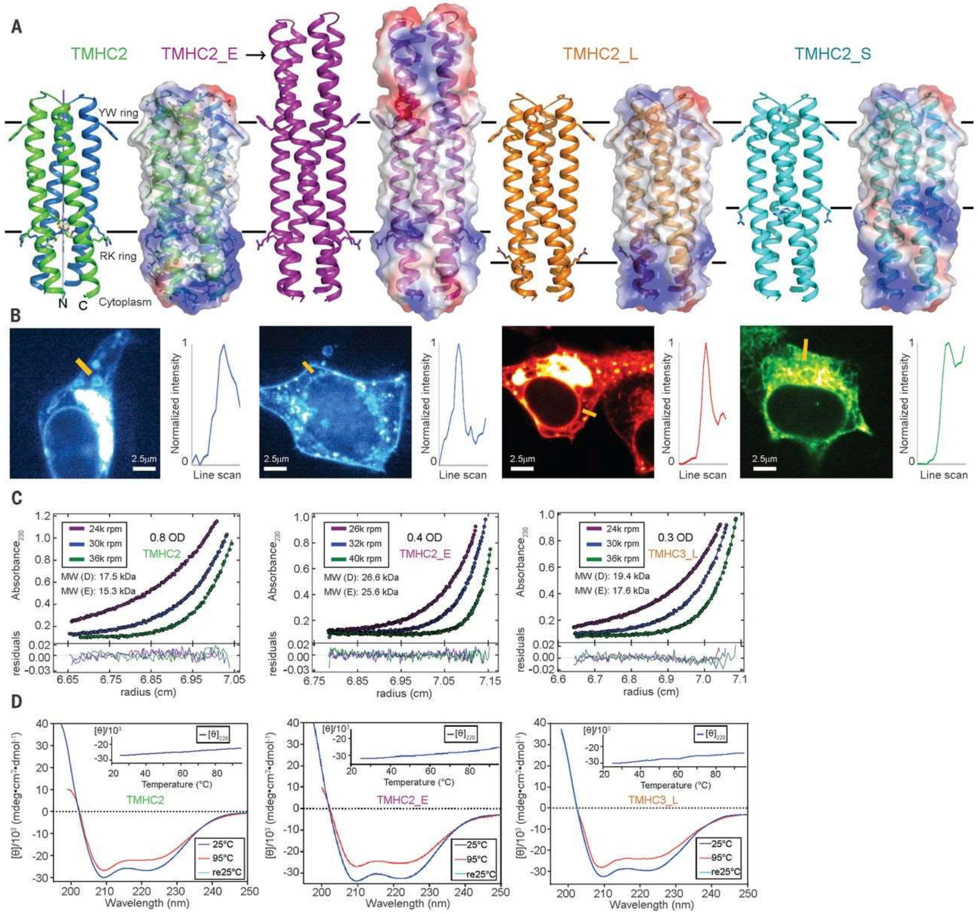
Encapsulin from *M. xanthus*

Encapsulin from *M. xanthus*

- Encapsulins are a new family of microbial proteinaceous compartments that have been engineered for nanoreactor construction. Typically, encapsulin has an overall size of about 20–40 nm (diameter), which is very similar to virus
- In fact, encapsulins are naturally occurring nanoreactors that encapsulate specific cargo proteins and are involved in diverse cell processes including iron mineralization, oxidative and nitrosative stress resistance, and anaerobic ammonium oxidation
- This example demonstrates that encapsulin compartments could be selected as a general platform for organelle construction in eukaryotes and has potential for wide application in synthetic biology

Membrane protein oligomers by design

Design and characterization of proteins with four transmembrane helices

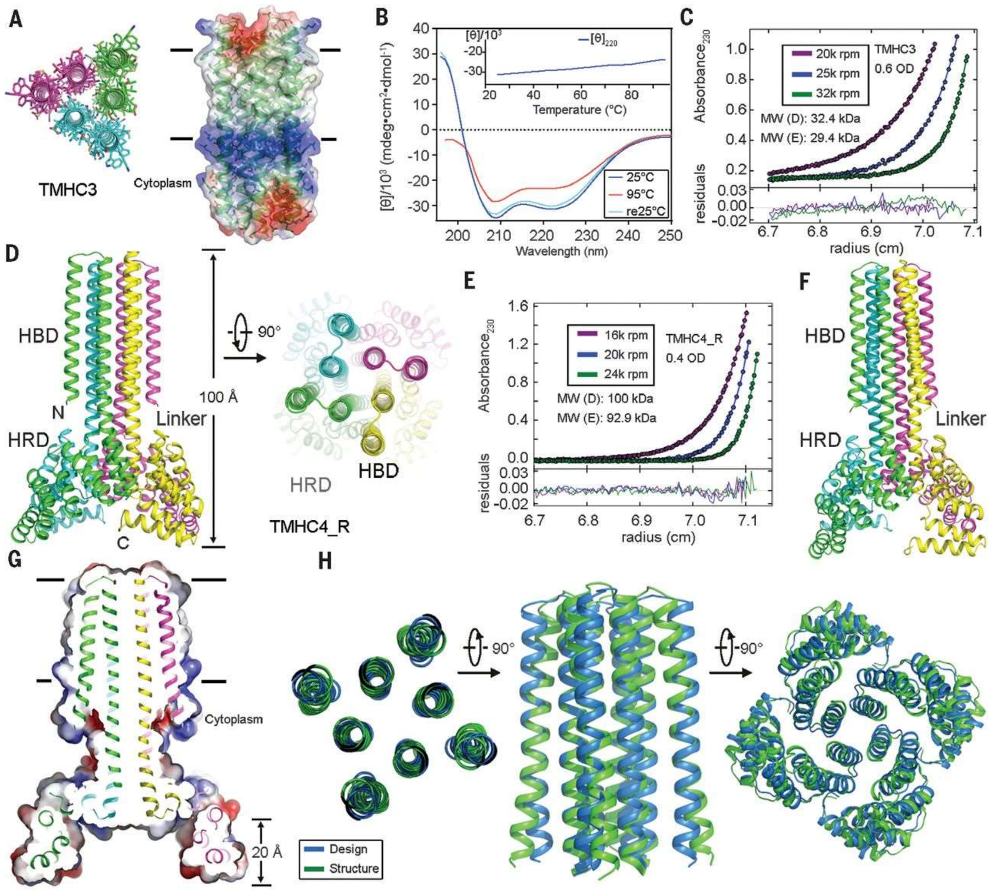


Science 2018: 359(6379):1042-1046, doi: 10.1126/science.aaq1739

A and **B**) From left to right, designs and data for TMHC2 (transmembrane hairpin C2), TMHC2_E (elongated), TMHC2_L (long span), and TMHC2_S (short span). (A) Design models with intra- and extramembrane regions with different lengths. Horizontal lines demarcate the hydrophobic membrane regions. Ribbon diagrams are at left, electrostatic surfaces are at right, and the neutral transmembrane regions are in gray. (B) Confocal microscopy images for HEK293T cells transfected with TMHC2 fused to mTagBFP, TMHC2_E fused to mTagBFP, TMHC2_L fused to mCherry, and TMHC2_S fused to enhanced green fluorescent protein. Line scans (yellow lines) across the membranes show substantial increase in fluorescence across the plasma membranes for TMHC2, TMHC2_E, and TMHC2_L, but less substantial increase for TMHC2_S. (**C**) Representative AUC sedimentation-equilibrium curves at three different rotor speeds. Each data set is globally well fit as a single ideal species in solution corresponding to the dimer molecular weight. “MW (D)” and “MW (E)” indicate the molecular weight of the oligomer design and that determined from experiment, respectively. (**D**) CD spectra and (inset) temperature melt. No apparent unfolding transitions are observed up to 95°C.

Membrane protein oligomers by design

Characterization of designs with six and eight membrane-spanning helices



Science 2018: 359(6379):1042-1046, doi: 10.1126/science.aaq1739

(**A**) Model of designed transmembrane trimer TMHC3 with six transmembrane helices. Stick representation from periplasmic side (left) and lateral surface view (right) are shown. (**B**) CD characterization of TMHC3. The design is stable up to 95°C. (**C**) Representative AUC sedimentation-equilibrium curves at three different rotor speeds for TMHC3. The data fit to a single ideal species in solution with molecular weight close to that of the designed trimer. (**D**) Model of designed transmembrane tetramer TMHC4_R with eight transmembrane helices. The four protomers are colored green, yellow, magenta, and blue, respectively. (**E**) AUC sedimentation-equilibrium curves at three different rotor speeds for TMHC4_R fit well to a single species, with a measured molecular weight of ~94 kDa. (**F**) Crystal structure of TMHC4_R. The overall tetramer structure is very similar to the design model, with a helical bundle body and helical repeat fins. The outer helices of the transmembrane hairpins tilt off the axis by ~10°. (**G**) Cross section through the TMHC4_R crystal structure and electrostatic surface. The HRD forms a bowl at the base of the overall structure with a depth of ~20 Å. The transmembrane region is indicated in lines. (**H**) Three views of the backbone superposition of TMHC4_R crystal structure and design model.

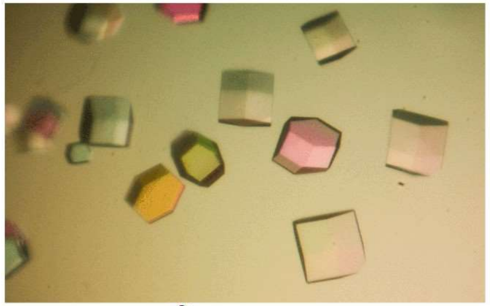
Design of protein nanomachines is enabled by advances in structural biology

Concepts
Methods
Applications

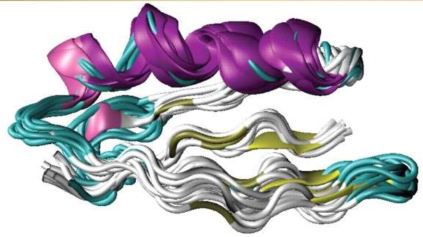
Structural biology of large macromolecular assemblies

- Determining 3D structure of proteins help protein engineers to understand molecular mechanism of protein action and its biological function.

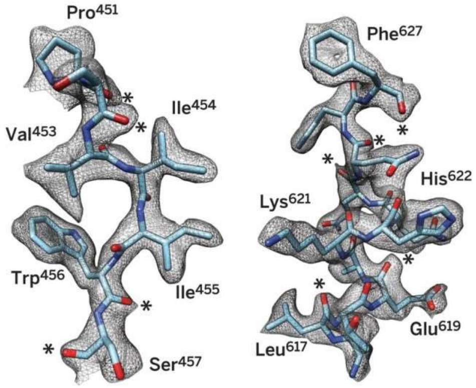
Structural biology techniques



- **X-ray crystallography**
  Crystallization required, no size limits, challenging for highly flexible proteins



- Nuclear magnetic resonance (NMR) spectroscopy
  Labelling required, suitable for smaller proteins, capturing protein motions

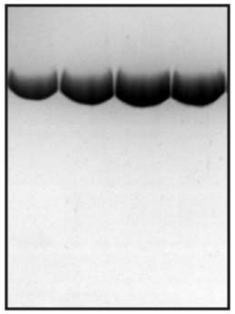


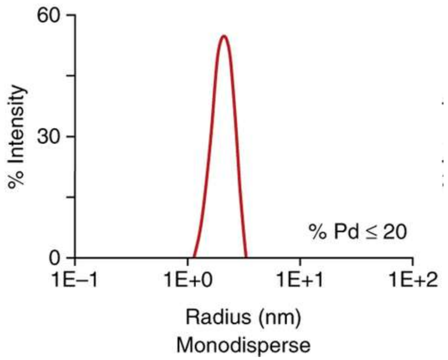
- **Cryo-electron microscopy**
  Automation, direct electron detectors, image processing suitable for large protein complexes

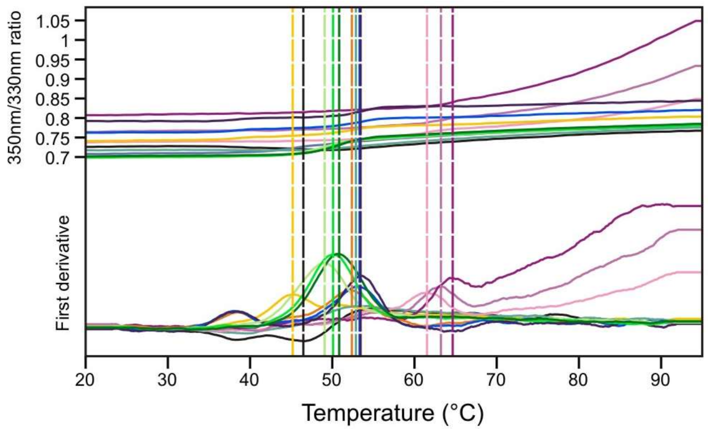
Macromolecular crystallography

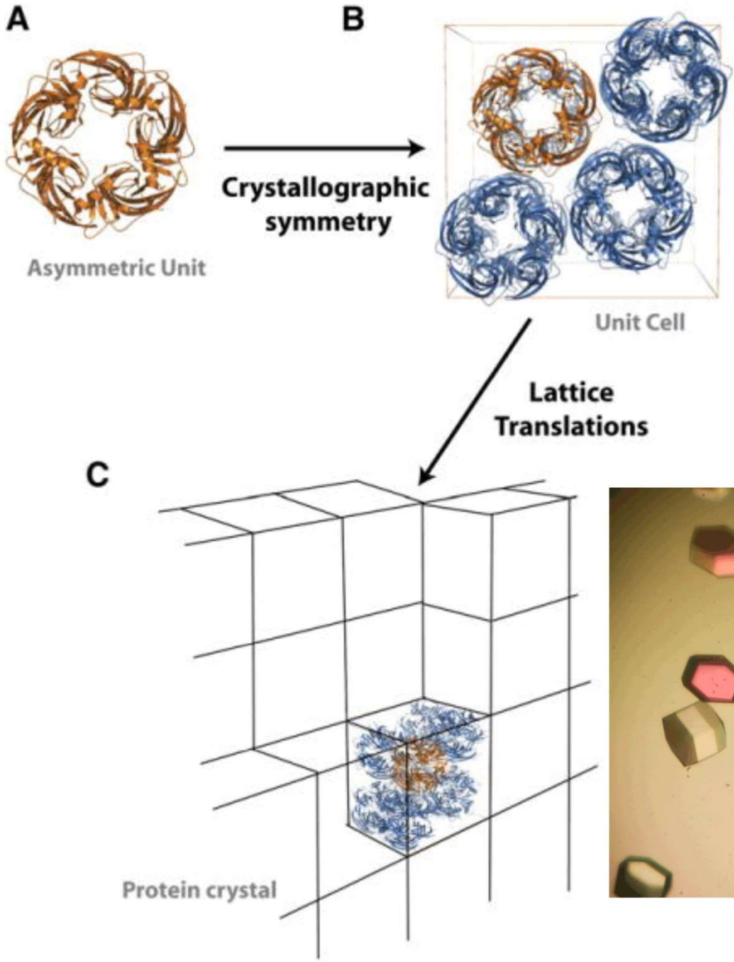
Prerequisite for successful protein crystallization

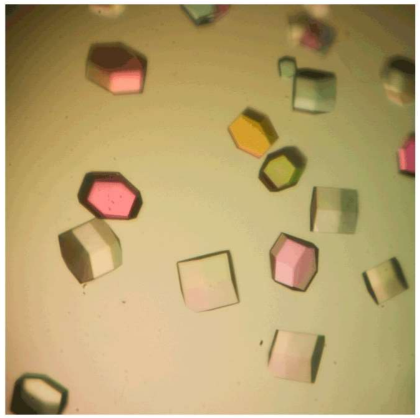
- Large quantities (few mgs)
- High purity
- Homogeneity
- Stability



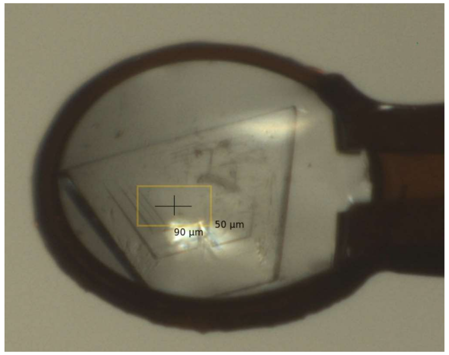


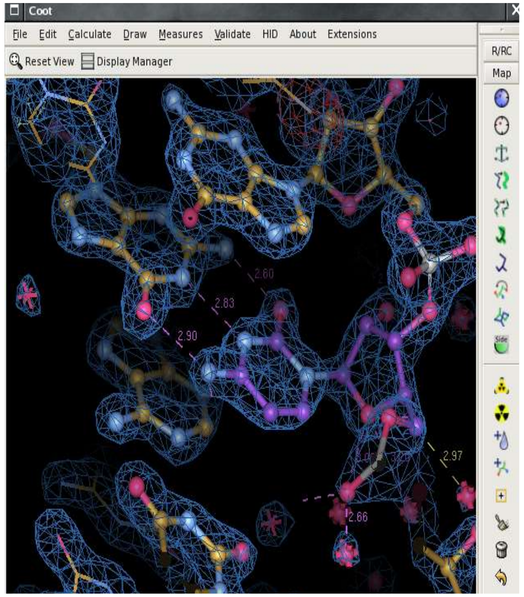


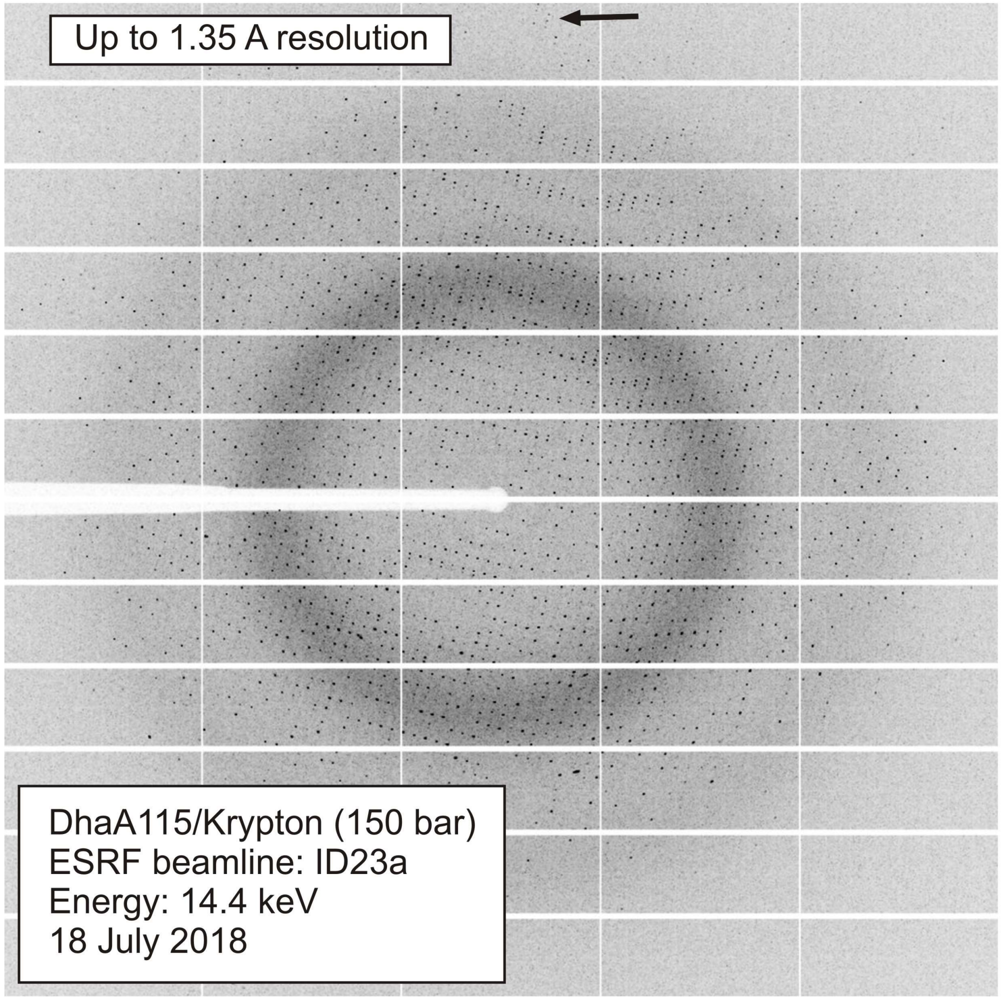




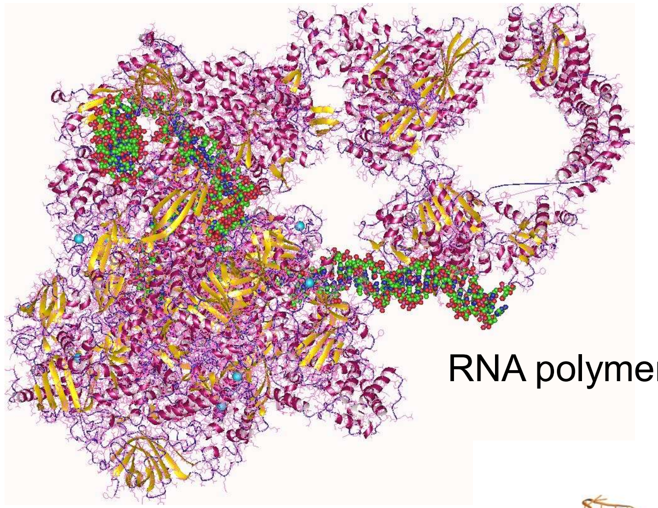
Crystal freezing, data collection, processing & model building



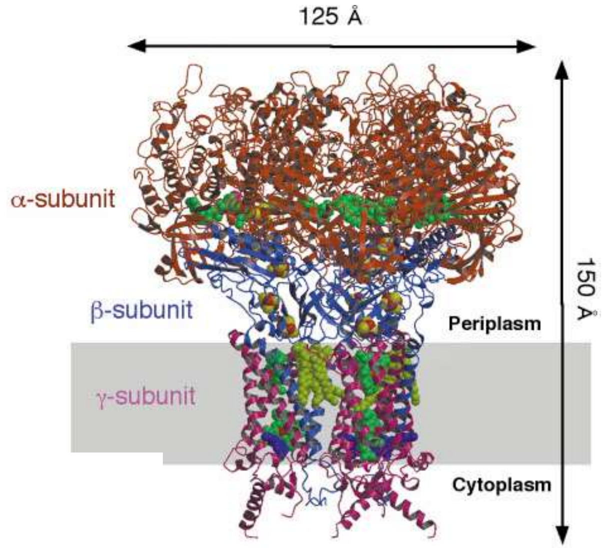




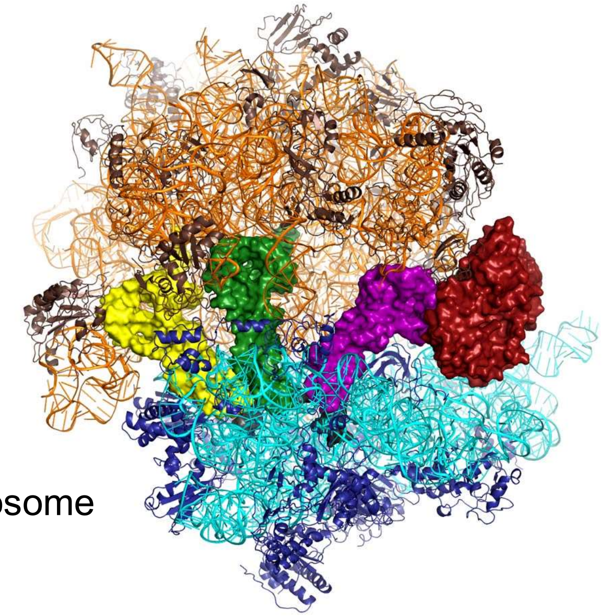
Examples of macromolecular X-ray structures



RNA polymerase



Membrane protein

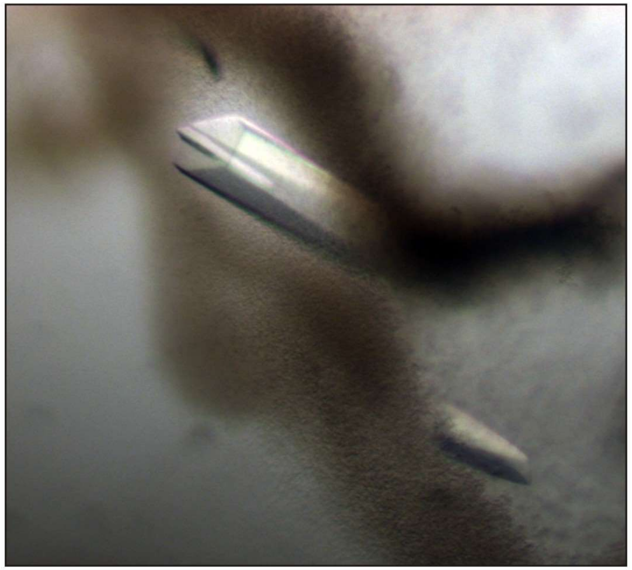


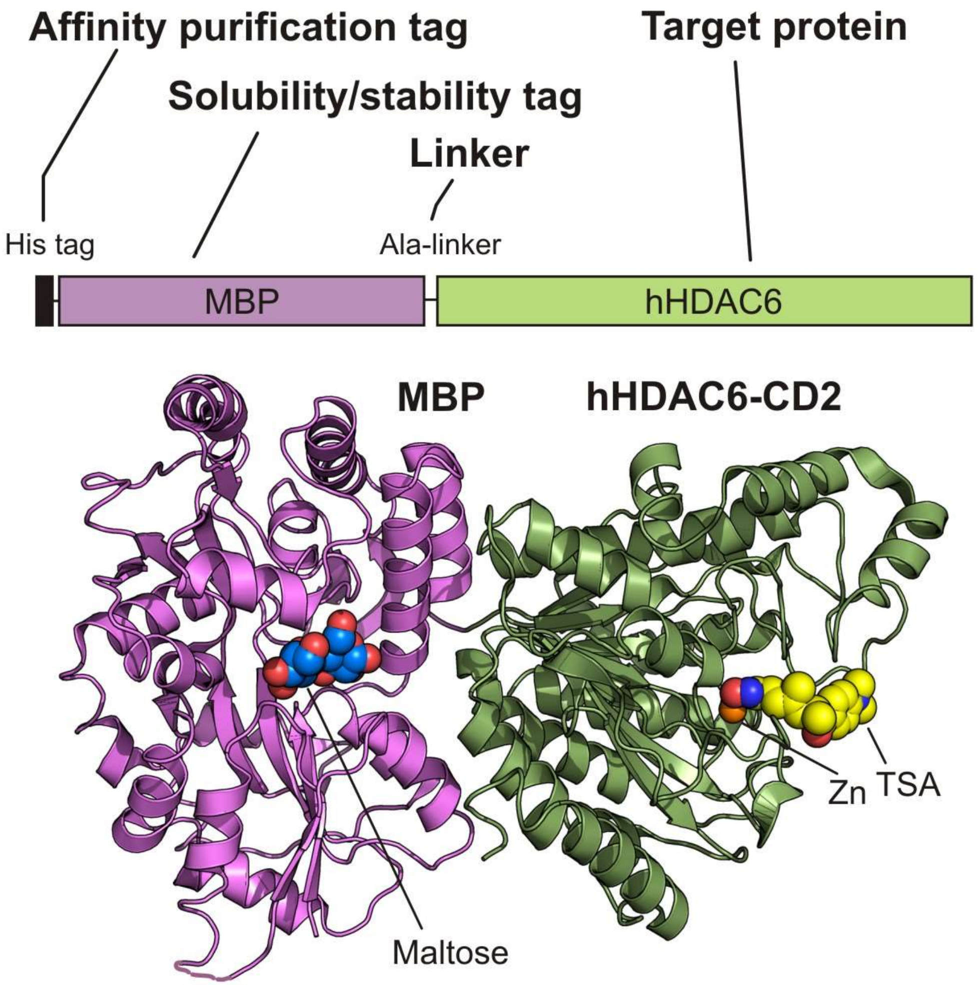
Ribosome

Engineered fusion proteins help protein crystallographers

Fusion tags used:

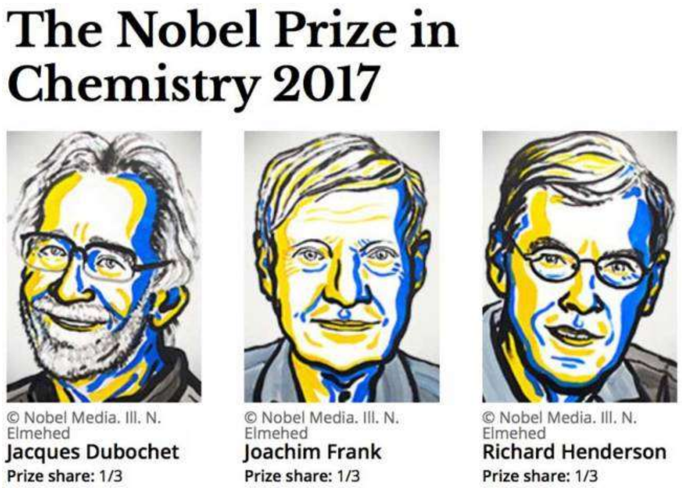
- Thioredoxin (Thx)
- Maltose-binding protein (MBP)
- Glutathione S-transferase (GST)
- Small ubiquitin-like modifier (SUMO)
- Polyhistidenes (6xHis, 12xHis)



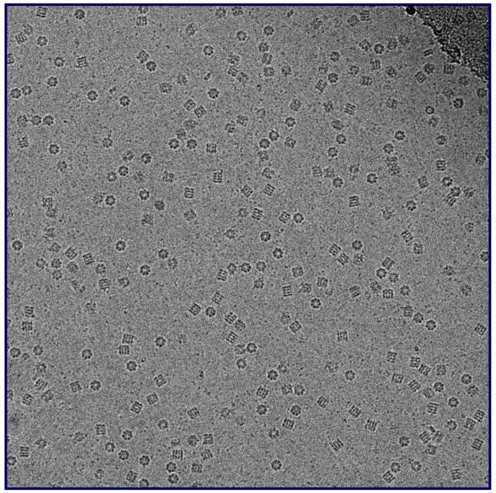


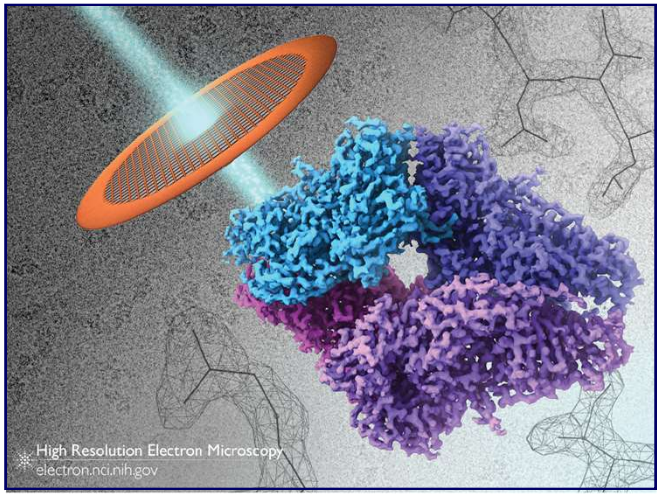
Hai & Christianson, Nat. Chem. Biol. 12:741-747 (2016)

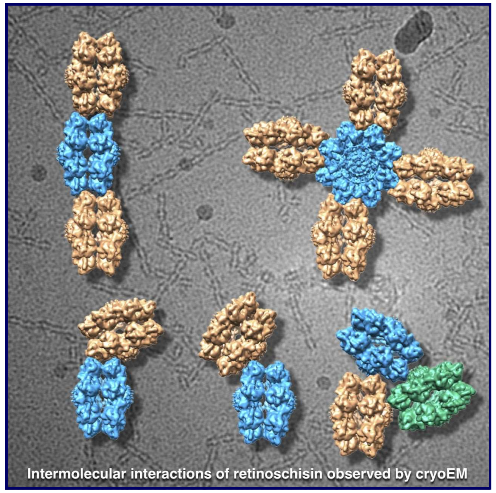
Cryo-electron microscopy: overview



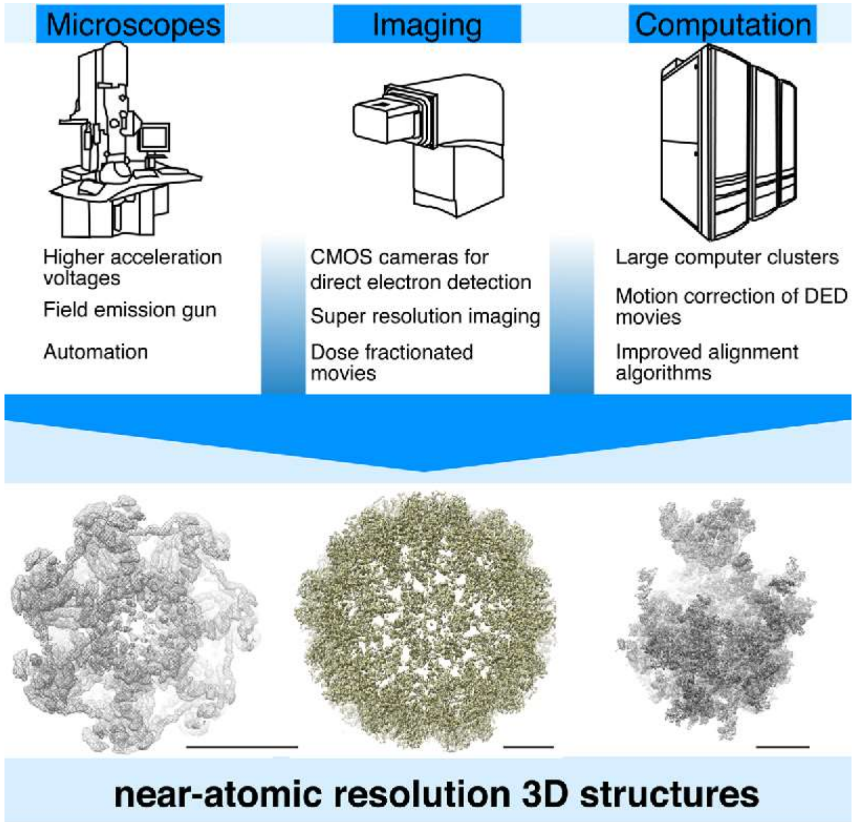
- **Cryo-electron microscopy** (**cryo-EM**) is an electron microscopy (EM) technique applied on samples cooled to cryogenic temperatures and embedded in an environment of vitreous water. An aqueous sample solution is applied to a grid-mesh and plunge-frozen in liquid ethane. While development of the technique began in the 1970s, recent advances in detector technology and software algorithms have allowed for the determination of biomolecular structures at near-atomic resolution.

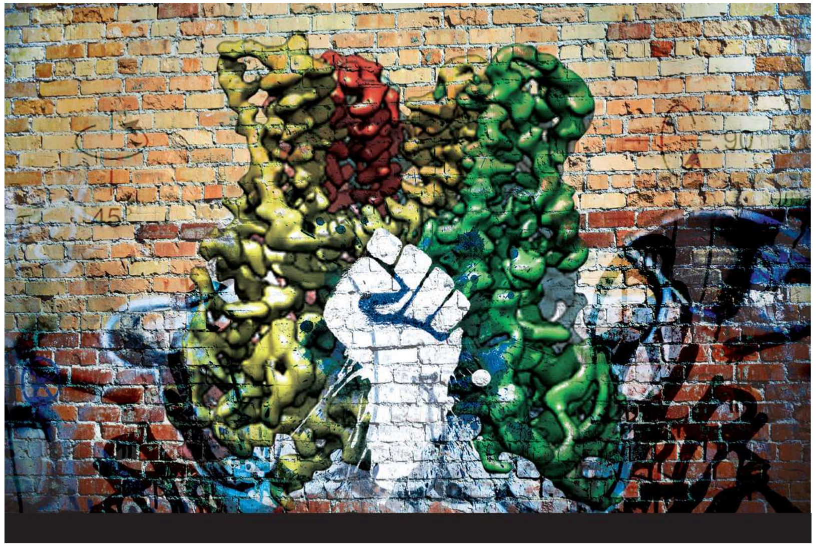






Recent major technical advances in cryo-EM





THE REVOLUTION WILL NOT BE CRYSTALLIZED

MOVE OVER X-RAY CRYSTALLOGRAPHY. CRYO-ELECTRON MICROSCOPY IS KICKING UP A STORM IN STRUCTURAL BIOLOGY BY REVEALING THE HIDDEN MACHINERY OF THE CELL.

BY EWEN CALLAWAY

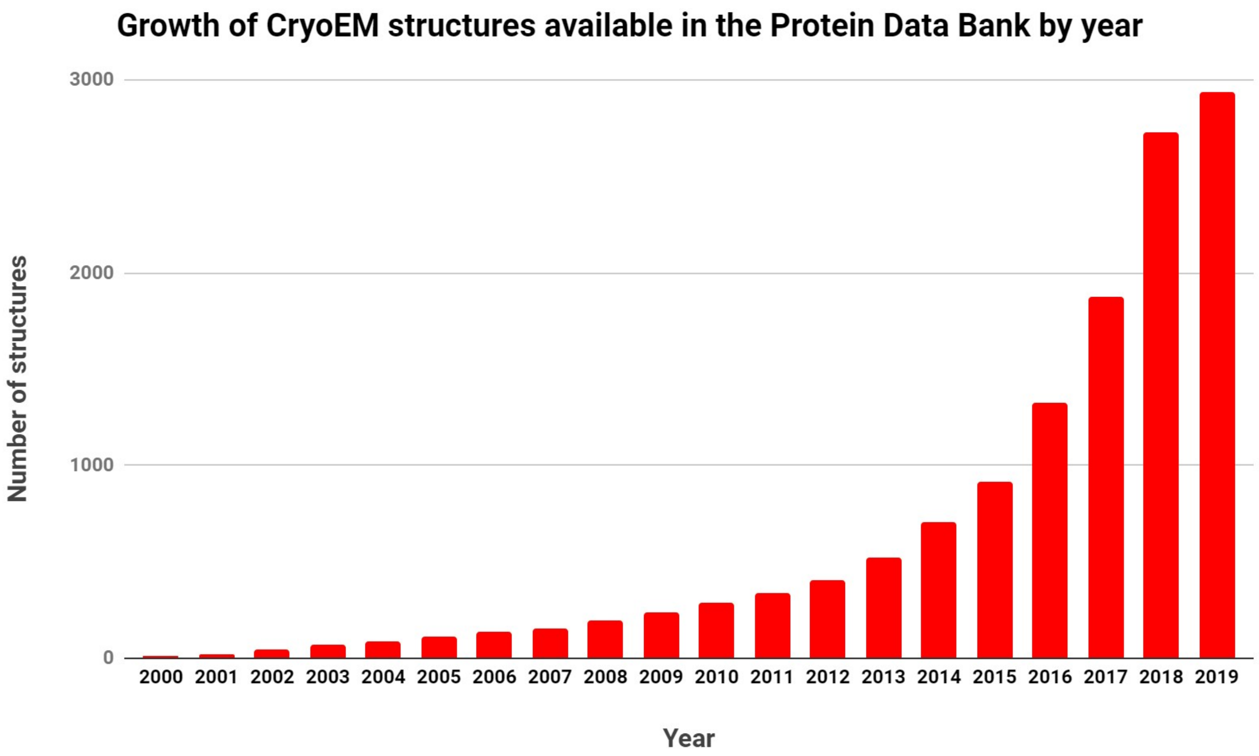
In a basement room, deep in the bowels of a steel-clad building in Cambridge, a major insurgency is under way.

A hulking metal box, some three metres tall, is quietly beaming terabytes' worth of data through thick orange cables that disappear off through the ceiling. It is one of the world's most advanced cryo-electron microscopes: a device that uses electron beams to photograph frozen biological molecules and lay bare their molecular shapes. The microscope is so sensitive that a shout can ruin an experiment, says Sjors Scheres, a structural biologist at the UK Medical Research Council Laboratory of Molecular Biology (LMB), as he stands dwarfed beside the £5-million (US$7.7-million) piece of equipment. "The UK needs many more of these, because there's going to be a boom," he predicts.

In labs around the world, cryo-electron microscopes such as this one are sending tremors through the field of structural biology. In the past three years, they have revealed exquisite details of protein-making ribosomes, quivering membrane proteins and other key cell molecules,

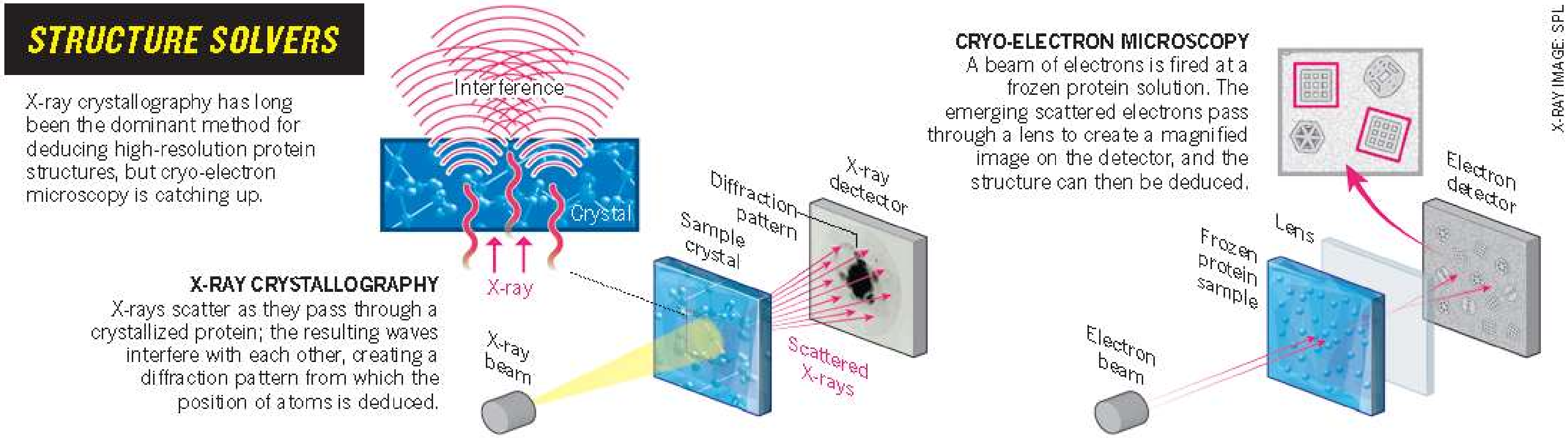
ILLUSTRATION BY VIKTOR KOEN

Cryo-EM structures in PDB database

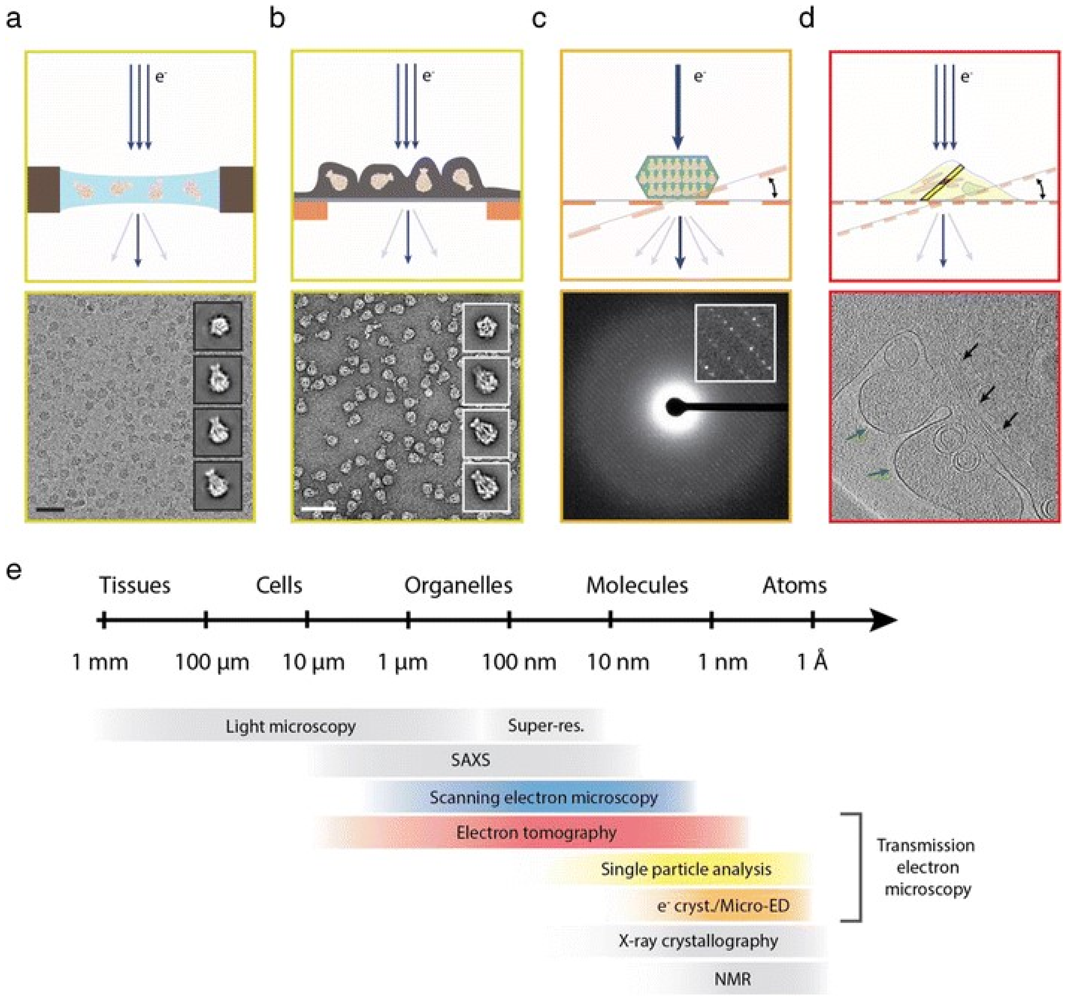


- The number of structures determined by cryo-EM is continuing to grow rapidly
- Computational methods are helping resolve conformational heterogeneity
- Improved biochemical methods are driving advances in cryo-EM of membrane proteins
- Novel approaches for cryo-EM specimen preparation are being explored

X-ray crystallography versus cryo-EM

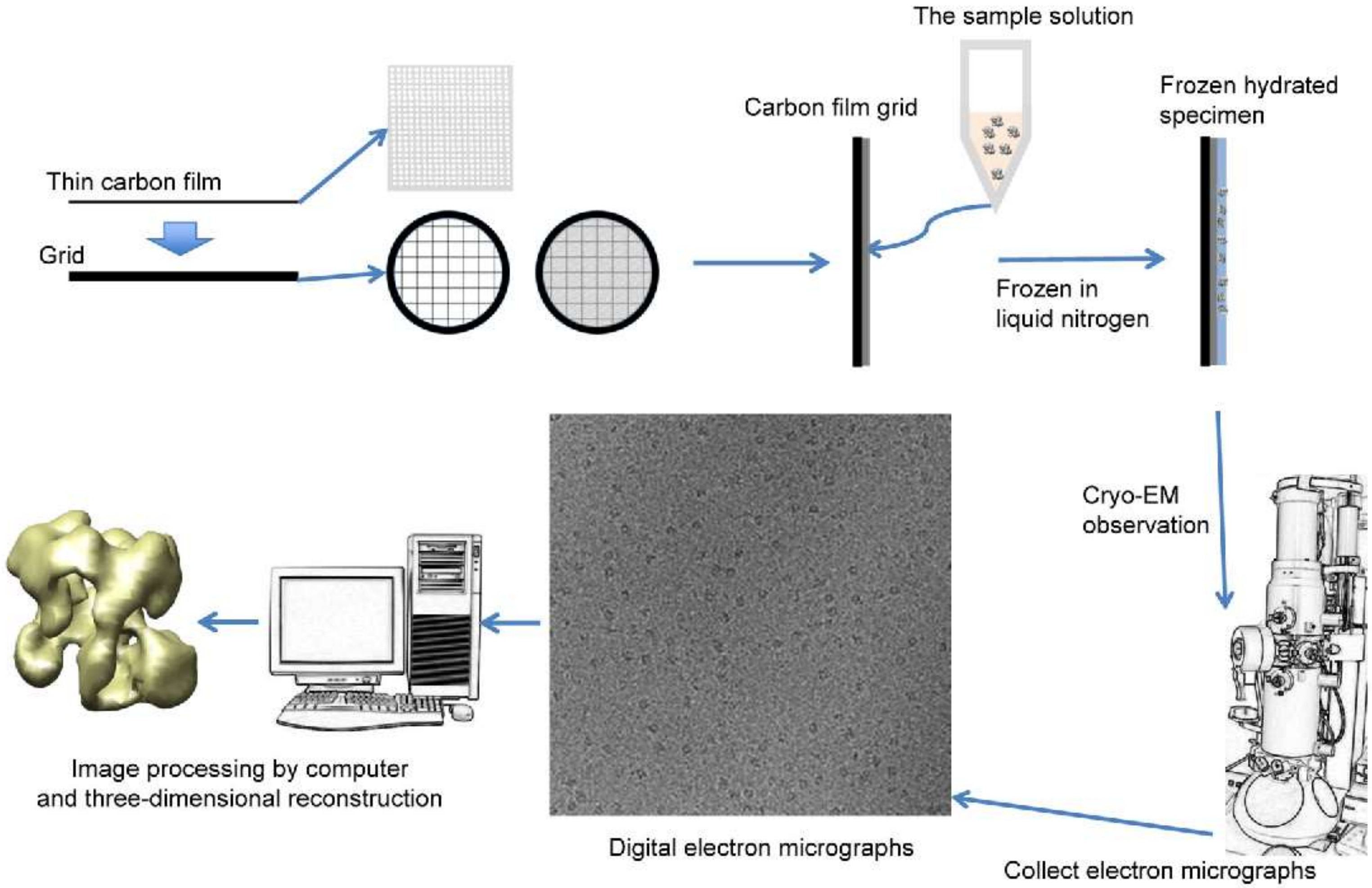


The most used cryo-EM techniques

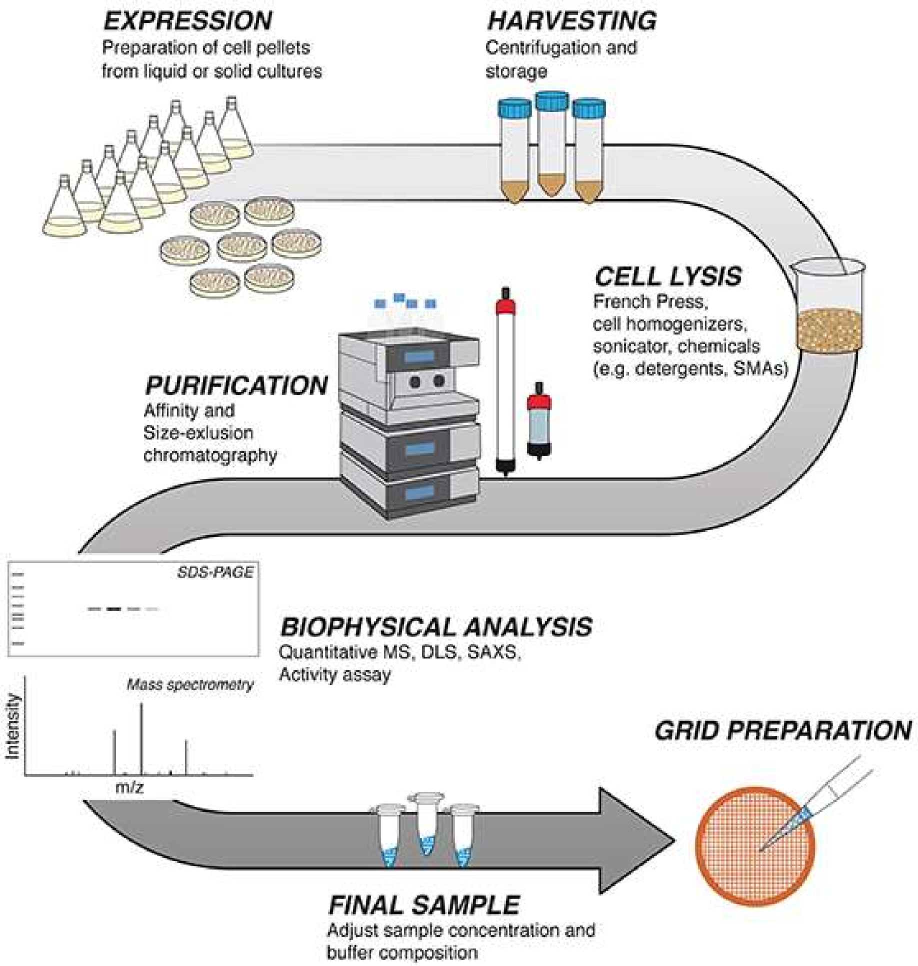


(**a**) **Single-particle cryo-EM**: particles are embedded in a thin layer of amorphous ice. Resulting representative class averages are shown as insets on the right. Scale bar, 50 nm. (**b**) **Single-particle negative-stain EM**: particles are embedded in a layer of heavy metal salts to increase the weak contrast of biological materials. Resulting representative class averages are shown as insets on the right. Scale bar, 50 nm. (**c**) **Micro-ED**: small 3-D crystals are hit with a focused electron beam and diffraction patterns are recorded at different tilt angles. Inset shows a small section of the diffraction image with individual diffraction spots at higher magnification. (**d**) **Cryo-ET**: the specimen is tilted within the microscope and images at different angles are recorded. A tomographic slice shows the cellular periphery with microtubule bundles (black arrows) and plasma membrane (green arrows). (**e**) Resolution range coverage of various methods in structural biology. Color code used for the TEM-based methods corresponds to (**a**)–(**d**). Yellow: single-particle analysis; orange: electron crystallography/micro-ED; red: electron tomography

Visualizing proteins by cryo-EM



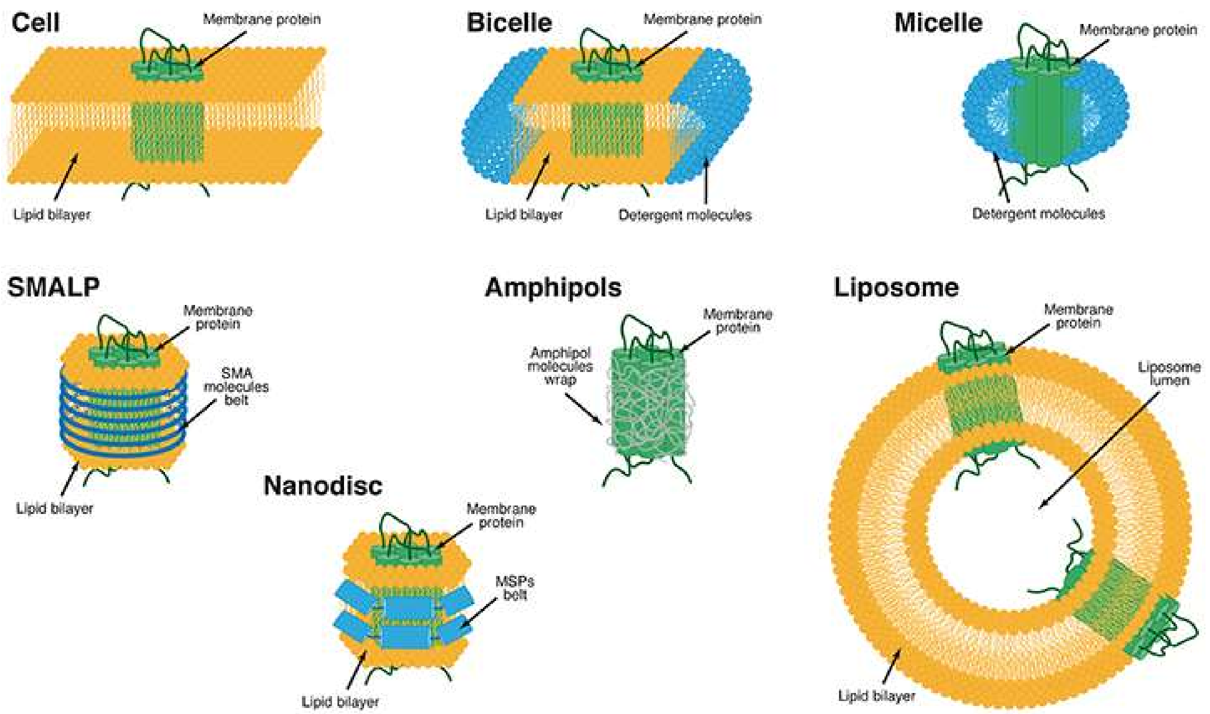
Protein sample preparation for cryo-EM



https://doi.org/10.3389/fmolb.2018.00074

- Cytoplasmic or membrane proteins are initially expressed in liquid or solid cultures, and pellets are stored after harvesting by centrifugation.
- Different physical or chemical cell disruption methods are utilized for releasing cytoplasmic proteins into solution or to obtain cell membrane extracts.
- Impure cytoplasmic proteins or solubilized cell membranes containing the protein of interest are purified by combination of different fast protein liquid chromatography (FPLC) methods.
- After protein stability, integrity and activity is verified by various biophysical techniques.
- The final sample concentration and buffer composition are adjusted before EM grid preparation.

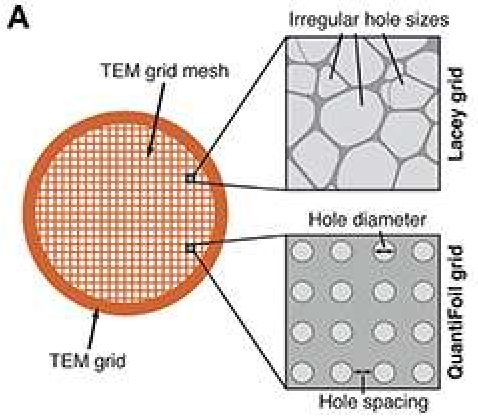
Membrane protein sample preparation and stabilisation

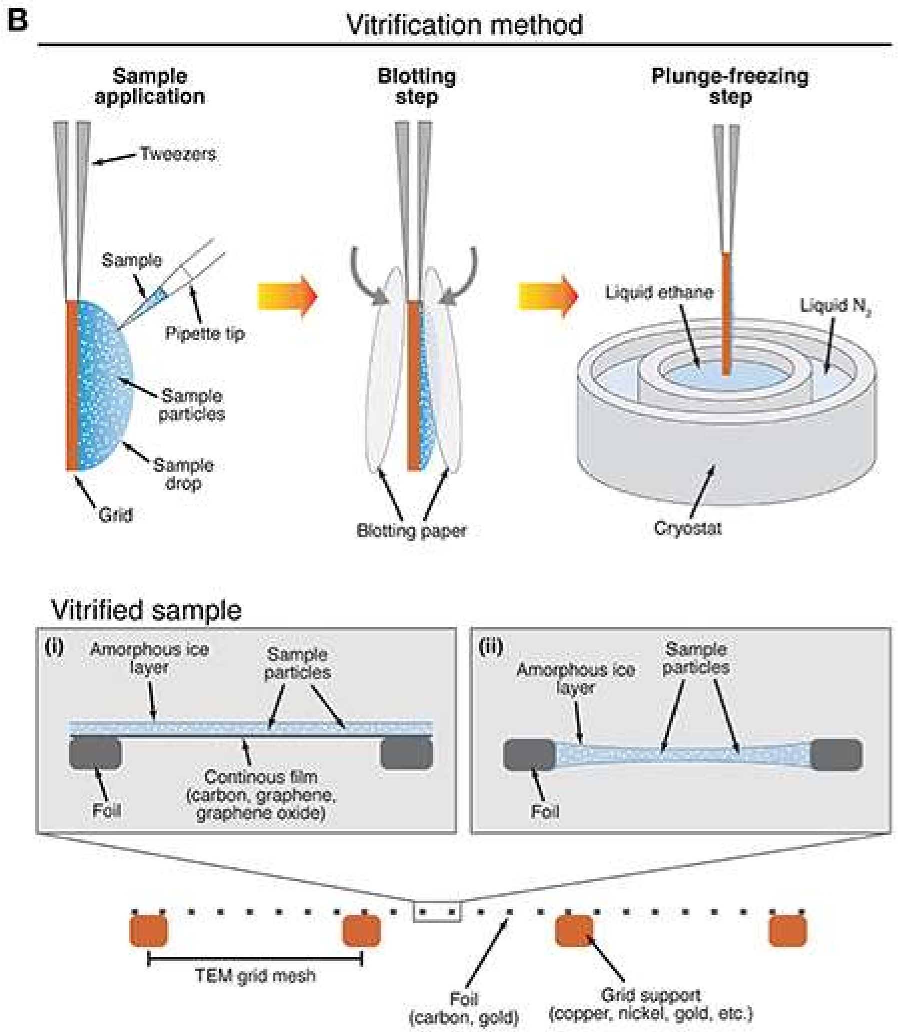


https://doi.org/10.3389/fmolb.2018.00074

Protein transmembrane domains are protected by the hydrophobic cell membrane phospholipid acyl chains. **Micelles** are spherical vesicles in which the detergent hydrophobic chains face inward and the hydrophilic polar heads face outward. **Bicelles** are obtained by a mixture of lipids and short chain detergents. The lipids will interact with the protein to form a lipid bilayer and the detergent will form the rim of the bicelle. Micelles will form after the solubilization of the membrane protein by detergents. **SMALP** (styrene-maleic acid lipid particles) are polymeric nanoparticles that protect the acyl chain of the lipid bilayer. **Nanodiscs** are lipid bilayers stabilized by wrapping a belt of amphipathic helix-rich membrane scaffold proteins (MSPs) around the detergent-solubilized membrane proteins. **Amphipol** polymers wrap around the hydrophobic patches of the membrane protein to form a stable complex in solution. **Liposomes** are artificial spherical lipid membranes where membrane proteins can assemble.

Cryo-EM grid preparation

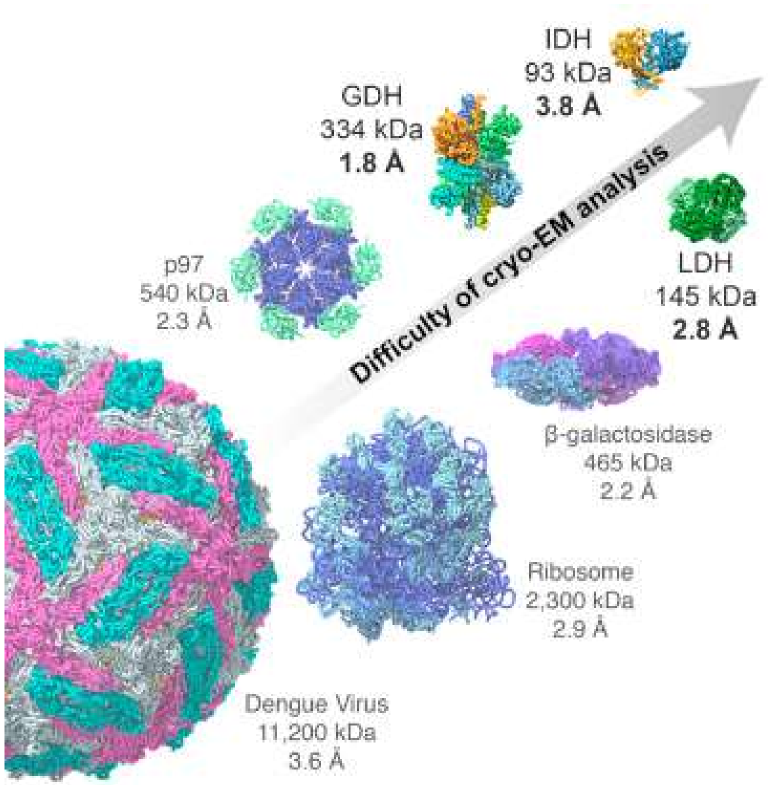


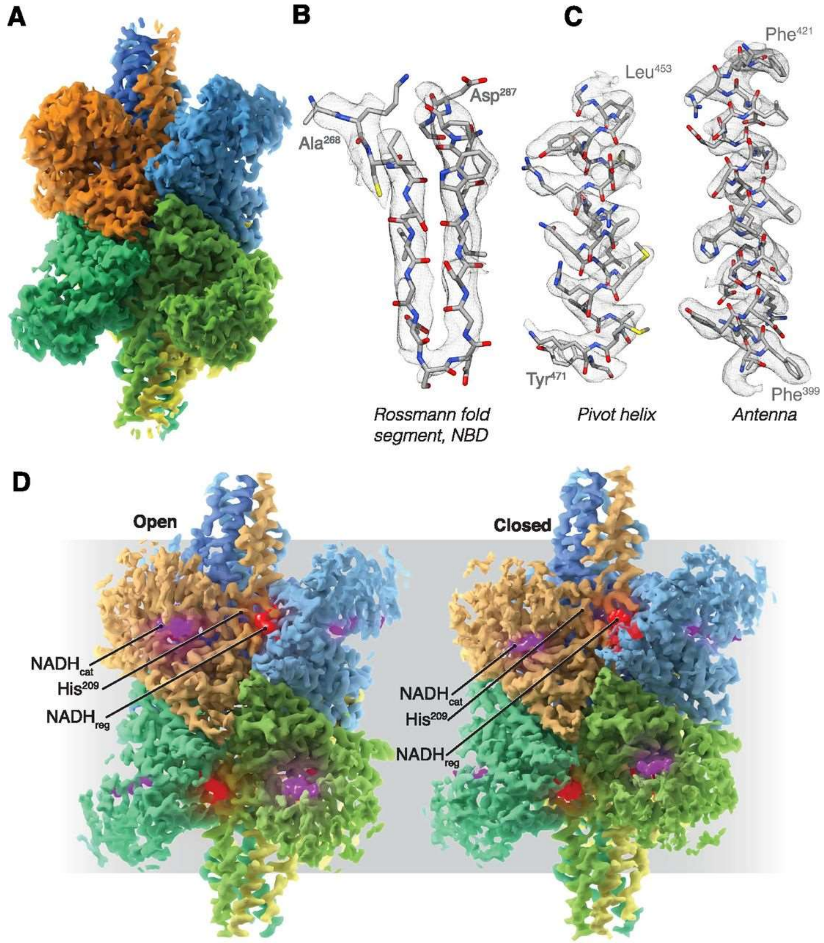


- **(A)** Examples of a TEM grid with irregular hole size foil (Lacey) or with defined hole diameter and spacing (Quantifoil).
- **(B)** An automated plunge-freezing device is commonly used for specimen vitrification. Sample is applied with a pipette at the surface of the cryo-EM grid and sample excess is removed by blotting with filter paper, followed by immediate freezing in liquid ethane. The specimen can be frozen on a grid with (i) or without (ii) a thin continuous film made of different materials. TEM grids with different grid mesh, foil and grid support materials can be used during specimen freezing.

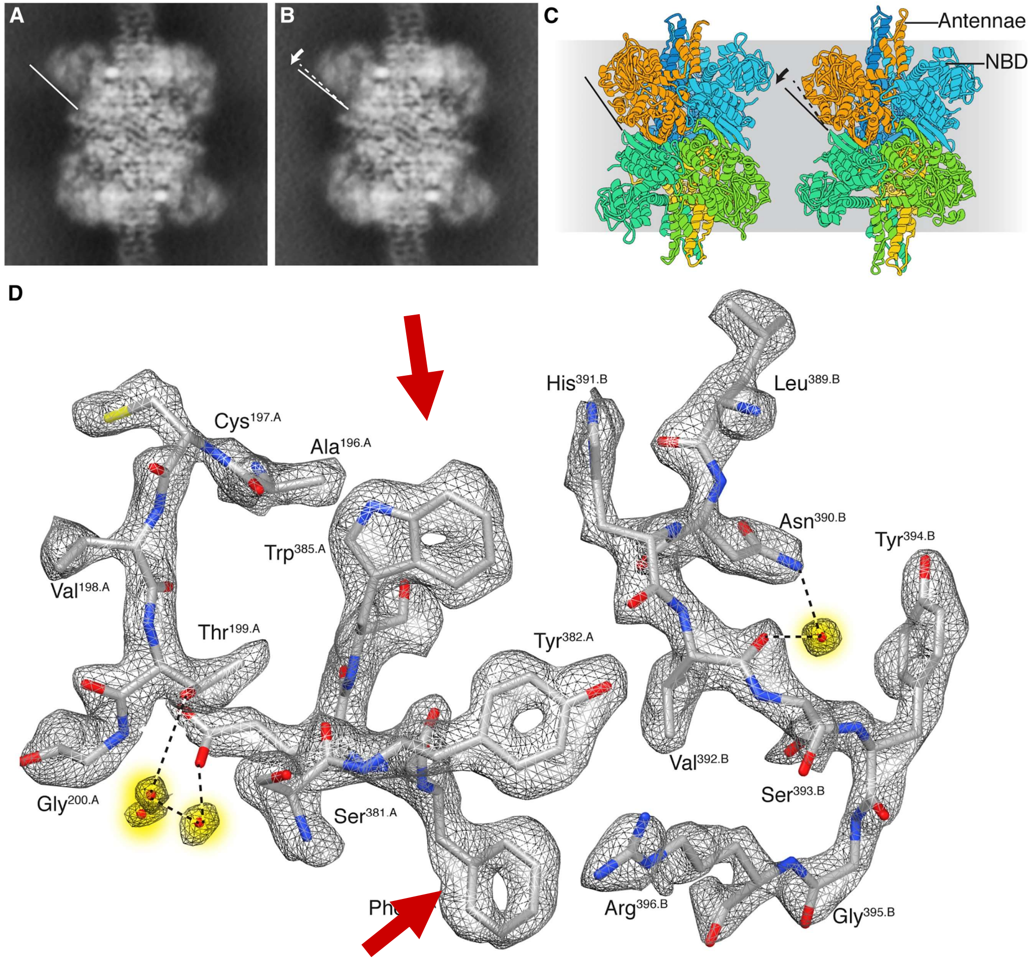
https://doi.org/10.3389/fmolb.2018.00074

Breaking cryo-EM resolution barriers





Cryo-EM structure of glutamate dehydrogenase (GDH) at 1.8 Å resolution



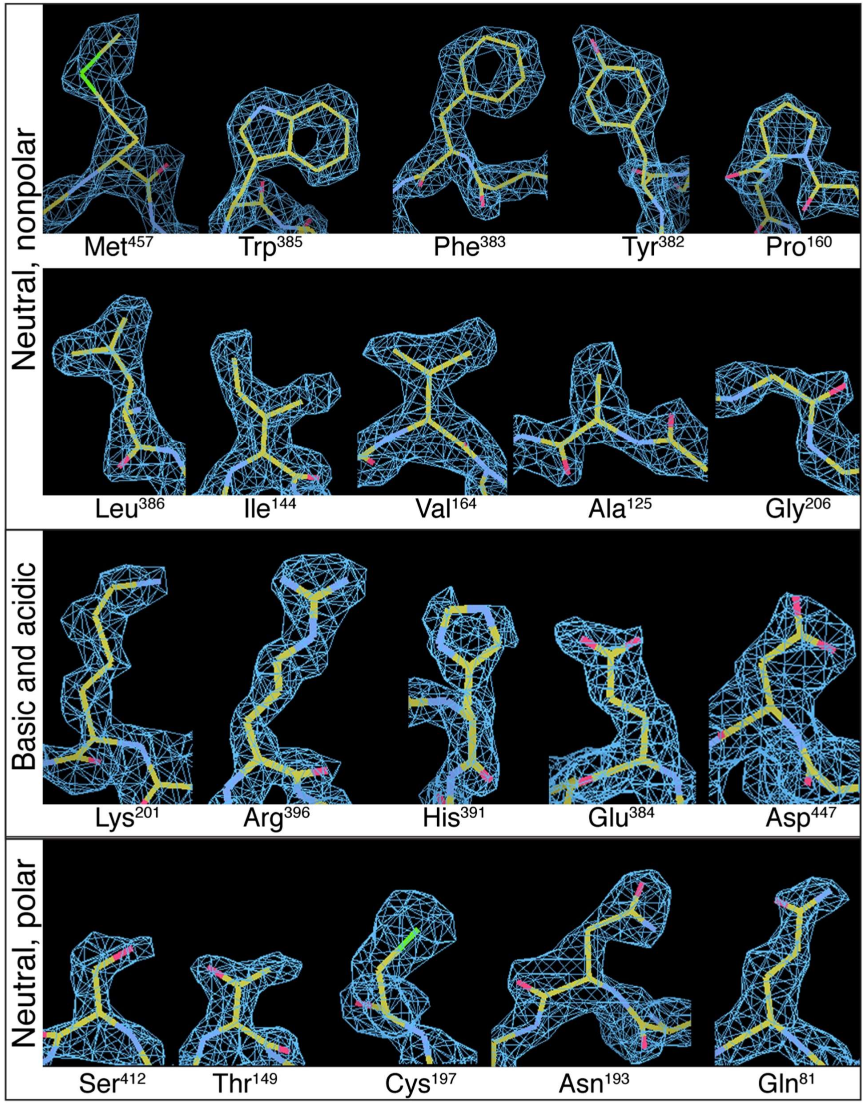
(A and B) Projection views of two 3D classes from cryo-EM analysis of GDH (glutamate dehydrogenase). In both classes, there is well-defined density at the core, but it is weakly defined at the peripheral nucleotide binding domain (NBD). The two classes display similar structures in the interior but differ slightly in the peripheral NBD. The two classes are likely to be subsets of a continuum of states with varying orientations for the outer domain relative to the core.

(C) Ribbon diagrams of the open and closed structures demonstrating the more extensive NBD movement associated with substrate binding and catalytic cleft closure.

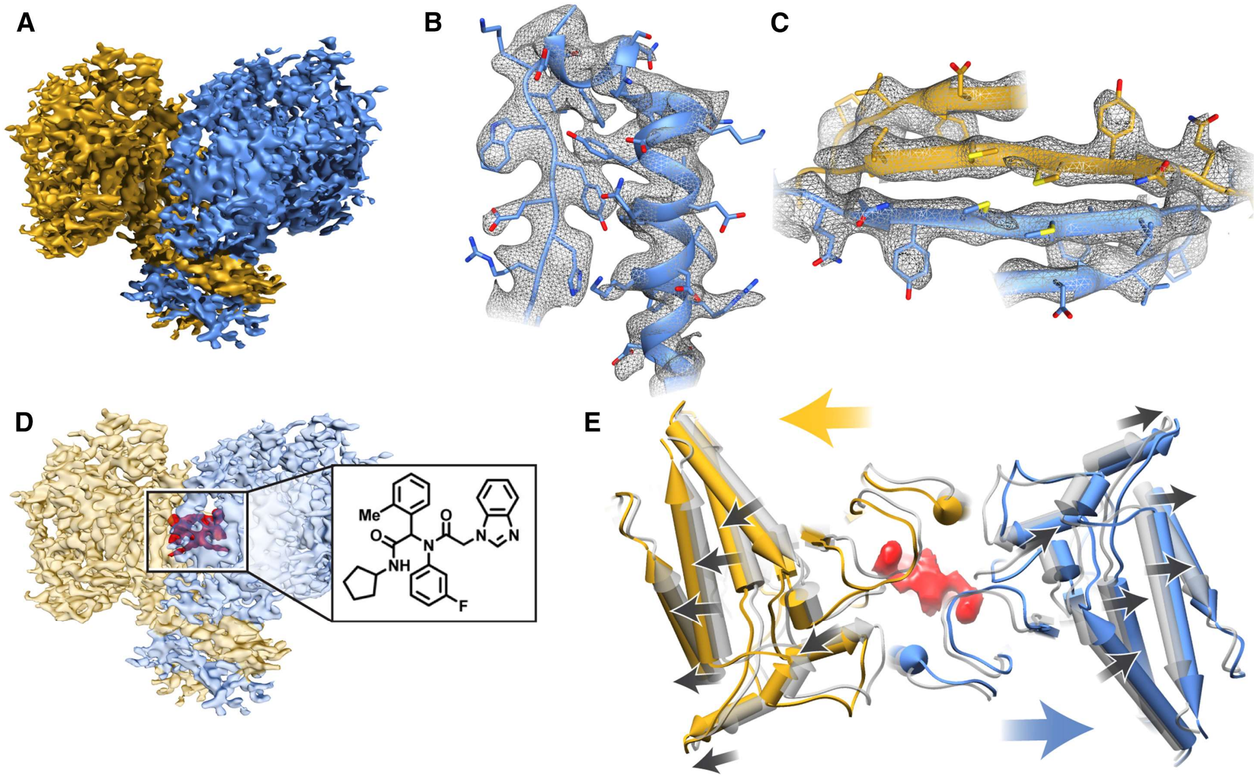
(D) A selected region of the cryo-EM map of the GDH structure, highlighting high-resolution features such as “holes” in the aromatic rings of Tyr382, Phe383, and Trp385, water molecules (shaded yellow), and well-resolved densities for carbonyl bonds.

https://doi.org/10.1016/j.cell.2016.05.040

Density representations for each of the 20 amino acid types from the 1.8 Å resolution cryo-EM structure of apo-GDH

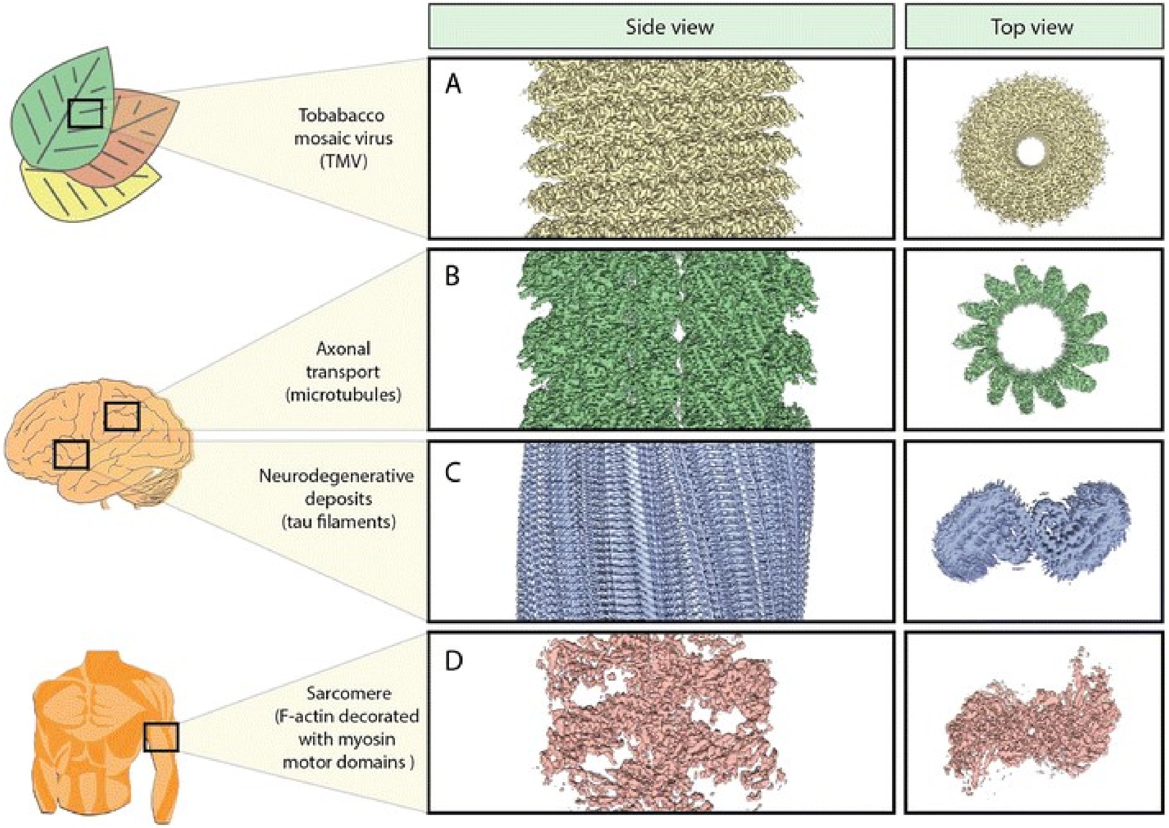


Cryo-EM analysis of isocitrate dehydrogenase (IDH1) in the absence and presence of the inhibitor ML309



(A) Cryo-EM map of the apo-IDH1 (isocitrate dehydrogenase) dimer, colored by subunit.
(B and C) Selected regions of the IDH1 map demonstrating density for side-chains in an α-helical region (B) and a β sheet region (C).
(D) Cryo-EM map of IDH1 in complex with ML309 showing density (red) for the inhibitor (inset) close to the dimer interface.
(E) Superposition of the apo- (gray) and ML309-bound (yellow/blue) IDH1 structures shows the outward movement of the subunits with ML309 binding (density shown in red). The black arrows indicate the direction of the changes in tertiary structure while the yellow and blue arrows show the overall movements at the level of quaternary structure.

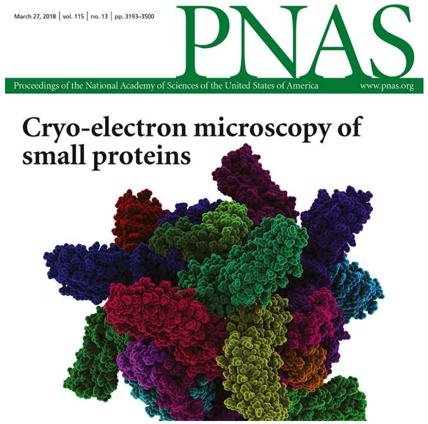
Cryo-EM of filamentous proteins

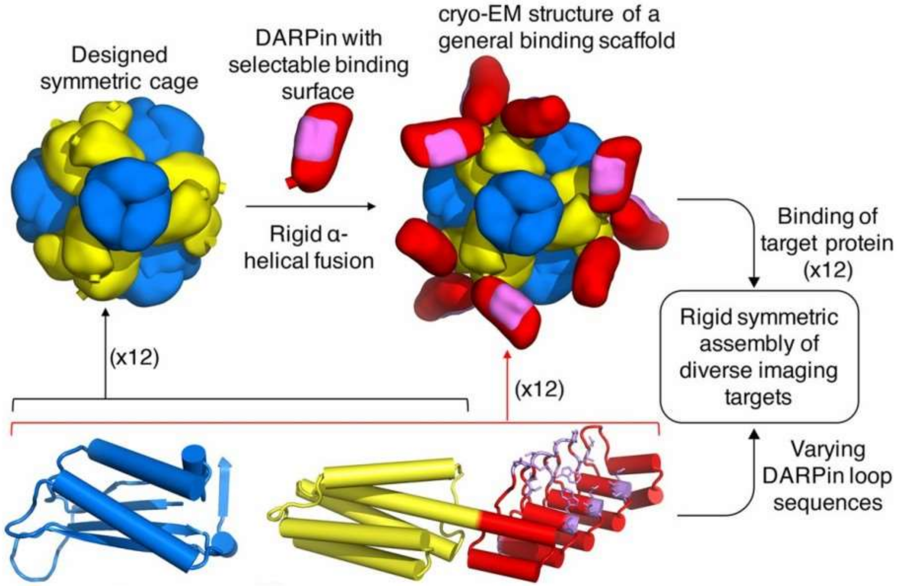


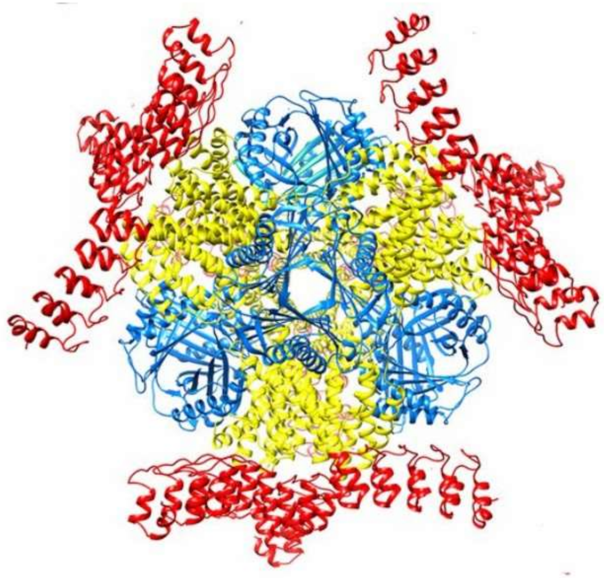
(A) Tobacco mosaic virus is one of the most widespread viruses around the world, being a prime example for plant pathogens that can have far-reaching consequences for the economy as well as food supply. (B) Microtubules are not only core components of the cytoskeleton, but are also essential in axonal transport along neurons, constituting the track for cargo-transporting motor proteins. (C) Tau filaments are neurodegenerative deposits that are found in the brains of AD patients. (D) The interaction of F-actin and myosin filaments is responsible for muscle contraction. Malfunctions can cause myopathies.

Imaging of small proteins displayed on protein scaffolds

- New electron microscopy (EM) methods are making it possible to view the structures of large proteins and nucleic acid complexes at atomic detail, but the methods are difficult to apply to molecules smaller than approximately 50 kDa.
- This limit can be successfully visualized when it is attached to a large protein scaffold designed to hold 12 copies of the attached protein in symmetric and rigidly defined orientations.







Liu *et al.*, PNAS 115: 3362-3367 (2018)

CEITEC Cryo-Electron Microscopy Core Facility

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Cryo-Electron Microscopy and Tomography Core Facility

- Profile
- News
- People
- Publications
- Equipment
- Jobs
- Partners' equipment

Booking systems
Direct access to booking system for registered users

Registration
Registration to booking system for unregistered users

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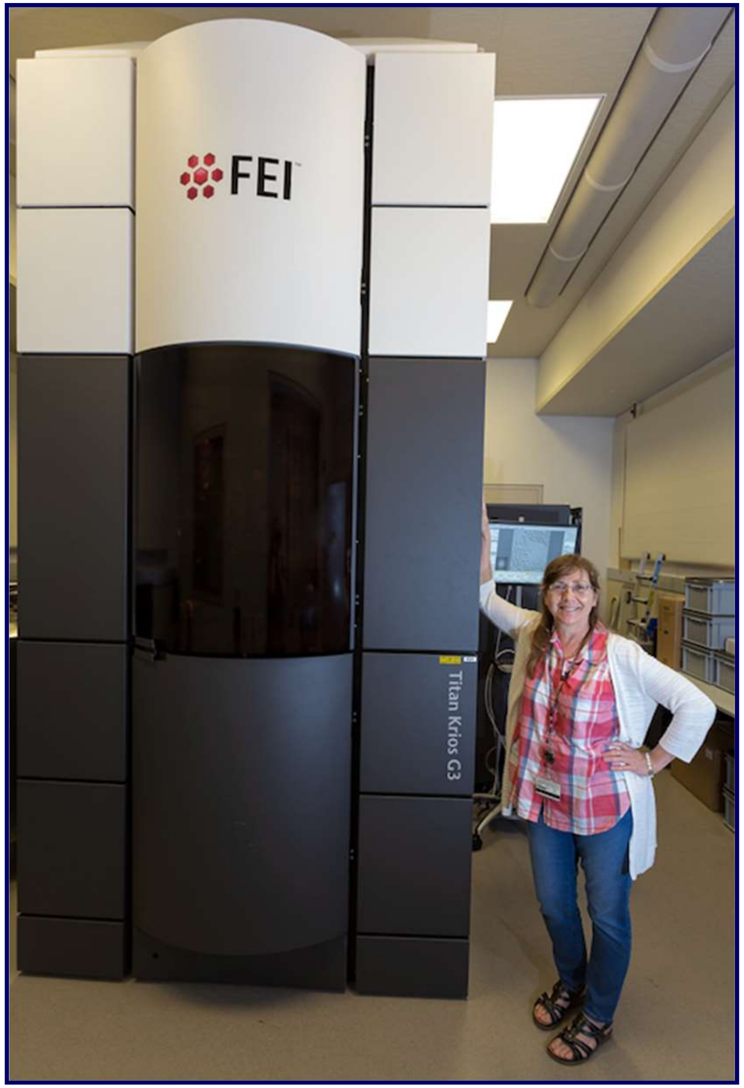
Main Activity

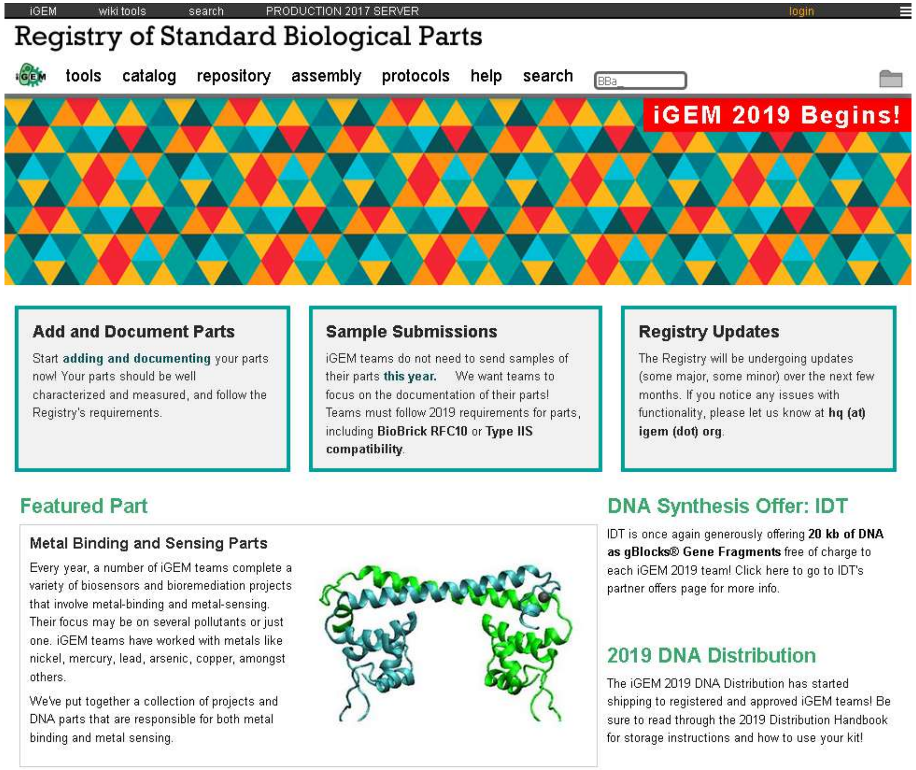
A high-end single particle cryo-EM microscope (3 metres tall)



Cryo-EM: take home message

- Cryo-EM can be used to determine structures at native state
- Prepare best sample possible before EM
- Use negative staining: initial screening, homogeneity assessment
- Use the high-tech technologies (microscope, camera & software)
- Be patient! And you will get your high-resolution structure with cryo-EM

Structure-based engineering and design of parts



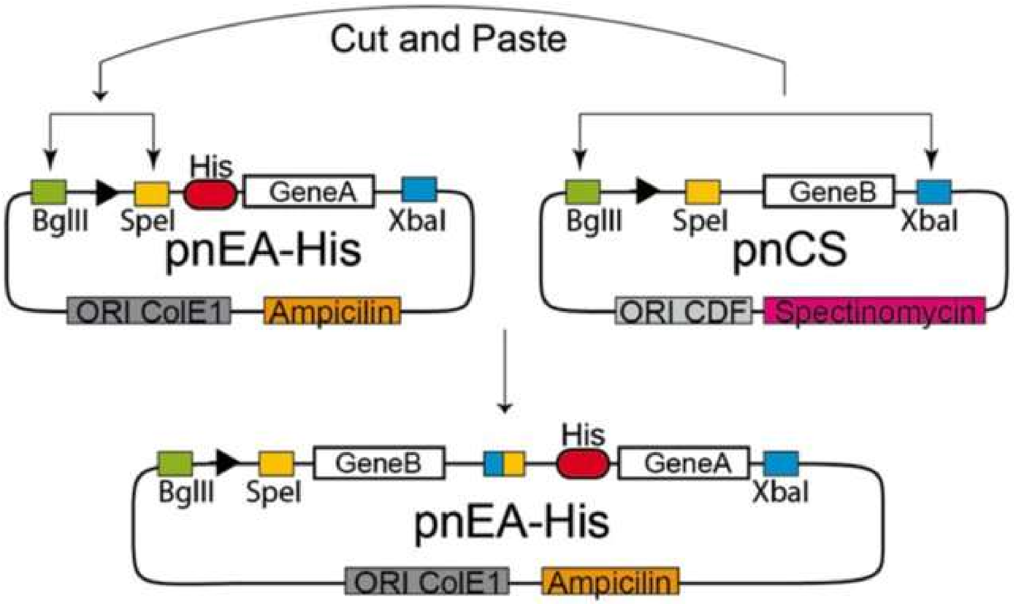
- Until now, structural biology has been mainly used in synthetic biology approaches to design new parts/components and tools
- Many of the characterized components can be found in the Registry of Standard Biological Parts (http://partsregistry.org/)
- Of special importance is the recently developed "SYNZIP protein toolbox" because it contains a complete biophysical quantitative description (i.e., affinities) of synthetic domains

Multi-expression technologies

Concepts
Methods
Applications

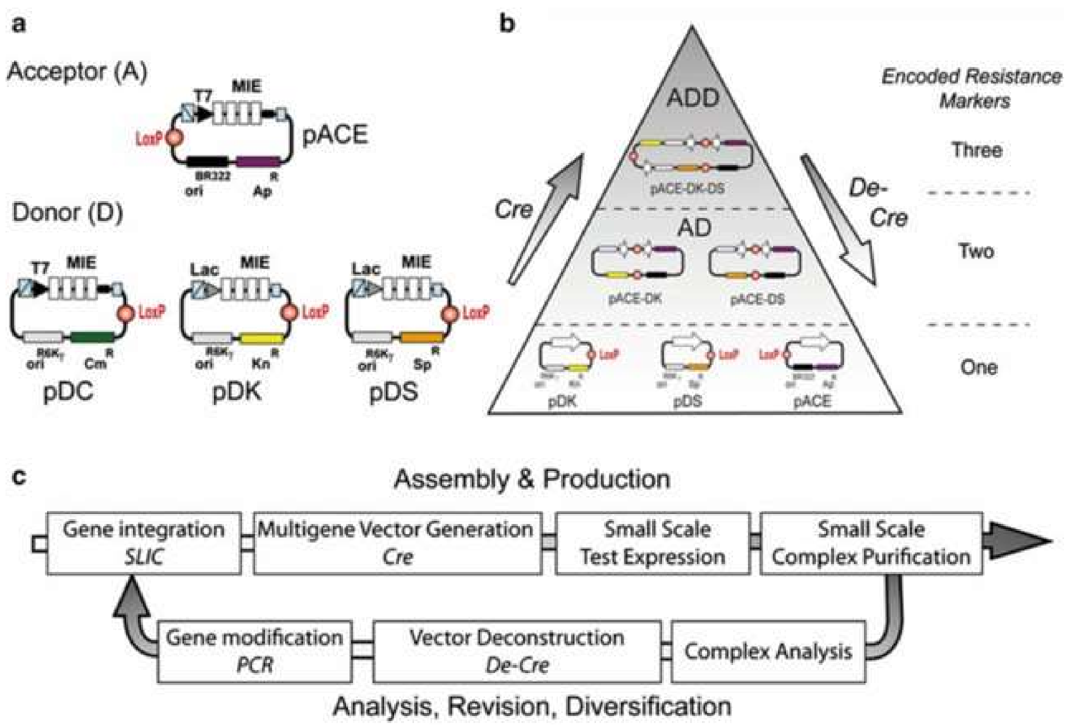
Co-expression of protein complexes in *E. coli*

The functional units within cells are often macromolecular complexes rather than single species. Production of these complexes as assembled homogenous samples is a prerequisite for their biophysical and structural characterization and hence an understanding of their function in molecular terms. Co-expression in *Escherichia coli* can decipher the subunit composition, assembly, and production of whole protein complexes.



- Example of the concatenation of two vectors of the pET-MCN vector series: pnEA-His (vector encoding an N-terminal poly-histidine tag in front of protein A) and pnCS (vector expressing the native protein B).
- The pnEA-His (acceptor vector) is linearized by removing with the restriction enzymes BglII and SpeI part of its promoter (T7 promoter and lacO).
- The full promoter of the pnCS (donor vector) is cut out with the restriction enzymes BglII and XbaI. After ligation of the pnCS promoter with the linearized pnEA-His vector, a new vector is obtained based on the backbone of the pnEA-His vector and whose promoter controls the genes of proteins A and B.

ACEMBL system for multiprotein production in *E. coli*

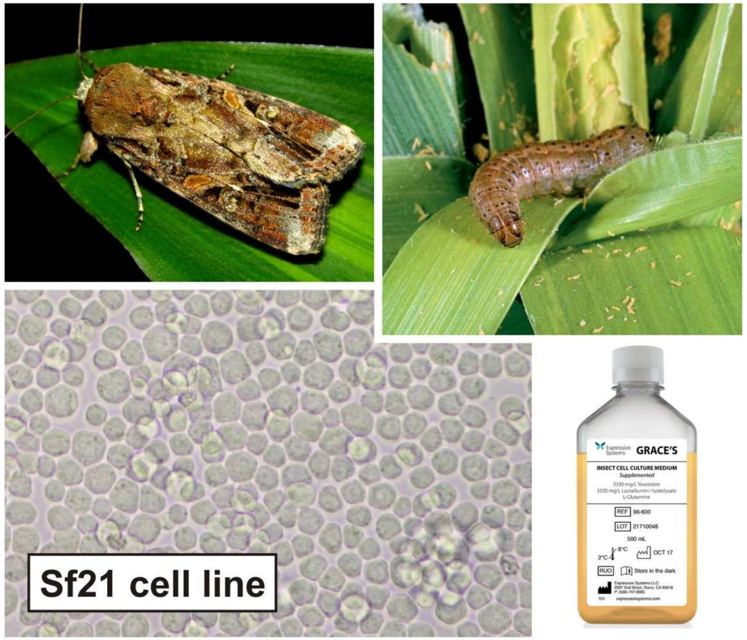


- (a) Donor and acceptor vectors contain LoxP sequences and a multiple insertion elements (MIE) for inserting one or several genes of interest. Acceptors have regular replicons (BR322). Donors have a conditional origin of replication derived from R6Kγ phage. Antibiotic resistance makers are as follows: Ap ampicillin, Cm chloramphenicol, Kn kanamycin, and Sp spectinomycin. Genes of interest are inserted into acceptor or donor vectors into the MIEs by SLIC.
- (b) Incubation of acceptor and donor constructs with Cre recombinase results in all combinations of fusions including acceptor-donor (AD) and acceptor-donor-donor (ADD) fusions.
- (c) The ACEMBL HT pipeline. Genes are integrated by ligation-independent methods (SLIC) followed by combinatorial multi-gene vector generation using Cre-LoxP fusion (tandem recombineering, TR), followed by protein expression and analysis of purified complex.

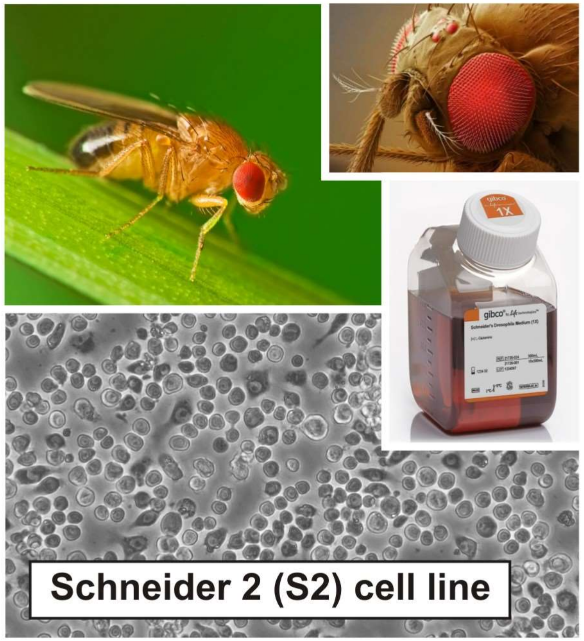
Production of protein complexes in insect cells

- Insect cells can efficiently express recombinant biologically active proteins and are mostly used for the development of virus-like particles and vaccines. It has been proven that insect cells are excellent platforms for the production of recombinant antibodies
- There are mainly three insect expression systems: baculovirus expression vector system (BEVS), InsectSelect (IS) system and *Drosophila* expression system (DES)

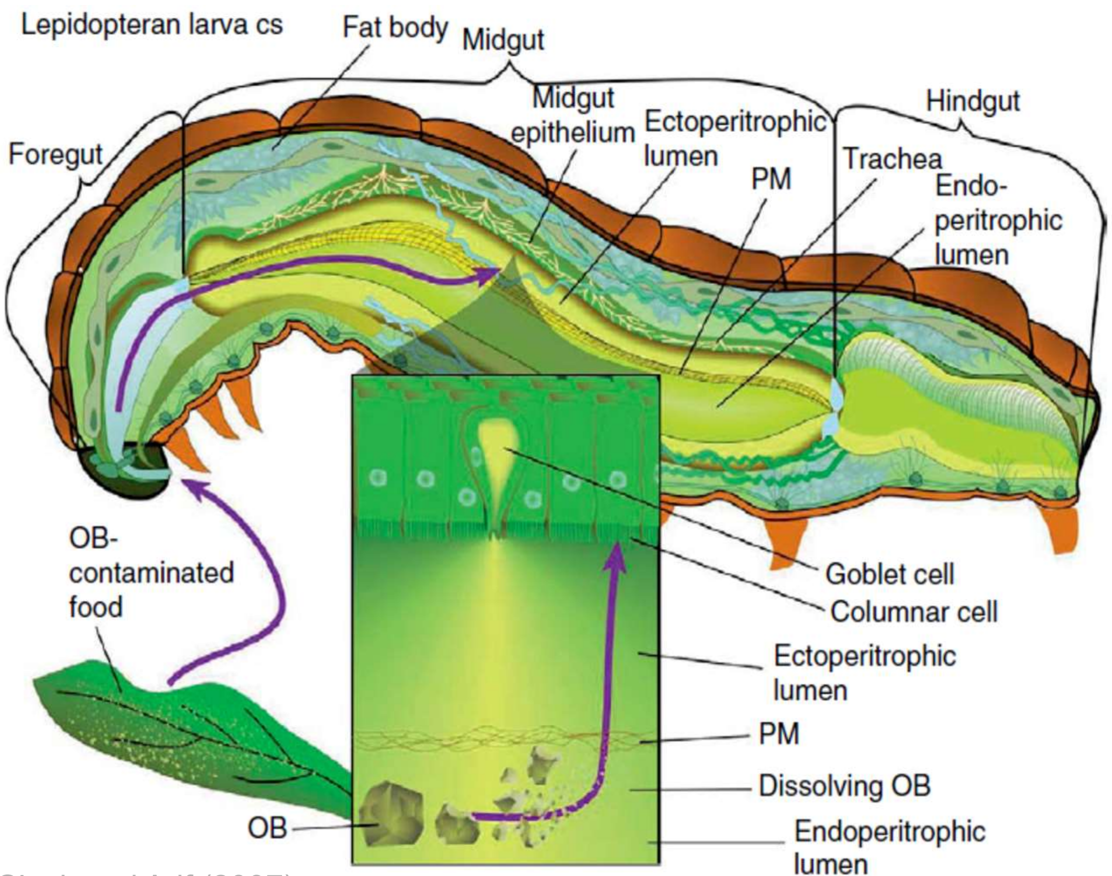
***Spodoptera frugiperda*, a fall army worm**



Drosophila melanogaster



Introduction to baculovirus biology

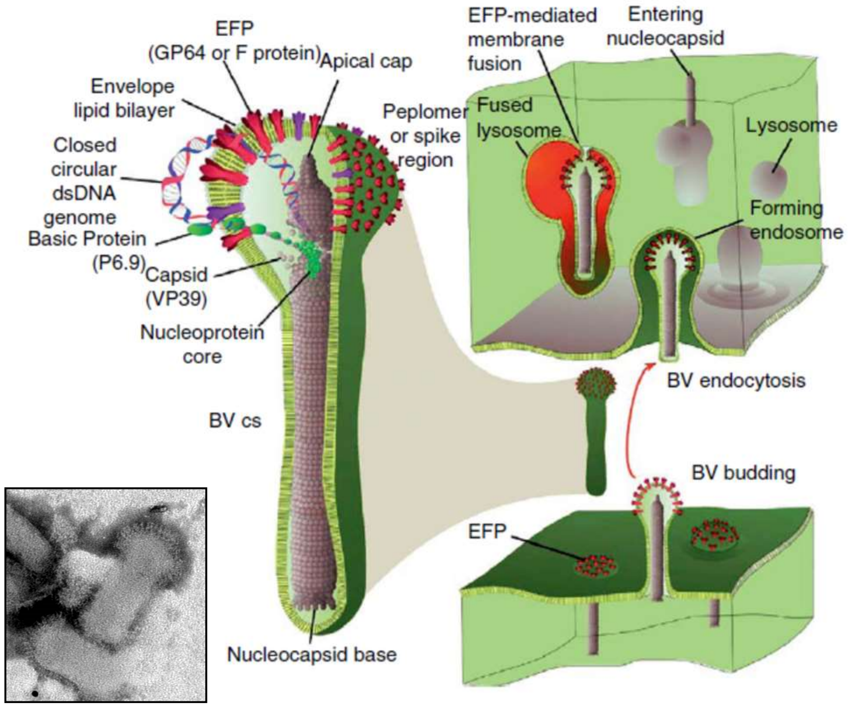


Slack and Arif (2007)

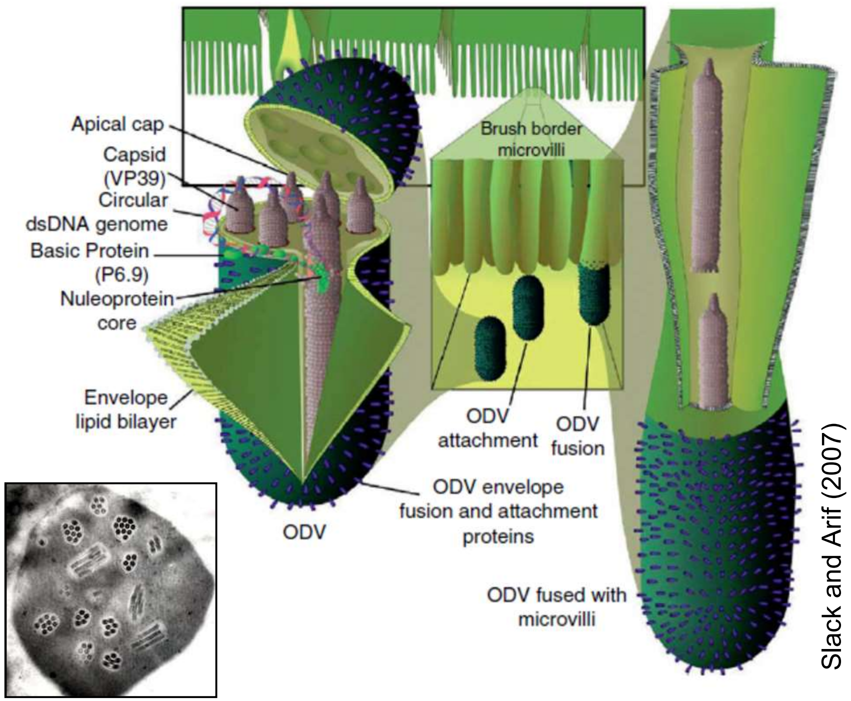
- Insect-infecting enveloped DNA viruses
- Baculoviruses are a very diverse group of viruses with double-stranded, circular, supercoiled genomes, with sizes varying from about 80 to over 180 kb, that encode between 90 and 180 genes
- The genome is packaged in rod-shaped nucleocapsids that are 230-385 nm in length and 40–60 nm in diameter
- In the most well characterized baculoviruses, the virions are present as two types, occluded virions (ODV) and budded virions (BV).
- Although these two types of virions are similar in their nucleocapsid structure, they differ in the origin and composition of their envelopes and their roles in the virus life cycle

Baculovirus morphogenesis: two types of virions

Budded virus (BV)

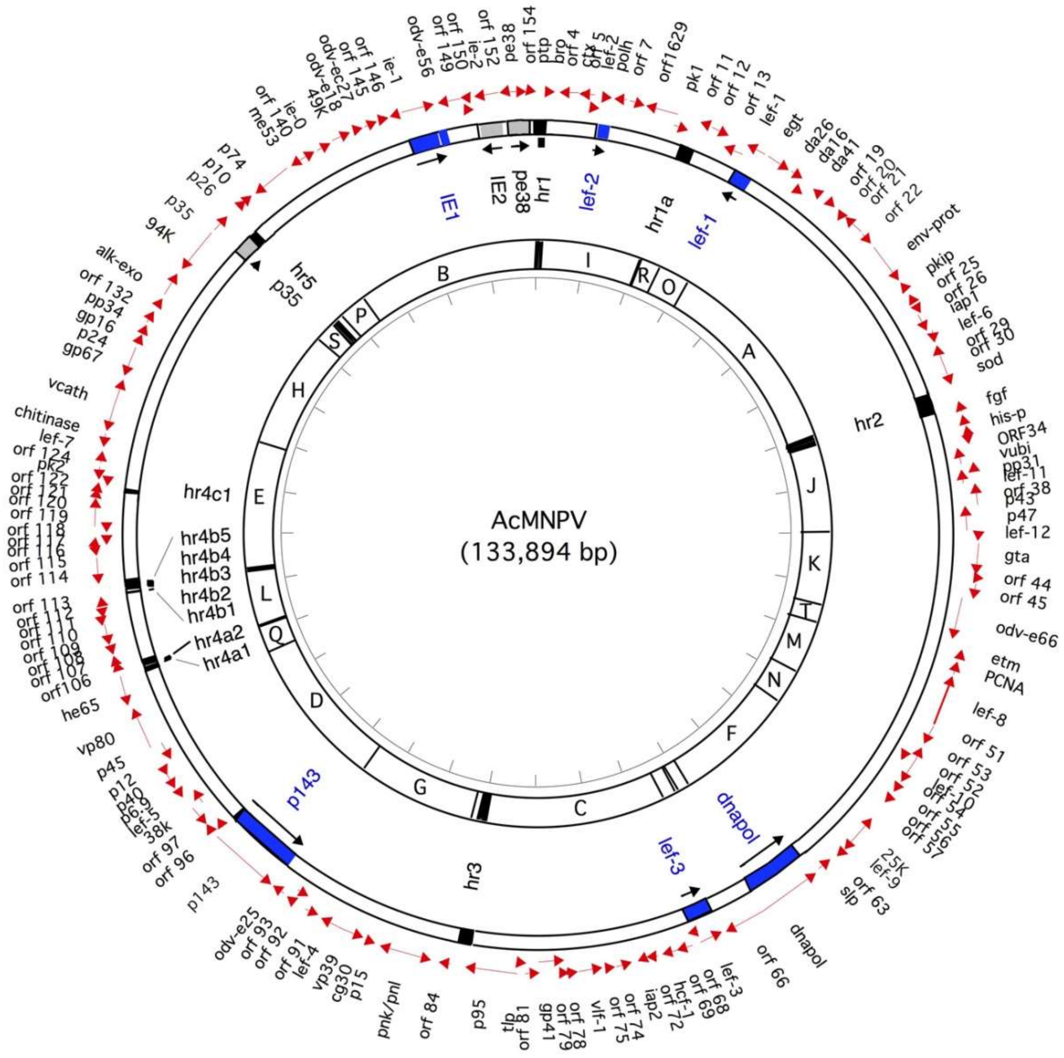


Occlusion-derived virus (ODV)



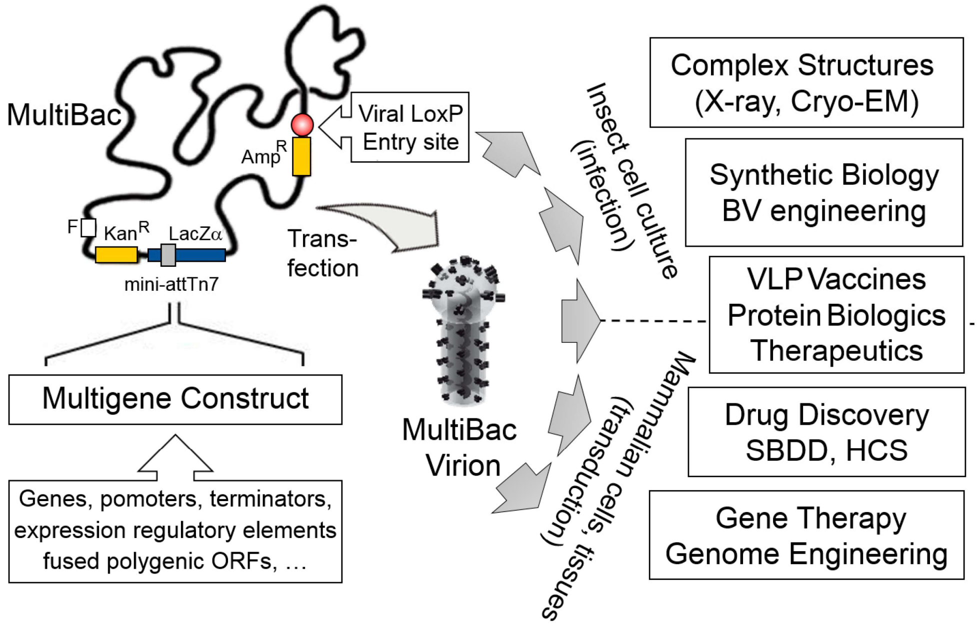
Budded virus (BV)	Occlusion-derived virus (ODV)
Circular dsDNA genome and associated proteins Capsid shell (VP39, etc.)	Circular dsDNA genome and associated proteins Capsid shell (VP39, etc.)
BV envelope (GP64, F)	ODV envelope (P74, ODV-E66, PIFs etc)
	Tegument (GP41), occlusion body (Polyhedrin)

The baculovirus genome



- Circular, supercoiled dsDNA genomes, with sizes varying from about 80 to over 180 kb, that encode between 90 and 180 genes
- The genome is packaged in rod-shaped nucleocapsids that are 230-385 nm in length and 40–60 nm in diameter

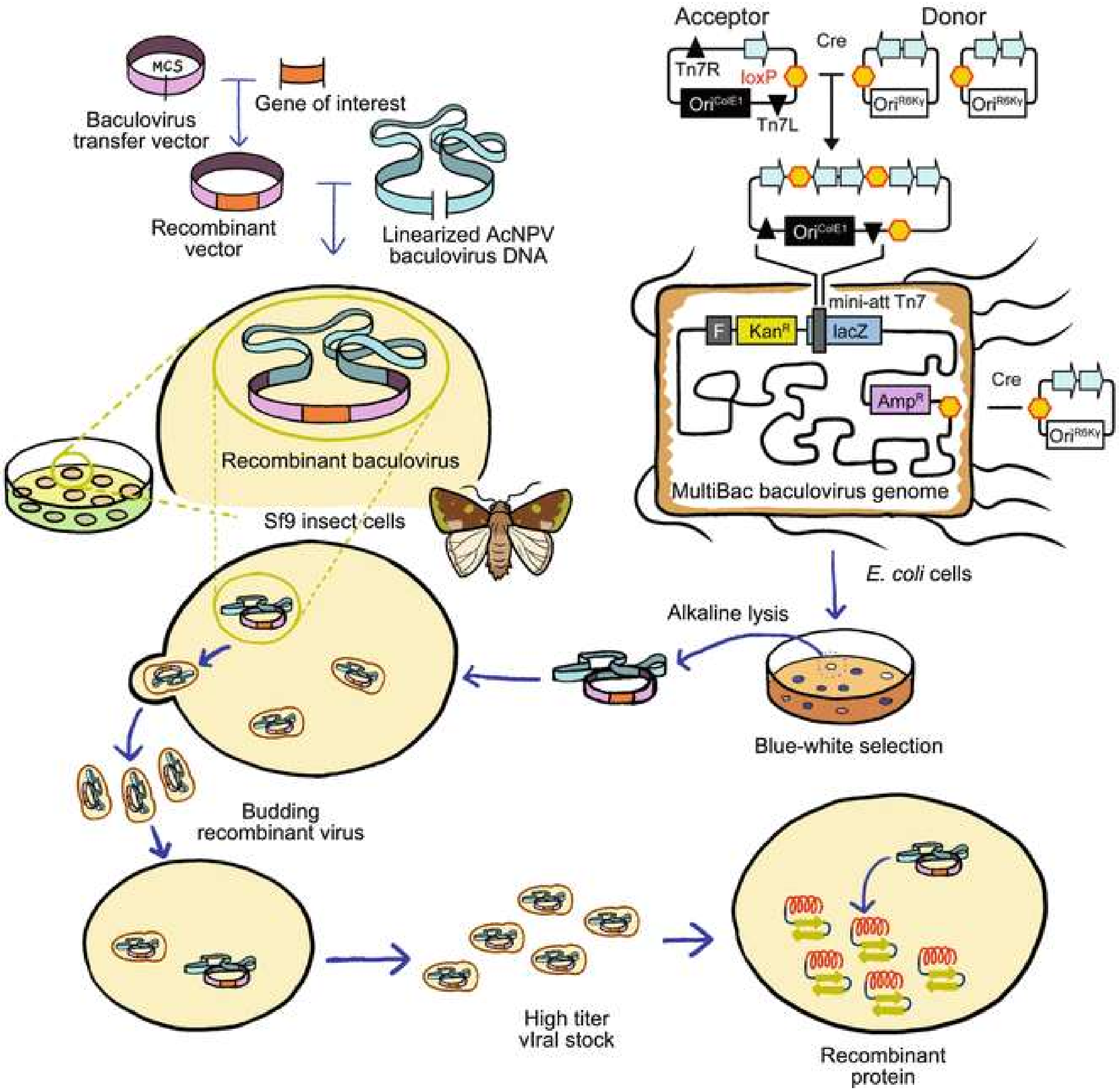
The MultiBac system



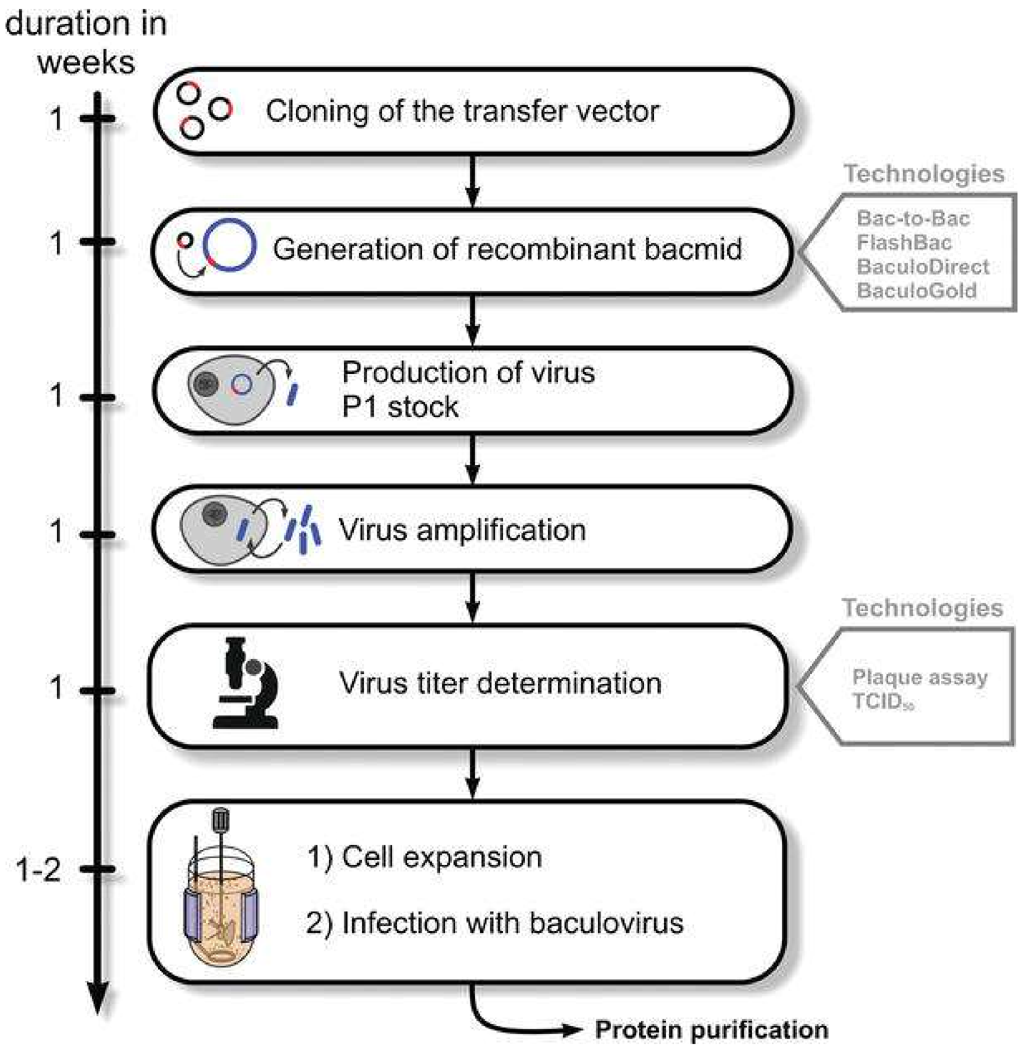
Prof. Imre Berger
School of Biochemistry & Bristol Synthetic Biology Centre, University of Bristol, UK

- MultiBac consists of a baculoviral genome optimized for multigene delivery and protein complex expression (left).
- The genome is present as a bacterial artificial chromosome (BAC) in *E. coli* cells supplying the Tn7 transposase.
- Expression cassettes are assembled into the multi-gene expression constructs and inserted into the MultiBac genome by Tn7 transposition.
- A second entry option into the viral backbone is provided distal from the Tn7 site, relying on Cre recombinase catalysed site-specific integration into a LoxP sequence (circle filled in red).
- Composite MultiBac baculoviral DNA containing all DNA elements of choice is extracted from *E. coli* cultures, followed by transfection into insect cell cultures to manufacture functional MultiBac virions.
- These are then used for a wide range of applications (right), by the infection of insect cell cultures or transduction of mammalian cells, tissues and organisms.

Application of MultiBac system to large complexes

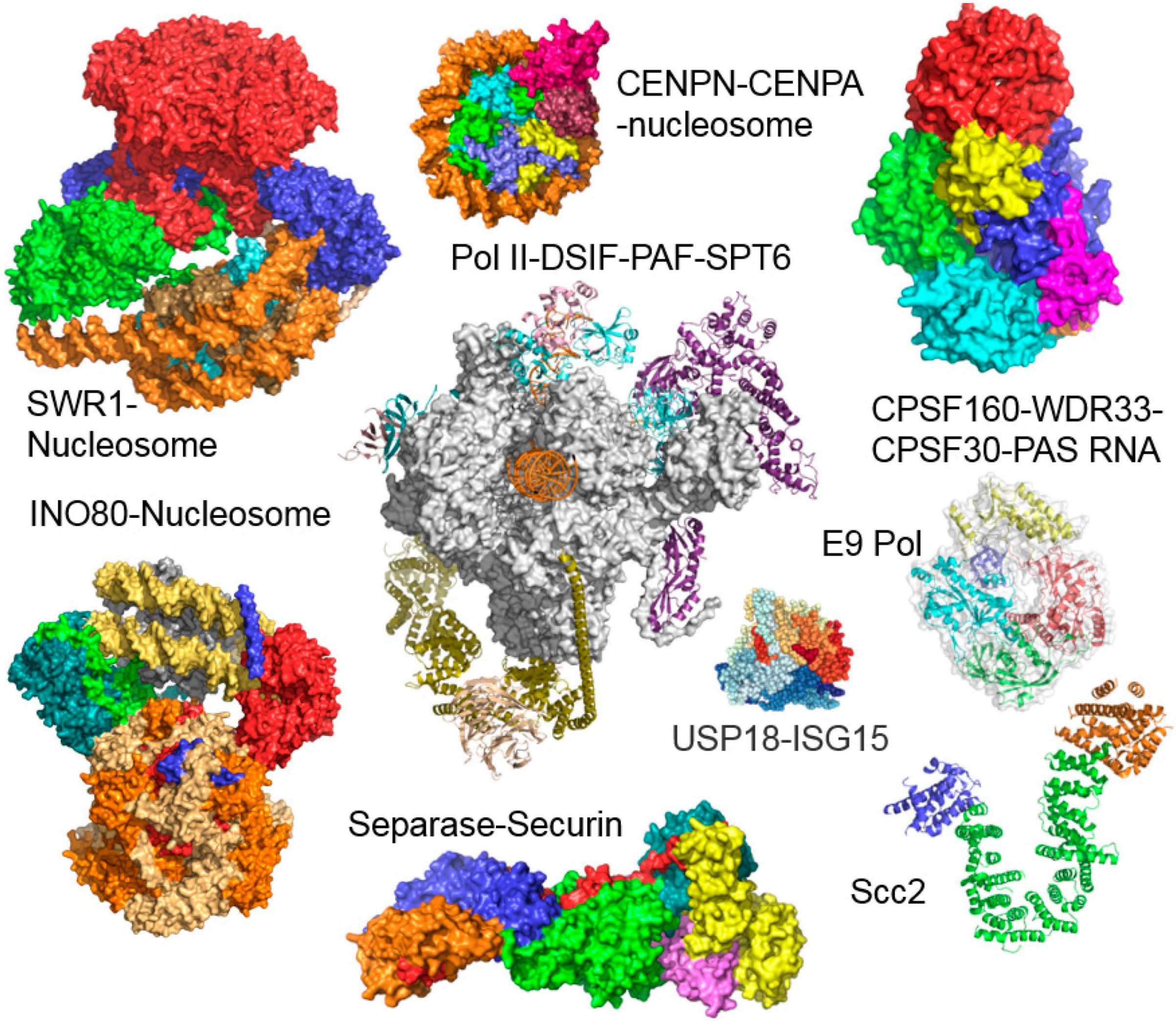


Workflow of protein production using BEVS



https://www.intechopen.com/books/new-insights-into-cell-culture-technology/process-optimization-for-recombinant-protein-expression-in-insect-cells

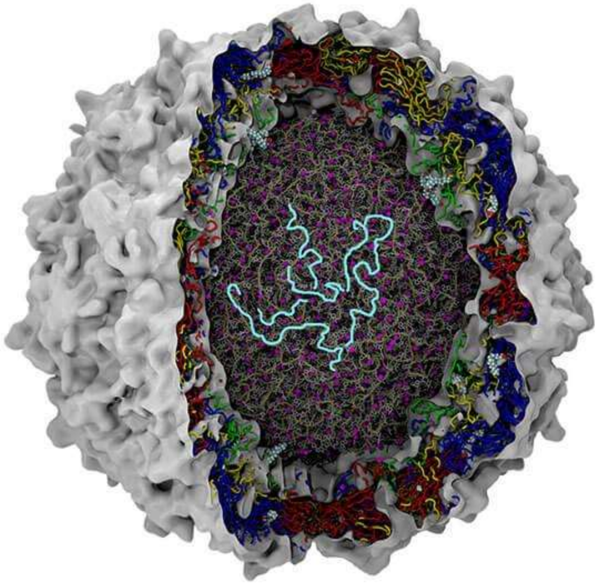
The MultiBac system: successful stories



- Chromatin remodeling enzymes SWR1 (14 subunits) and INO80 (11 subunits)
- The yeast polII DSIF-PAF-Spt6 cryo-EM structure
- The CENPN-CENPA-nucleosome complex
- The human CPSF-160–WDR33–CPSF-30–PAS RNA quaternary complex
- The E9 polymerase
- The USP18-ISG15 complex
- The Separase-Securin complex
- The cohesion loader Scc

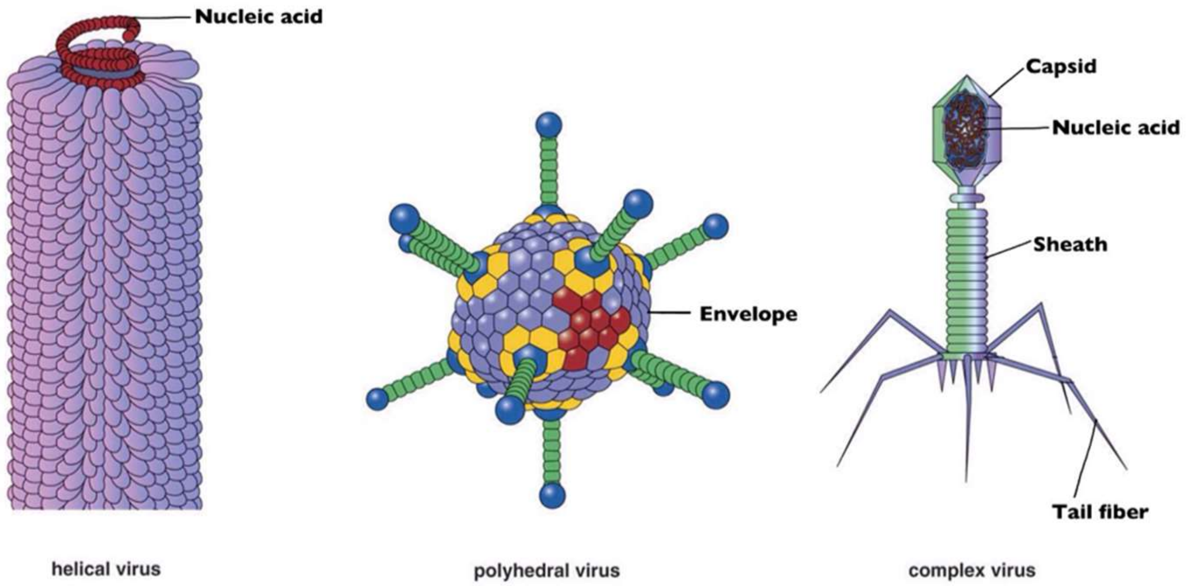
Synthetic virology, immunotherapy & gene therapy

Concepts
Methods
Applications



What are viruses?

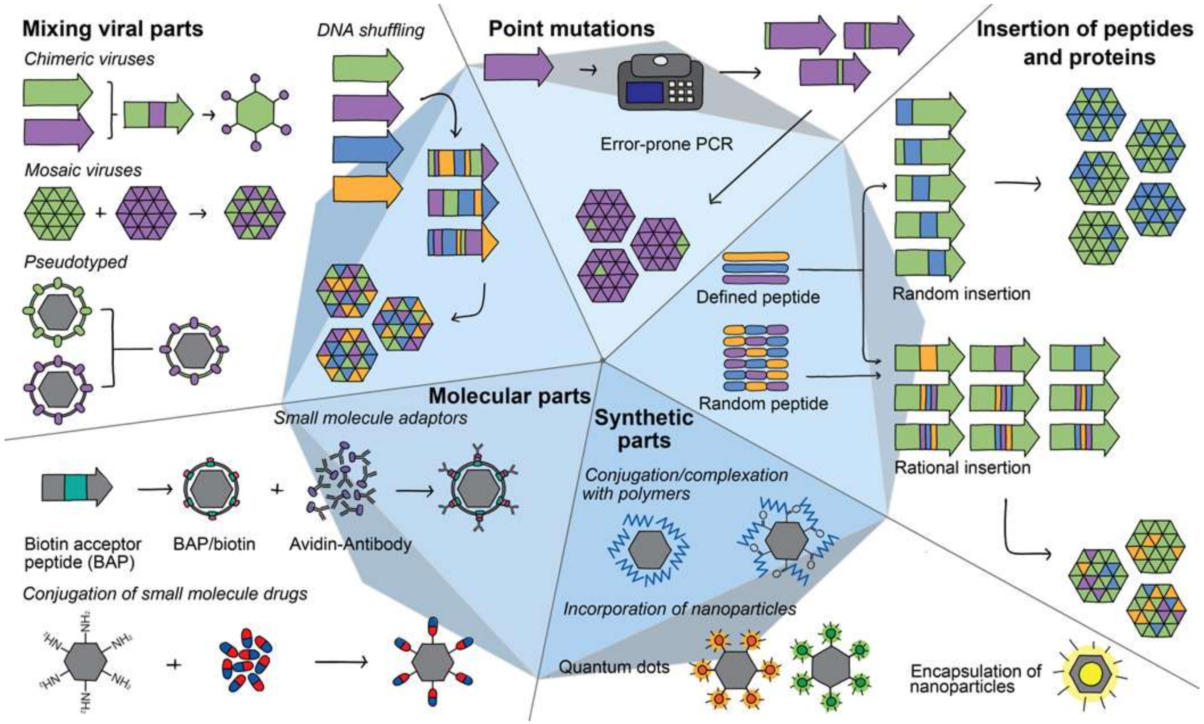
- A **virus** is an infectious particle that reproduces by "commandeering" a host cell and using its machinery to make progeny viruses
- A virus is made up of a RNA or DNA genome inside a protein shell called a **capsid**. Some viruses have an external membrane **envelope**
- Viruses are very diverse. They come in different shapes and structures, have different kinds of genomes, and infect different hosts
- Viruses reproduce by **infecting** their host cells and reprogramming them to become virus-making "factories"



Constructing *de novo* synthetic viruses for biomedicine

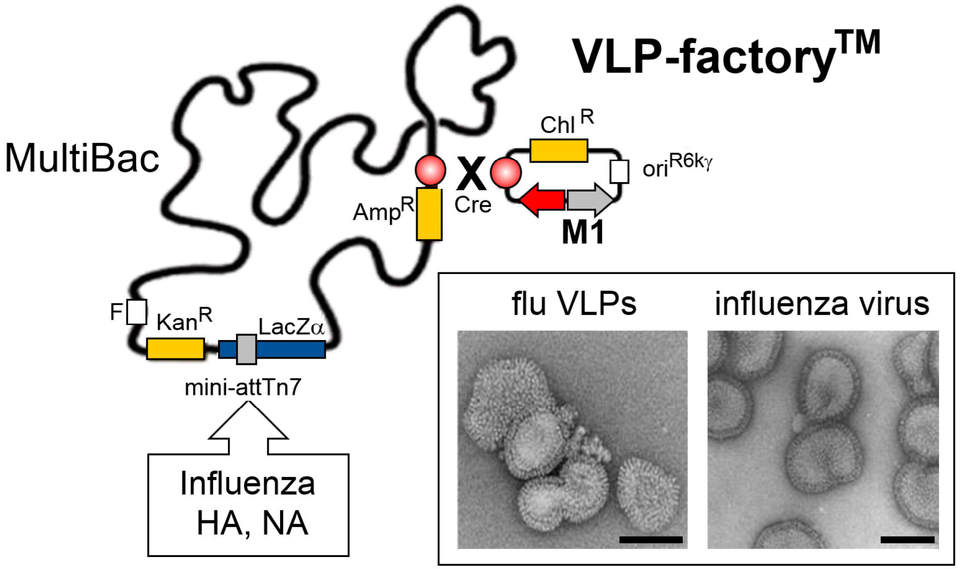
- Synthetic virology aims to reprogram naturally occurring viruses into controllable and predictable devices
- The field can be divided into two main endeavours:

  1) engineering of the virus capsid (immunotherapy, vaccines)
  2) engineering of the genetic programs encoded by the viral genome (gene therapy)

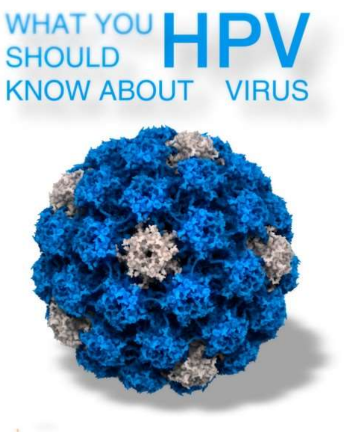


Synthetic viruses can be created by mixing pre-existing viral parts, resulting in formation of chimeric or mosaic capsids. Molecular parts, such as biotin or small molecule drugs, can be attached to virus capsids to act as adaptors or to carry out therapeutic action, respectively. Synthetic parts, such as man-made polymers and inorganic nanoparticles, can be incorporated into viruses to endow functionalities new to viruses in general. Genetically encoded peptides and proteins can be inserted into viruses, either in rationally chosen sites or randomly throughout the capsid, to impart new functions. Finally, viral properties can be altered through introduction of point mutations scattered throughout the capsid or concentrated in specific capsid domains.

The MultiBac: a factory for synthetic virus-like particles



- A plasmid module comprising expression cassettes for the capsid-forming influenza H1N1 M1 protein (colored in grey) and a fluorescent protein marker, mCherry (colored in red), was introduced into the MultiBac baculoviral genome by Cre recombinase enzyme mediated plasmid fusion into the LoxP site (circle filled in red, gradient)
- Co-expression of HA, NA and M1 yields synthetic influenza virus-like particles (VLPs) resembling live influenza virus

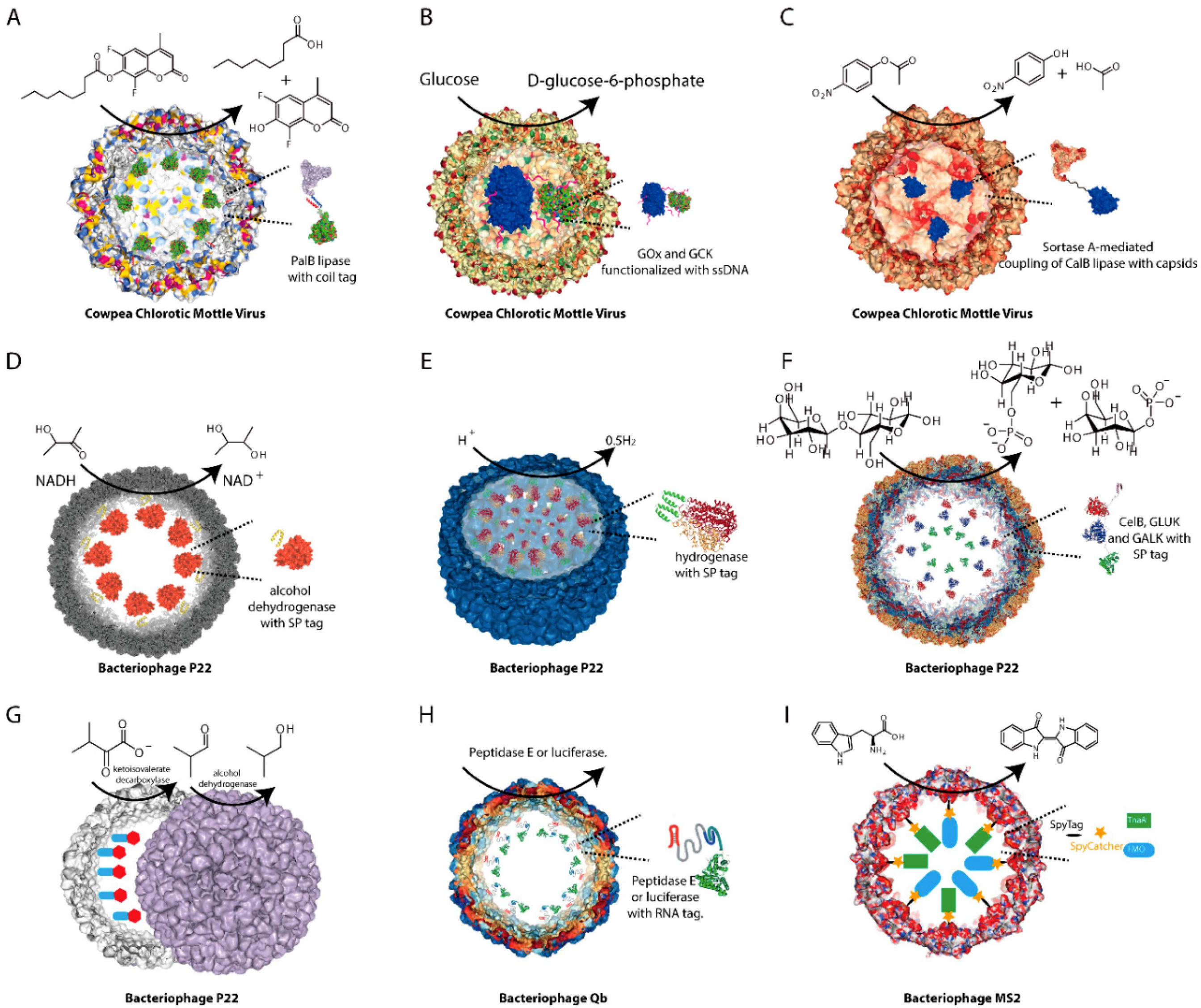


Human papillomavirus-based vaccine produded by baculovirus expression system

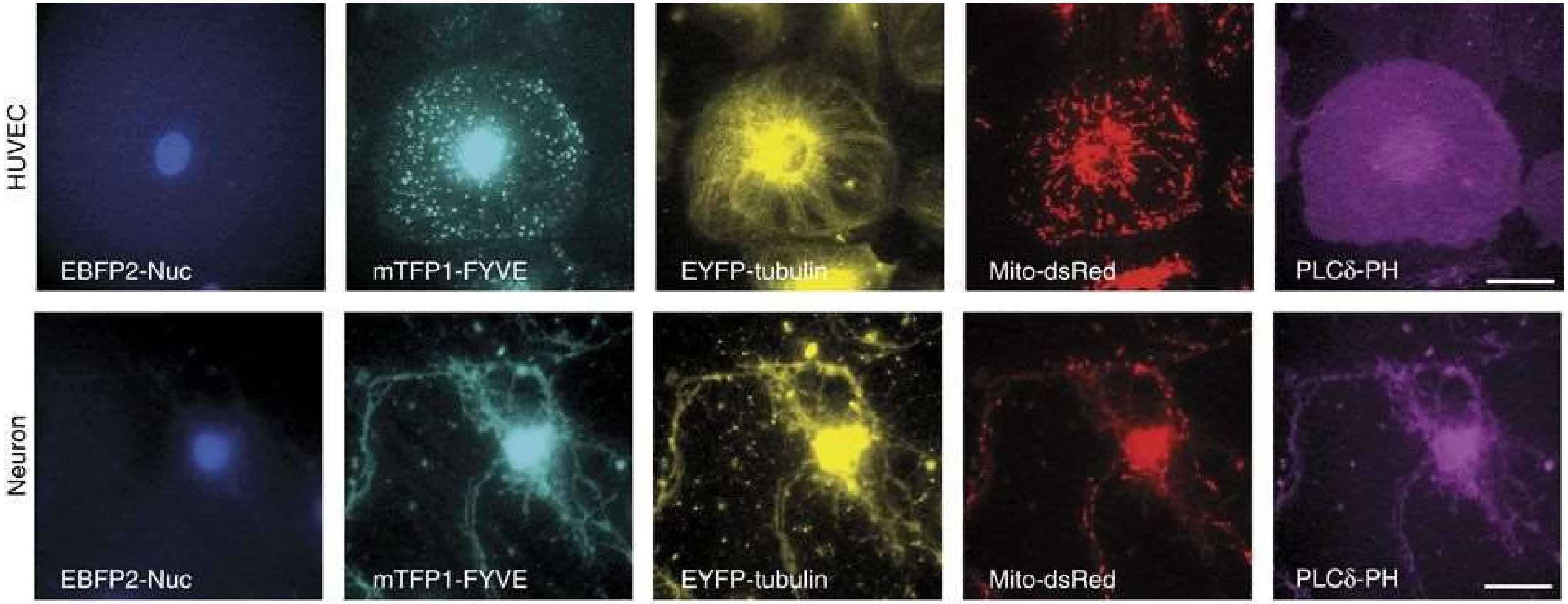
Selected vaccines produced by BEVS

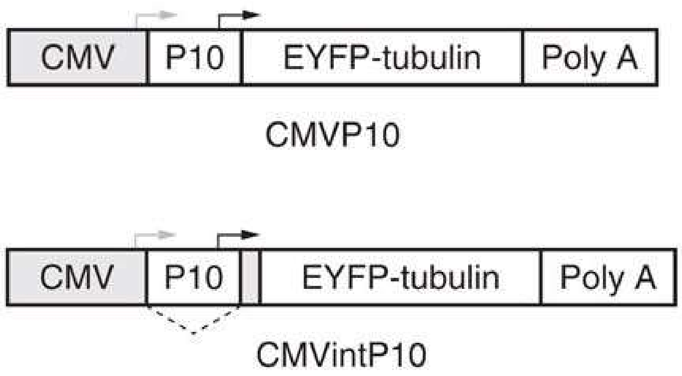
Application	Product name	Company	Stage	References
For human use				
Cervical cancer	CERVARIX®	GSK	Approved	[27]
Prostate cancer	PROVENGE®	Dendreon	Approved	[28]
Influenza	FluBlok®	Protein Sciences	Approved	[29, 30]
Influenza	A/H5N1 Virus-like particle	Novavax	Phase I (NCT01596725)	[31]
For veterinary use				
Procrine circovirus 2 (PCV2)	Porcilis® PCV	Merck	Approved	[32]
PCV2	CircoFLEX®	Boehringer Ingelheim	Approved	[33]
Swine fever	Porcilis Pesti®	Merck	Approved	[34]

Viral capsid-based nanocontainers as nanoreactors



Baculovirus-mediated multigene delivery in cells

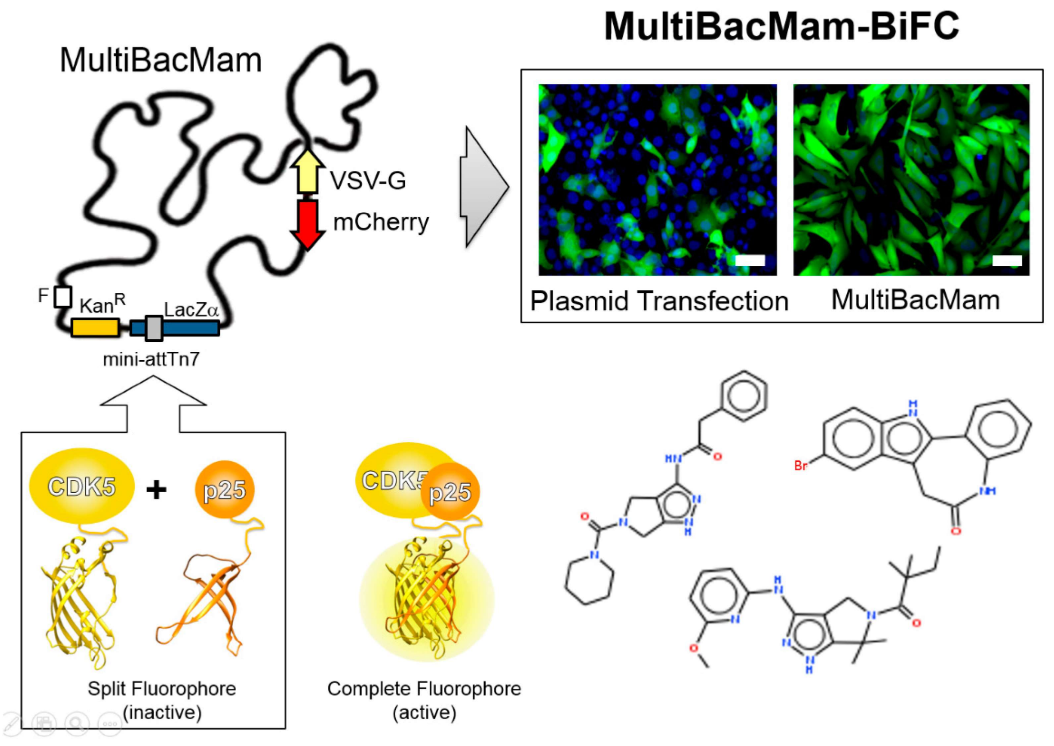




- HUVEC and REF cells were infected with a MultiPrime baculovirus encoding EBFP2-Nuc (labelling the nucleus), mTFP1-FYVE (PI-3-P containing endosomes), tubulin-EYFP (cytoskeleton), Mito-dsRed (mitochondria) and PLCδ-PH (PI-4,5-P2; plasma membrane)
- Structure of the tested dual promoters. In CMVP10, the baculoviral very late promoter p10 was inserted downstream of the CMV promoter. In CMVintP10, the p10 promoter was placed within an intron and is spliced out from the transcript of the CMV promoter

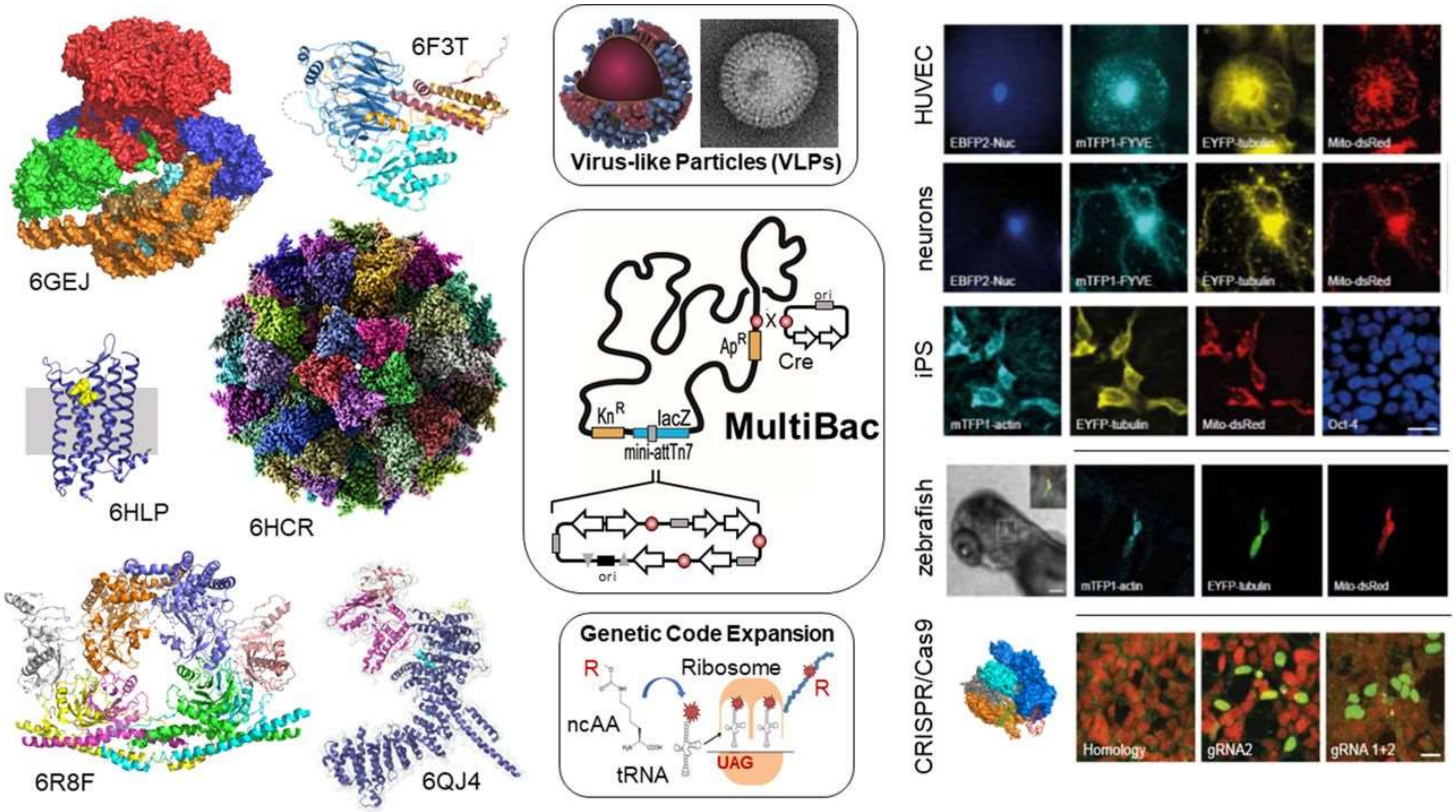
Application of MultiBac in drug discovery

MultiBacMam-BiFC for compound screening



- The MultiBacMam baculovirus is shown (top left). Genes encoding for vesicular stomatitis virus glycoprotein (VSV-G) and mCherry to track virus performance during manufacturing have been integrated into the baculoviral backbone, each controlled by baculoviral late promoters
- MultiBacMam was outfitted in the Tn7 site with genes encoding for CDK5 and p25, each fused to complementary fragments of a split fluorescent protein, which, upon CDK5-p25 complex formation reconstitute complete, active fluorophore (bottom left)
- Composite MultiBacMam baculovirus is produced in insect cells and then used to transduce mammalian cells with superior efficacy as compared to plasmid transfection (top right)
- A selection of chemical compounds inhibiting the CDK5-p25 PPI is depicted (bottom right), identified by using our MultiBac-BiFC cell-based screening assay.

The MultiBac system: a perspective



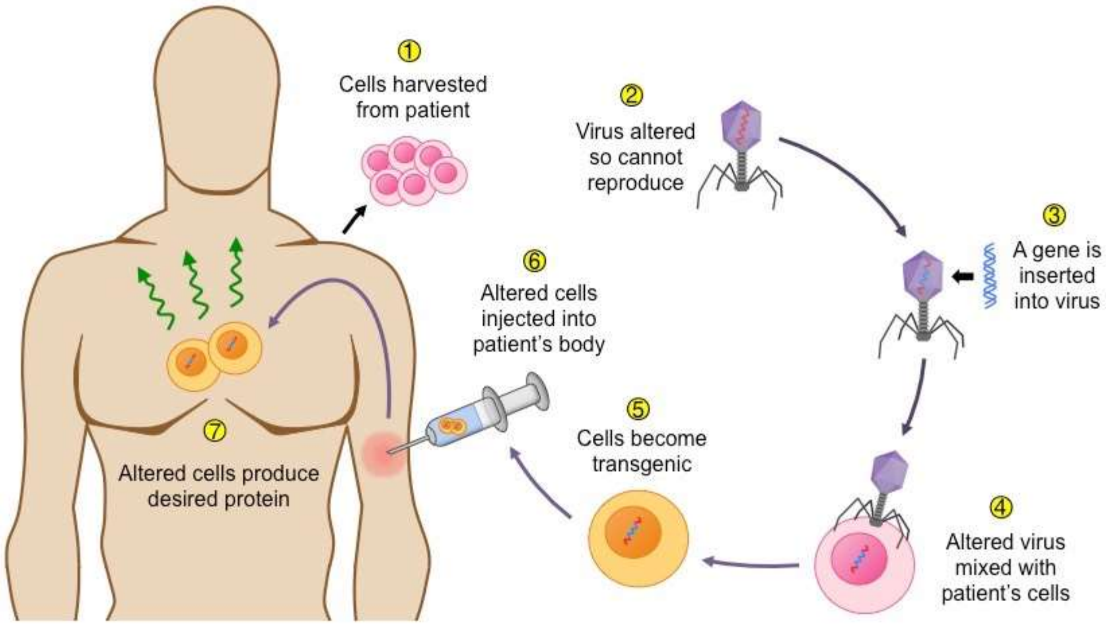
Gene therapy comes of age

Nearly five decades ago, scientists hypothesized that genetic modification by exogenous DNA might be an effective treatment for inherited human diseases. This "gene therapy" strategy offered the theoretical advantage that a durable and possibly curative clinical benefit would be achieved by a single treatment. Although the journey from concept to clinical application has been long, gene therapy is now bringing new treatment options to multiple fields of medicine.

Dunbar *et al.*,Science 359: eaan4672 (2018), DOI: 10.1126/science.aan4672

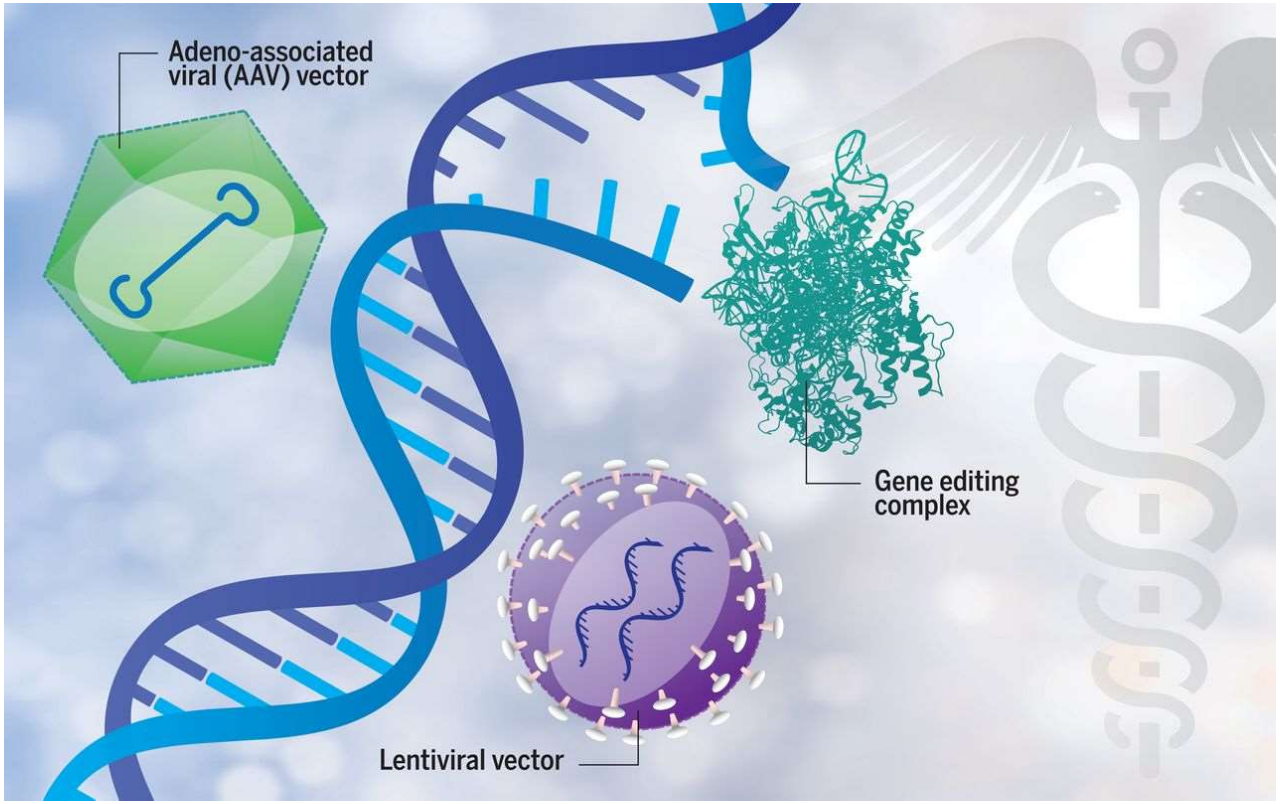
Overview of gene therapy

Gene therapy is the insertion of genes into an individual's cells to treat hereditary diseases by replacing defective alleles



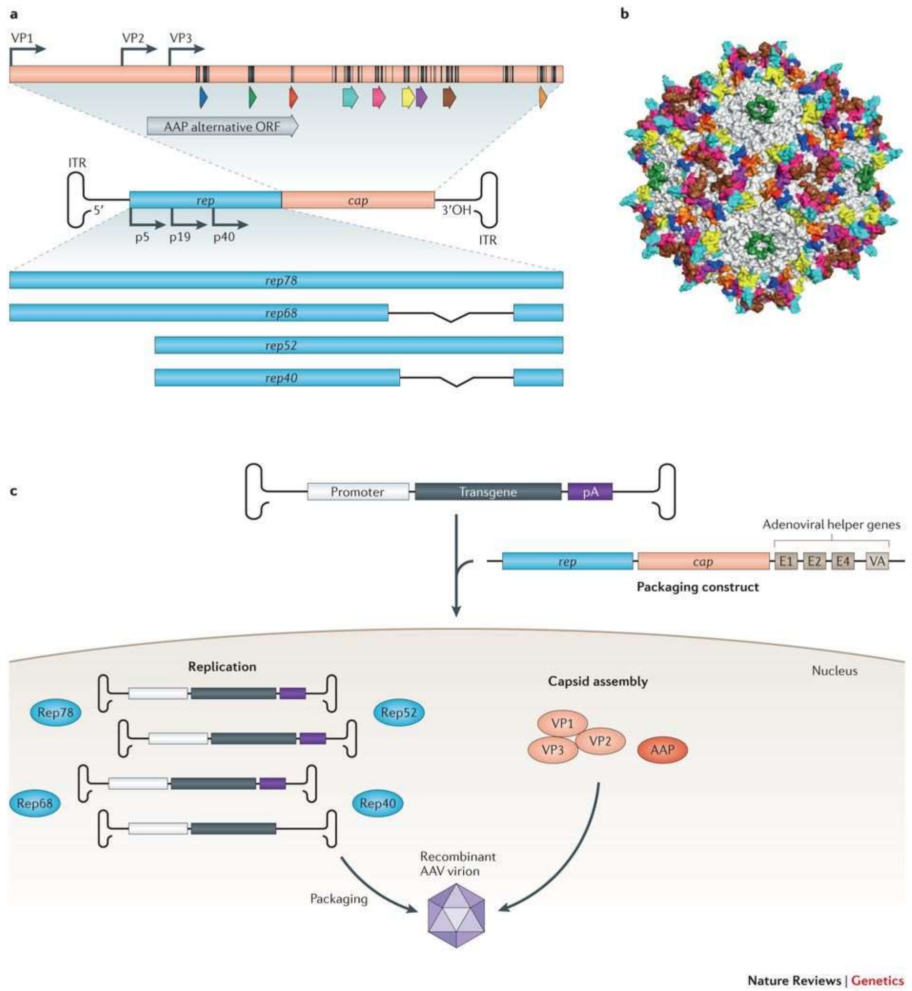
- Viral vectors are typically used, due to their ability to integrate DNA into the host's genome
- The process involves removing cells from a patient and using a viral vector to introduce a functional copy of the defective gene
- When the cells are transplanted back into the patient, they should begin expressing the missing protein to restore normal health

Three essential tools for human gene therapy



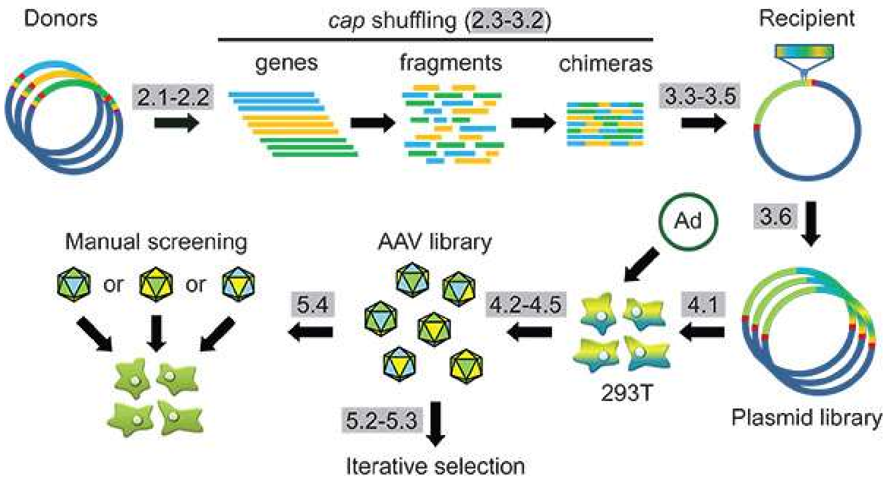
- AAV and lentiviral vectors are the basis of several recently approved gene therapies.
- Gene editing technologies (CRISPR/Cas9, TALENs etc.) are in their translational and clinical infancy but are expected to play an increasing role in the field.

AAV biology and vector manufacturing

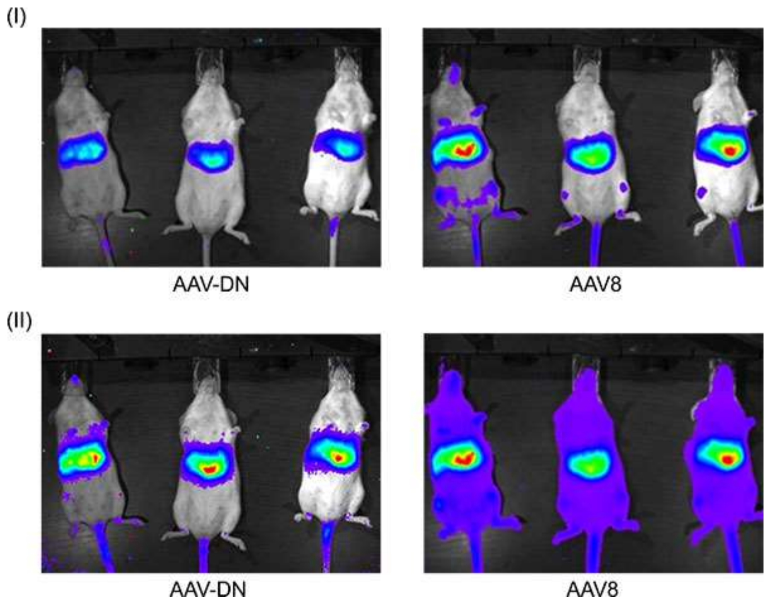


- Adeno-associated virus (AAV) is a small (25-nm) virus
- It is composed of a non-enveloped icosahedral capsid (protein shell) that contains a linear single-stranded DNA genome of about 4.7 kb
- The AAV genome encodes for several protein products, namely, four non-structural Rep proteins, three capsid proteins (VP1–3), and the recently discovered assembly-activating protein (AAP)
- The AAV genes are required for its biological cycle and are flanked by two AAV-specific palindromic inverted terminal repeats (ITRs; 145 bp)
- AAV viruses infect both dividing and non-dividing cells, and remain latent in the host cell DNA by integration into specific chromosomic loci (AAVS) unless a helper virus activates its replication
- AAV viruses naturally infect humans; an exposure to the wild-type virus occurs at around 1–3 years of age, and is not associated with any known disease
- Importantly, the timing of human exposure to AAV viruses determines the host immunological response to the recombinant AAV vectors

Engineering and evolution of AAV vectors by DNA shuffling



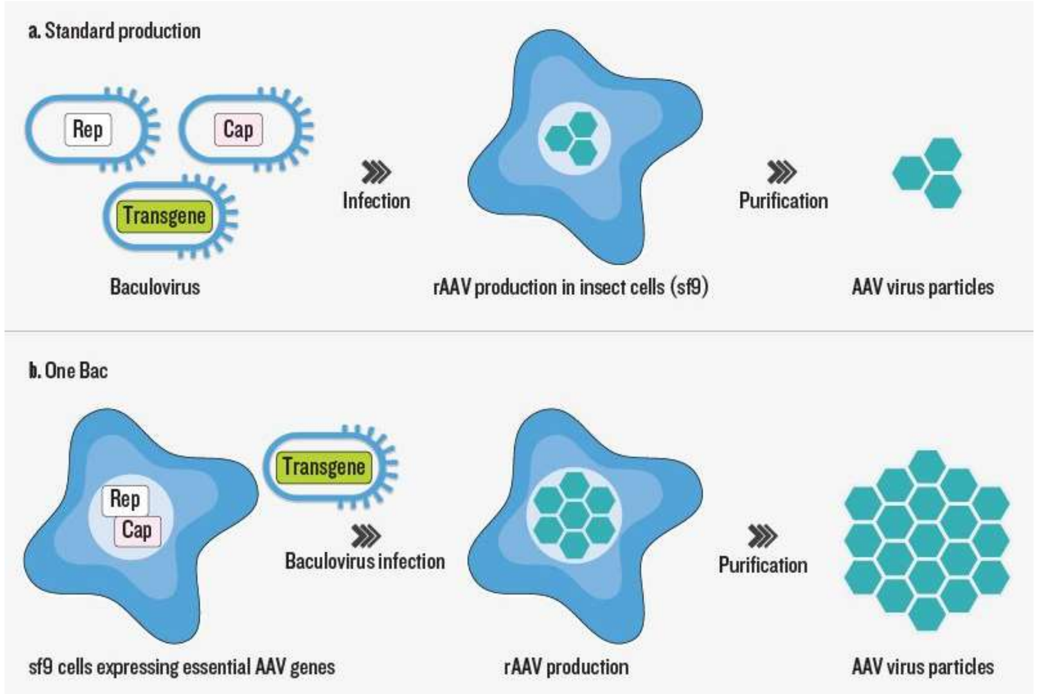
Synthetic AAV capsid engineering via DNA family shuffling and subsequent selection in cells or in animals.

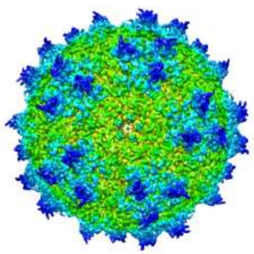


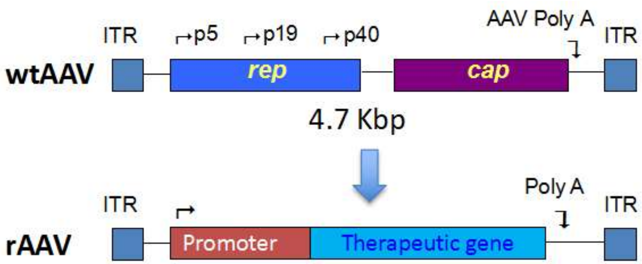
Example for analysis of a selected AAV chimera in mouse liver. Clone AAV-DN was selected on murine hepatoma cells and then used to produce *luciferase*-expressing recombinant vectors. Note that while the AAV-DN clone gives slightly less overall expression in the liver (panel **I**), it is more specific for this organ since it exhibits substantially less off-targeting in non-hepatic tissues (panel **II**).

Baculovirus-mediated production of gene therapy vectors

Baculovirus-mediated production of adeno-associated viral (AAV) vectors as gene therapy vehicles



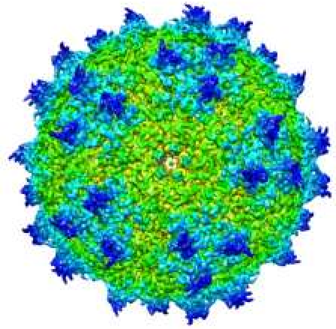


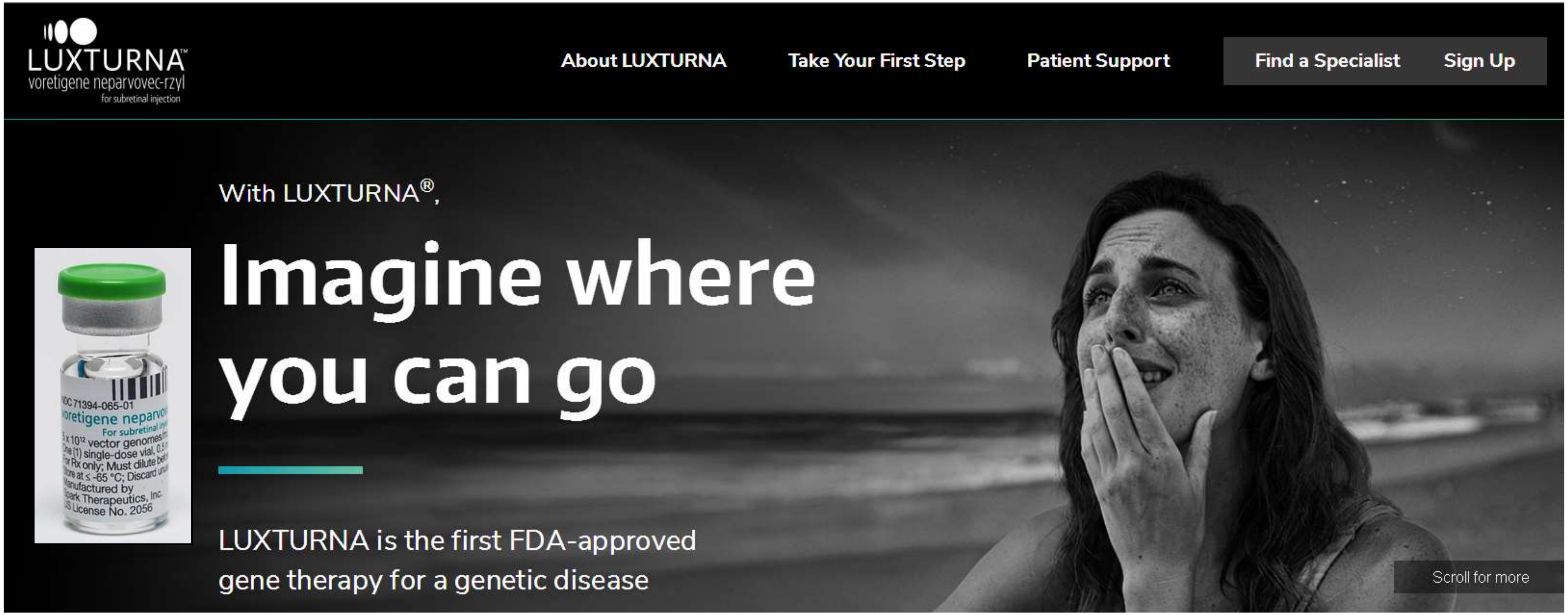


- In 2002, Prof. Robert Kotin and colleagues at the US National Heart, Lung, and Blood Institute first demonstrated its suitability for AAV manufacturing
- They infected Sf9 cell lines — derived from the fall armyworm — with three different baculoviruses: two containing essential genes for AAV particle production (rep and cap), and one containing the transgene sequence intended for delivery
- In this manufacturing process, the baculoviruses play a dual role, functioning as the ‘helper’ virus normally required for replication, as well as the vehicle for AAV genetic material
- In their initial demonstration, Kotin’s team achieved levels of productivity comparable with existing AAV manufacturing approaches — on the order of 50,000 functional viral particles per cell

Development of AAV-based gene therapy products

- In 2017, FDA-approval of the first gene therapy product targeting a disease caused by mutations in a single gene
- This product, LUXTURNA™ (voretigene neparvovec-rzyl; Spark Therapeutics, Inc., Philadelphia, PA), delivers a normal copy of the *RPE65* gene to retinal cells for the treatment of *RPE65* mutation–associated retinal dystrophy, a blinding disease
- Many additional gene therapy programs targeting both inherited retinal diseases and other ocular diseases are in development

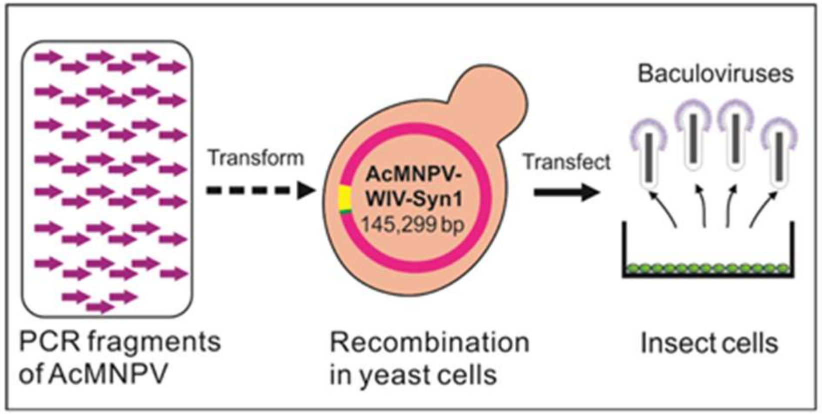


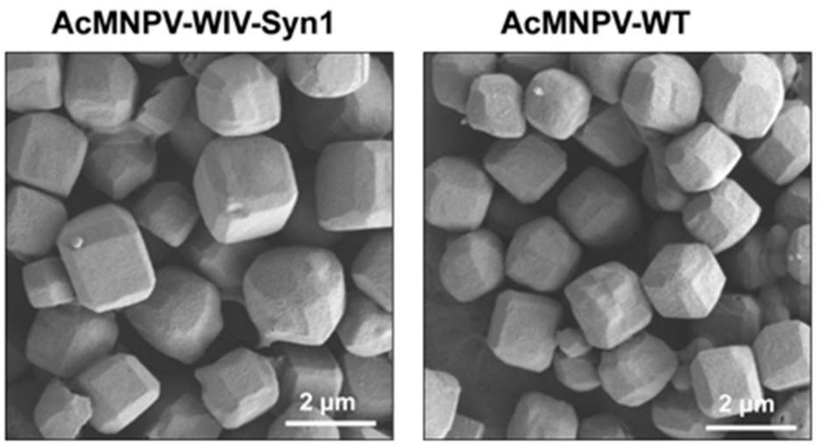


World's most efficient large-scale AAV manufacturing

- Virovek has developed a patented BAC-to-AAV technology that utilizes the baculovirus expression system to produce AAV vectors in insect cells under serum-free condition
- The capability to generate over 3e+16vg of AAV vectors with a single production run, which is unmatched by any other AAV production system

Synthesis of a functional baculovirus reported in 2017



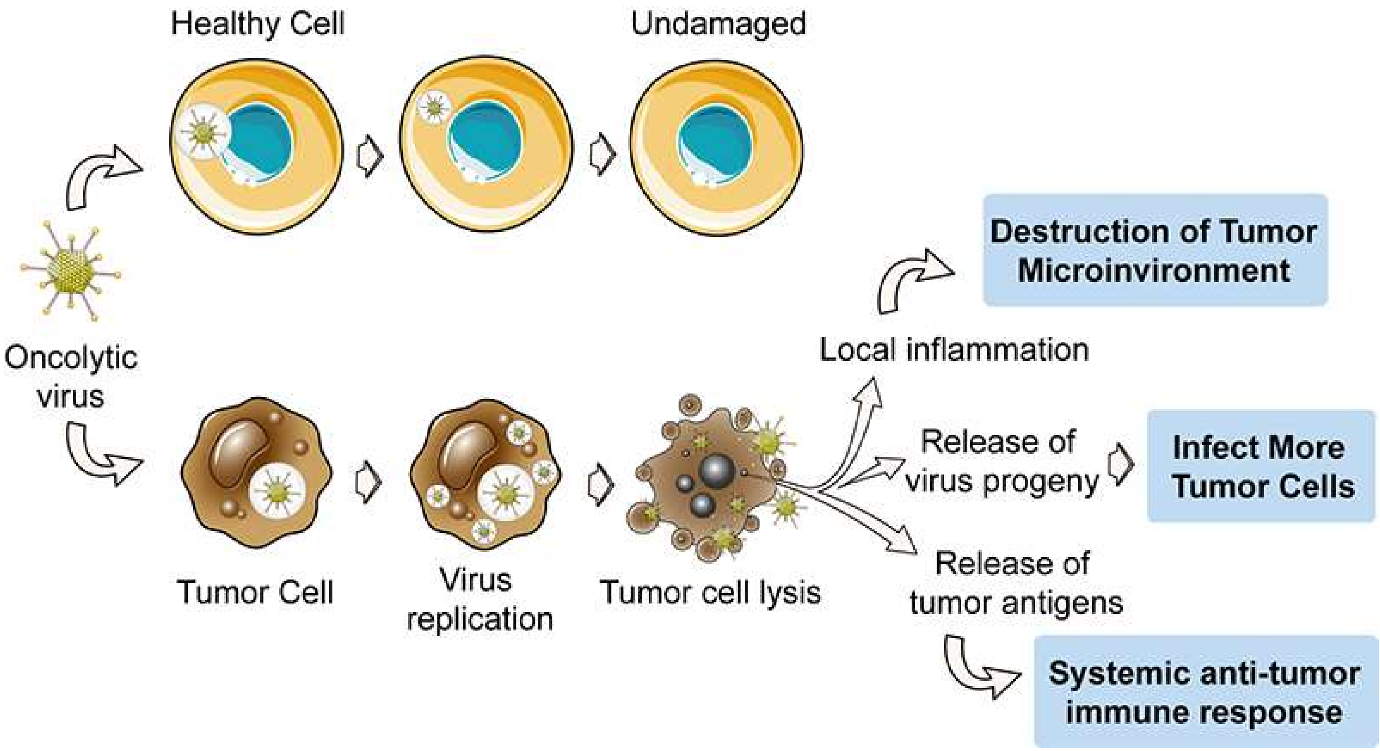


- So far, most synthetic viruses have been RNA viruses (<30 kb) and small DNA viruses
- Baculoviruses contain a large circular dsDNA genome of 80–180 kb and have been used as biocontrol agents and protein expression vectors
- First synthesis of a baculovirus genome by a combination of PCR and transformation-associated recombination in yeast
- The synthetic genome (145,299 bp) comprising the complete genome of AcMNPV except for the *hr4a* locus that was replaced with an 11.5 kb cassette of bacterial and yeast artificial chromosomal elements and an *egfp* gene
- Insect cells were transfected with the synthetic baculovirus genome and progeny virus was examined by electron microscopy
- The results showed that the rescued virus had structural and biological properties comparable to the parental virus
- This is the largest DNA virus synthesized so far, and its success will stimulate the fields of other large DNA viruses such as herpesviruses and poxviruses

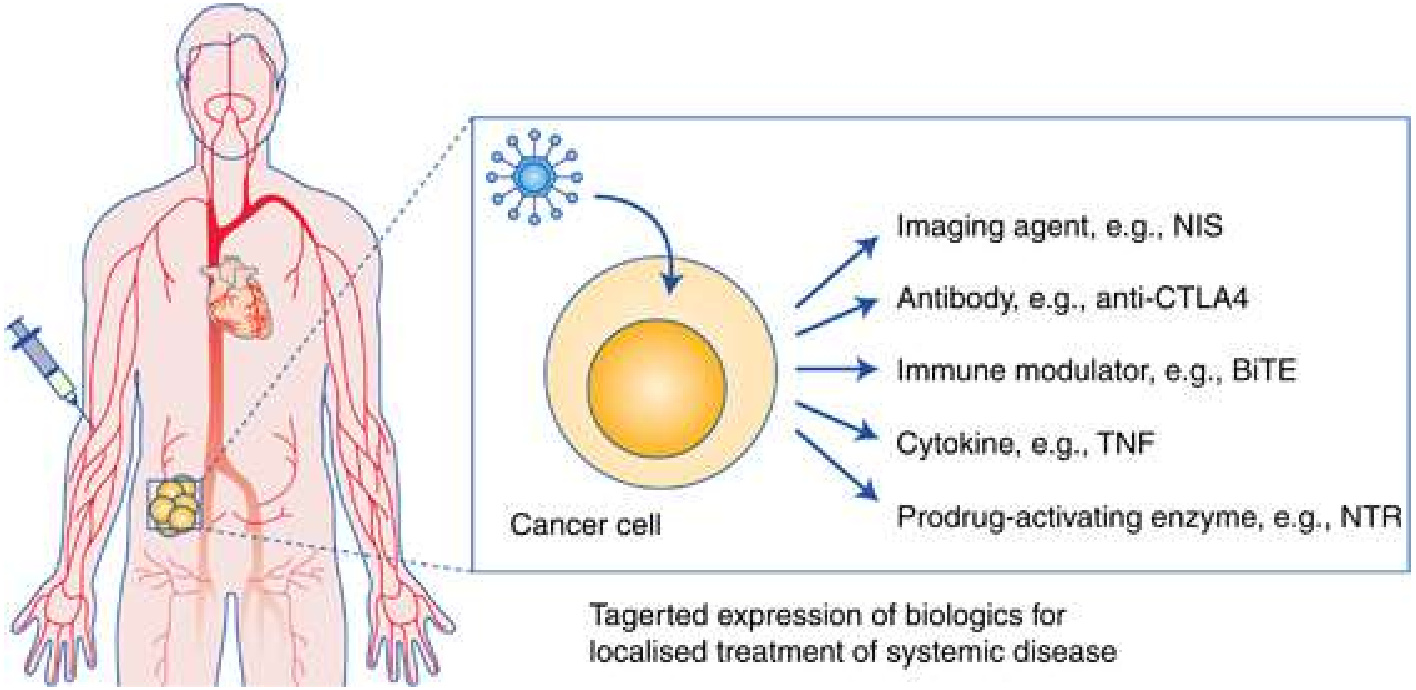
https://pubs.acs.org/doi/abs/10.1021/acssynbio.7b00028?journalCode=asbcd6

Oncolytic viruses as synthetic platforms for cancer therapy

- Oncolytic viruses (OVs) selectively replicate in and kill cancer cells, and spread within the tumor, while not harming normal tissue
- In addition to this direct oncolytic activity, OVs are also very effective at inducing immune responses to themselves and to the infected tumor cells
- OVs encompass a broad diversity of DNA and RNA viruses that are naturally cancer-selective or can be genetically-engineered



Cancer therapy: armed oncolytic viruses



Oncolytic viruses can be ‘armed’ to express therapeutic proteins within infected tumour cells, and secrete them into the tumour microenvironment. This can provide high-level tumour-selective expression of biologics, maximising local activity and minimising systemic toxicities. If the virus is suitable for systemic delivery, this provides the intriguing concept of localised therapy in disseminated tumours following intravenous delivery. AntiCTLA4=checkpoint inhibitor antibody; BiTE=Bi-specific T-cell engager, for modulating activity and tropism of T cells; NIS=sodium iodide symporter (for SPECT imaging of virus activity); NTR=nitroreductase TNF=tumour necrosis factor.

Seymour and Fisher
British Journal of Cancer (2016) 114, 357-361 doi:10.1038/bjc.2015.481

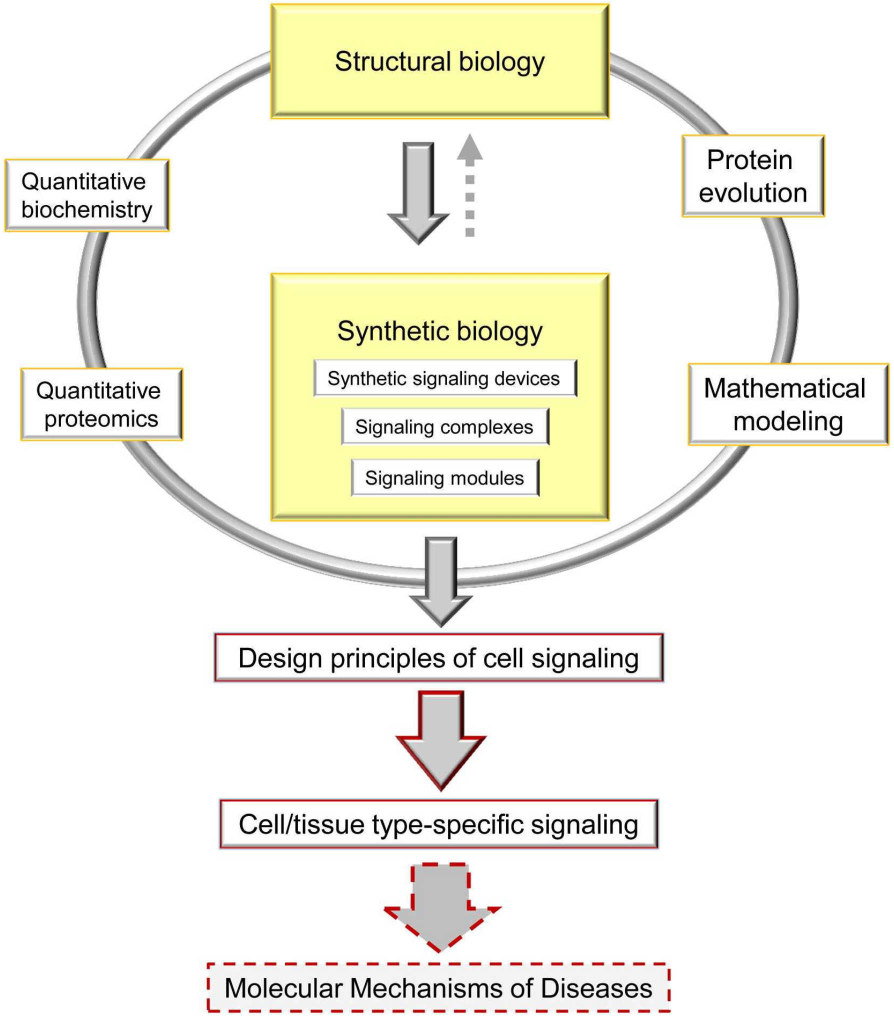
Industrial large-scale production of oncolytic viruses

https://www.halix.nl/vectors-and-vaccines/?gclid=EAIaIQobChMIsJvE_6Wc5QIVRrTtCh174QBIEAAYASAAEgKpFPD_BwE

Questions

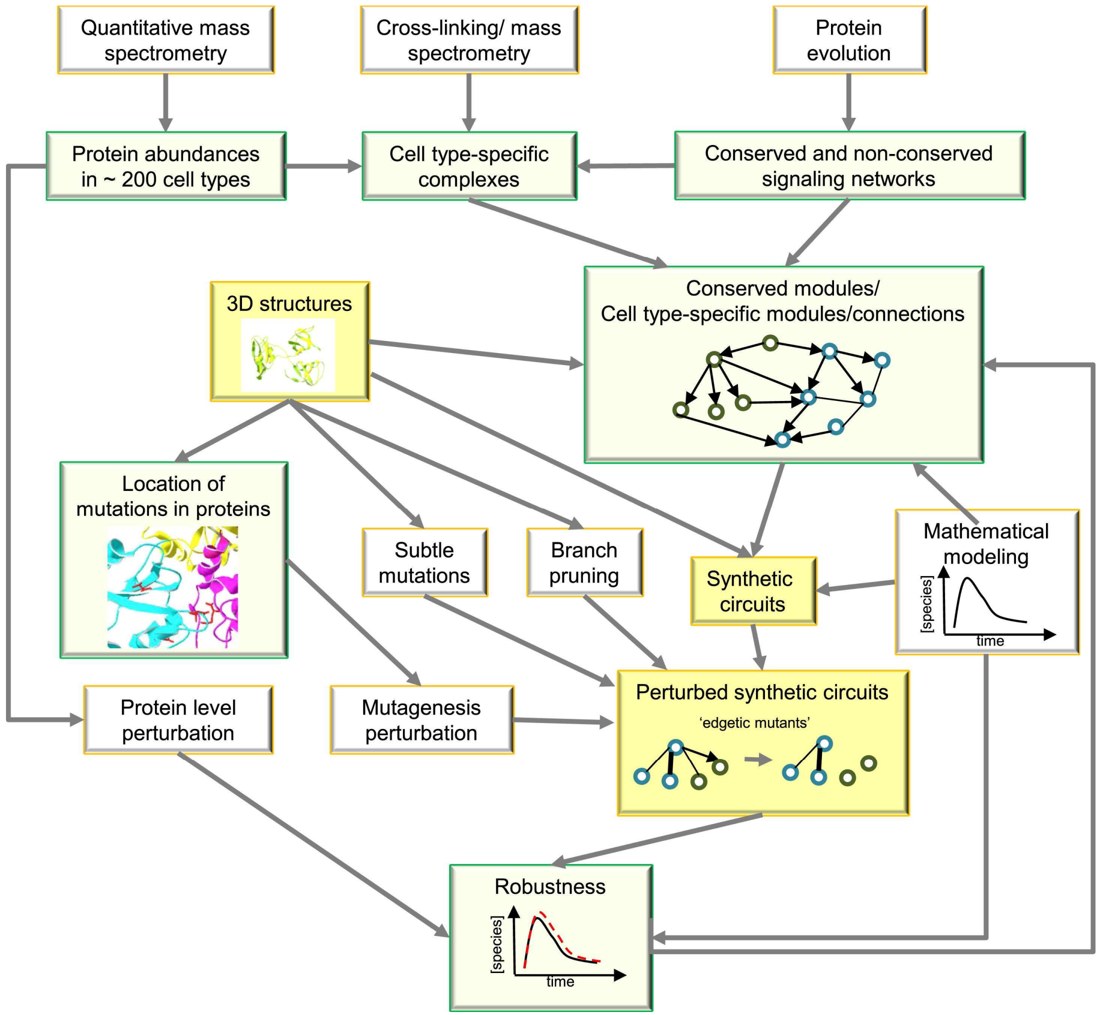
Supplementary materials

Combining structural biology with synthetic biology to provide insights into cell signalling



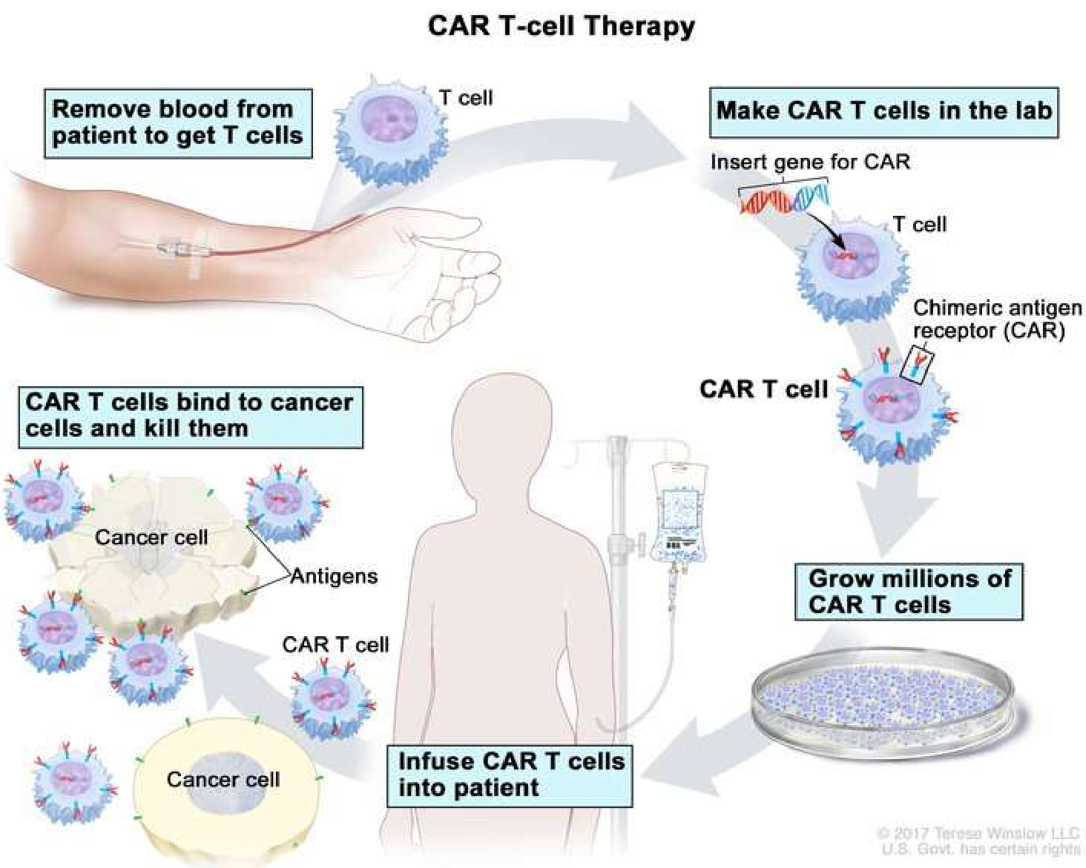
- Structural information provides a valuable tool in engineering synthetic signalling devices
- Combined with additional information from quantitative biochemistry and proteomics, gene evolution, and mathematical modelling, this can provide insights into signalling modules and the general design principles of cell signalling
- Altogether, this will improve our understanding of cell- and tissue-type-specific signal transduction
- In the future, this knowledge can be the basis for understanding the molecular mechanisms of disease by predicting the effect of disease mutation using mathematical modelling

Flow chart of combining different disciplines to study cellular design principles and cell-type-specific signalling



- Structural information is a key component in proposing network modules, predicting the localization and effect of disease mutations, and designing perturbed protein complexes to be used in synthetic biology approaches

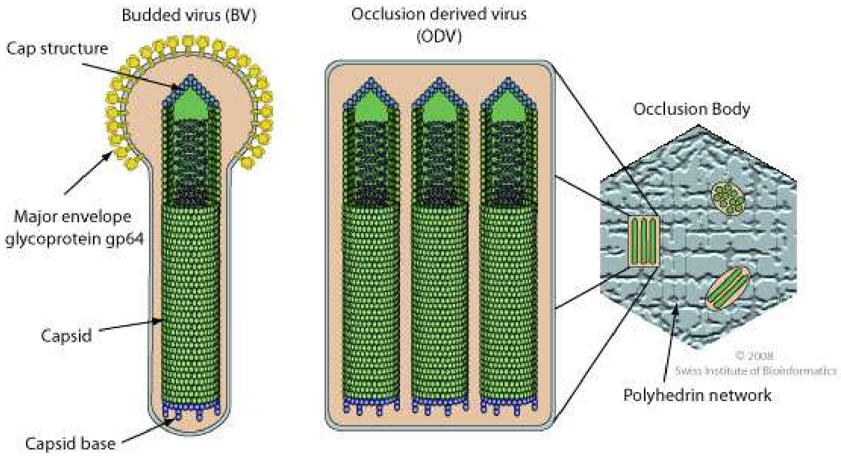
Synthetic biology approaches to improving immunotherapy



- One of the major drivers of cancer immunotherapy interest is CAR-T (CAR stands for Chimeric Antigen Receptor and T is for T-cells) cell therapy.
- This is a cell-based therapy in which T-cells are taken from the patient and then modified to have specific receptors that help it recognize and attack the cancer.
- The chimeric antigen receptor works by fusing a target-binding domain that recognizes the cancer and an activation domain that turns on the T-cell into attack mode against the cancer.
- Plenty of these modified cells are then grown up in the lab and returned to the patient.
- The first CAR-T treatment was approved by FDA in 2017 and many more are in clinical trials.

In 1959 Richard Feynman delivered what many consider the first lecture on nanotechnology. This lecture, presented to the American Physical Society at the California Institute of Technology, prompted intense discussion about the possibilities, or impossibilities, of manipulating materials at the molecular level. Although at the time of his presentation, the manipulation of single molecules and single atoms seemed improbable, if not impossible, Feynman challenged his audience to consider a new field of physics, one in which individual molecules and atoms would be manipulated and controlled at the molecular level (Feynman 1960). As an example of highly successful machines at the “small scale,” Feynman prompted his audience to consider the inherent properties of biological cells. He colorfully noted that although cells are “very tiny,” they are “very active, they manufacture various substances, they walk around, they wiggle, and they do all kinds of wonderful things on a very small scale” (Feynman 1960). Of course, many of these “wonderful things” that he was referring to are a result of the activities of proteins and protein complexes within each cell.
The field of nanotechnology has indeed emerged and blossomed since Feynman's 1959 lecture, and scientists from many disciplines are now taking a careful look at the protein “machines” that power biological cells (Drexler 1986). These “machines” are inherently nanoscale, ranging in width from a few nanometers (nm) to over 20 nm, and have been carefully refined by millions of years of evolution.

Baculovirus biology



- Extracellular enveloped baculovirus virions can be found in two forms: OV (occluded virus) and BV (budded virus). The nucleocapsid is about 21 nm x 260 nm.
- Circular dsDNA genome , 80-180 kb in length, encoding for 100 to 180 proteins.

BACULOVIRUS INFECTION CYCLE

- Attachment of the viral glycoproteins to host receptors mediates endocytosis of the virus into the host cell.
- Fusion with the plasma membrane.
- The DNA genome is released into the host nucleus.
- Immediate early phase: host RNA polymerase transcribes viral genes involved in the regulation of the replication cascade, prevention of host responses and viral DNA synthesis.
- Late phase: The virally encoded RNA polymerase expresses late genes.
- Replication of the genome by rolling circle in nuclear viral factories.
- Nucleocapsids are formed which can either bud out through the cellular membrane and disseminate the infection or be occluded for horizontal transmission.
- Occlusion phase: the virus becomes occluded in the protein polyhedrin and the polyhedral envelope (calyx) is produced.
- Lysis of the cell releases the occluded virus.

Engineering and evolution of AAV vectors by DNA shuffling

- Adeno-associated viral (AAV) vectors represent some of the most potent and promising vehicles for therapeutic human gene transfer due to a unique combination of beneficial properties.
- These include the apathogenicity of the underlying wildtype viruses and the highly advanced methodologies for production of high-titer, high-purity and clinical-grade recombinant vectors.
- A further particular advantage of the AAV system over other viruses is the availability of a wealth of naturally occurring serotypes which differ in essential properties yet can all be easily engineered as vectors using a common protocol.
- Development of strategies to use these natural viruses as templates for the creation of synthetic vectors which either combine the assets of multiple input serotypes, or which enhance the properties of a single isolate.
- The respective technologies to achieve these goals are either DNA family shuffling, *i.e.* fragmentation of various AAV capsid genes followed by their re-assembly based on partial homologies (typically >80% for most AAV serotypes), or peptide display, *i.e.* insertion of usually seven amino acids into an exposed loop of the viral capsid where the peptide ideally mediates re-targeting to a desired cell type.
- For maximum success, both methods are applied in a high-throughput fashion whereby the protocols are up-scaled to yield libraries of around one million distinct capsid variants. Each clone is then comprised of a unique combination of numerous parental viruses (DNA shuffling approach) or contains a distinctive peptide within the same viral backbone (peptide display approach).
- The subsequent final step is iterative selection of such a library on target cells in order to enrich for individual capsids fulfilling most or ideally all requirements of the selection process. The latter preferably combines positive pressure, such as growth on a certain cell type of interest, with negative selection, for instance elimination of all capsids reacting with anti-AAV antibodies.
- This combination increases chances that synthetic capsids surviving the selection match the needs of the given application in a manner that would probably not have been found in any naturally occurring AAV isolate.

Baculovirus-mediated production of AAV vectors

Gene transfer and gene therapy are powerful approaches for many biological research applications and promising avenues for the treatment of many genetic or cancer diseases. The most efficient gene transfer tools are currently derived from viruses. Among them, the recombinant **adeno-associated viruses (AAVs)** are vectors of choice for many fundamental and therapeutic applications. The increasing number of clinical trials involving AAVs demonstrates the need to implement production and purification processes to meet the quantitative and qualitative demands of regulatory agencies for the use of these vectors in clinical trials. In this context, the rise of production levels on an industrial scale appeared essential. The introduction, in 2002, of an AAV process using a baculovirus expression vector system (BEVS) has circumvented this technological lock. The advantage of BEVS in expanding the AAV production in insect cells has been to switch the process to bioreactor systems, which are the ideal equipment for scaling up. We describe here a method for producing AAV vectors using the BEVS which can be easily used by research laboratories wishing to overcome the difficulties associated with the scaling up of production levels. The method provides sufficient quantities of AAV vectors to initiate preclinical projects in large animal models or for research projects where a single batch of vectors will consolidate the repeatability and reproducibility of in vitro and especially in vivo experimental approaches.

Methods Mol. Biol. 2019; 1937: 91-99. doi: 10.1007/978-1-4939-9065-8_5

Baculovirus-mediated multigene delivery in cells

- Multigene delivery and subsequent cellular expression is emerging as a key technology required in diverse research fields including synthetic and structural biology, cellular reprogramming and functional pharmaceutical screening.
- Current viral delivery systems such as retro- and adenoviruses suffer from limited DNA cargo capacity, thus impeding unrestricted multigene expression.
- The MultiPrime, a modular, non-cytotoxic, non-integrating, baculovirus-based vector system expediting highly efficient transient multigene expression from a variety of promoters.
- MultiPrime viruses efficiently transduce a wide range of cell types, including non-dividing primary neurons and induced-pluripotent stem cells (iPS).
- The MultiPrime can be used for reprogramming, and for genome editing and engineering by CRISPR/Cas9.
- Implementation of dual-host-specific cassettes enabling multiprotein expression in insect and mammalian cells using a single reagent.
- The MultiPrime as a powerful and highly efficient tool, to deliver multiple genes for a wide range of applications in primary and established mammalian cells.

Applications of MultiBac system

The MultiBac system is depicted schematically in the centre. It consists of an engineered baculoviral genome which exists as a BAC in specialized bacterial cells. Foreign DNA can be introduced into this BAC using a set of helper plasmids comprising the multigene circuitry of choice, by a variety of means including Tn7 mediated transposition and/or site-directed insertion by Cre recombinase. Originally, we designed the MultiBac system for expressing heterologous multiprotein complexes in insect cells. A selection of recent high-impact structures of samples produced using MultiBac is shown on the left, marked by their Protein Data Bank identifier (PDB-ID). 6GEJ, SWR1-nucleosome complex; 6F3T, human TFIID subcomplex TAF5–TAF6–TAF9; 6HLP, human neurokinin 1 receptor GPCR [36]; 6R8F, human BRISC–SHMT2 complex; 6QJ4, Ycs4–Brn1/Smc4hd–Brn1C complex; 6HCR, ADDomer virus-like particle (VLP). MultiBac is successfully used for a range of applications beyond producing protein complexes for structural and mechanistic studies. For example, customized MultiBac baculoviral genomes were prepared to express virus-like particles (VLPs), which are promising vaccine candidates as they resemble live viruses but do not contain genetic material and are thus safe. A different example is MultiBacTAG, a customized MultiBac baculovirus capable of genetic code expansion. MultiBacTag exploits an orthogonal tRNA/tRNA synthetase pair to insert artificial amino acids (ncAA) into polypeptide chains at defined sites by means of AMBER codon (UAG) suppression. By altering the tropism of the baculovirion, MultiBac can be turned into a powerful high-capacity DNA delivery device into mammalian cells, tissues and even organisms (right), to faithfully deliver multicomponent DNA circuitry transiently or stably by genomic insertion using CRISPR/Cas technology. For a more detailed conspectus of MultiBac applications see recent review articles by our group.

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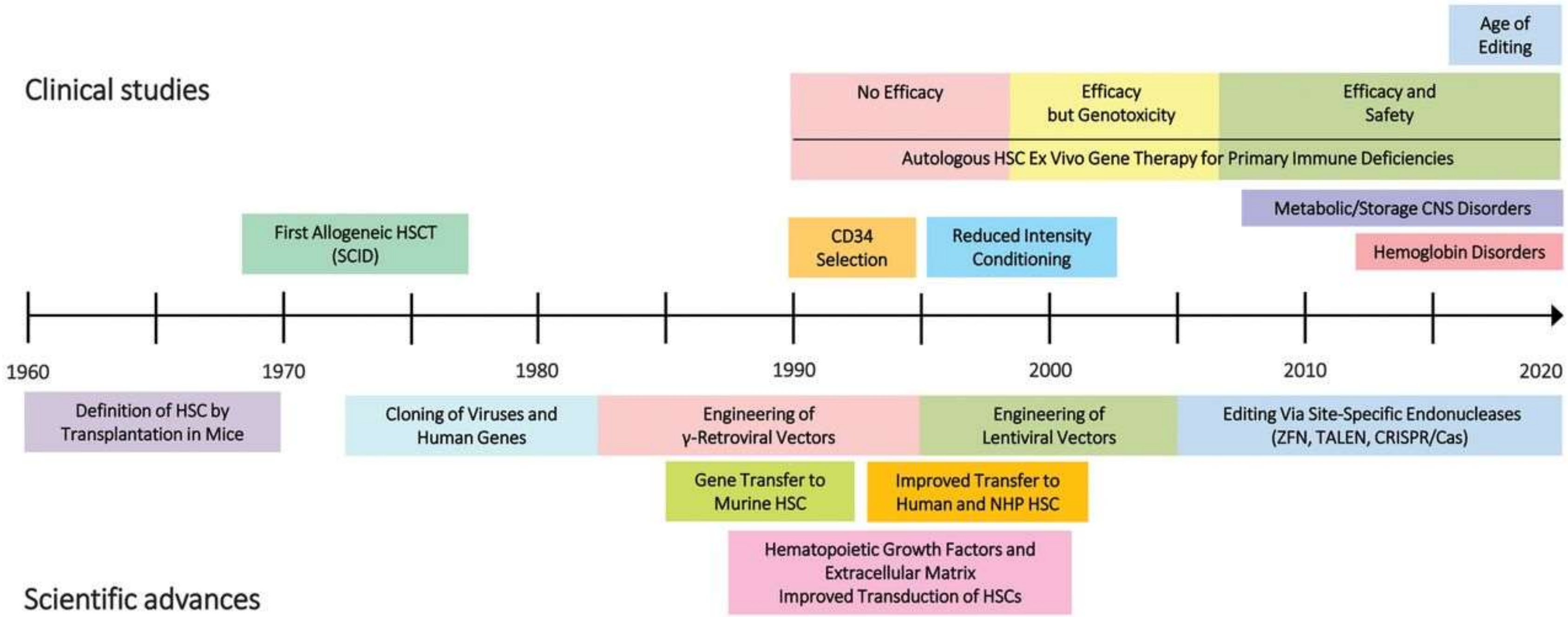
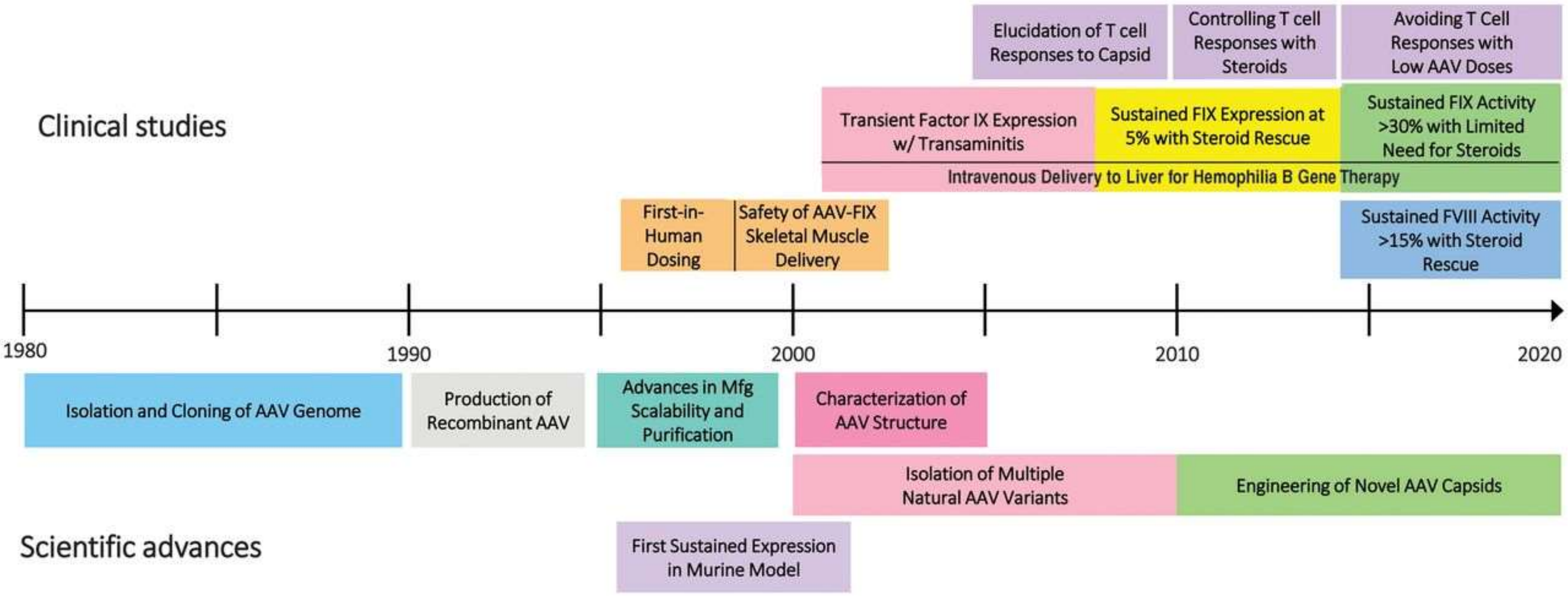
Historical overview of gene therapy



Abbreviations: HSCT: hematopoietic stem cell transplantation; HSC: Hematopoietic stem cell; SCID: severe combined immunodeficiency; NHP: nonhuman primate; ZFN: zinc finger nuclease; TALEN: transcription activator–like effector nuclease; CRISPR/Cas9: clustered regularly interspaced short palindromic repeat (CRISPR)–CRISPR-associated 9 (Cas9) nucleases.

Dunbar *et al.*, Science 359: eaan4672 (2018), DOI: 10.1126/science.aan4672

Historical overview of AAV gene therapy for hemophilia



Abbreviations: AAV: adeno-associated viral vector; FVIII: factor VIII; FIX: factor IX; Mfg: Manufacturing.

Dunbar *et al.,*Science 359: eaan4672 (2018), DOI: 10.1126/science.aan4672

Oncolytic viruses

- The activity of OVs is very much a reflection of the underlying biology of the viruses from which they are derived and the host-virus interactions that have evolved in the battle between pathogenesis and immunity
- Typically, OVs fall into 2 classes:

  (i) viruses that naturally replicate preferentially in cancer cells and are non-pathogenic in humans often due to elevated sensitivity to innate antiviral signalling or dependence on oncogenic signalling pathways (parvoviruses, poxviruses, paramyxoviruses, reoviruses, picornaviruses)

  (ii) viruses that are genetically-engineered with mutations/deletions in genes required for replication in normal, but not cancer cells (adenoviruses, herpesviruses, rhabdoviruses)

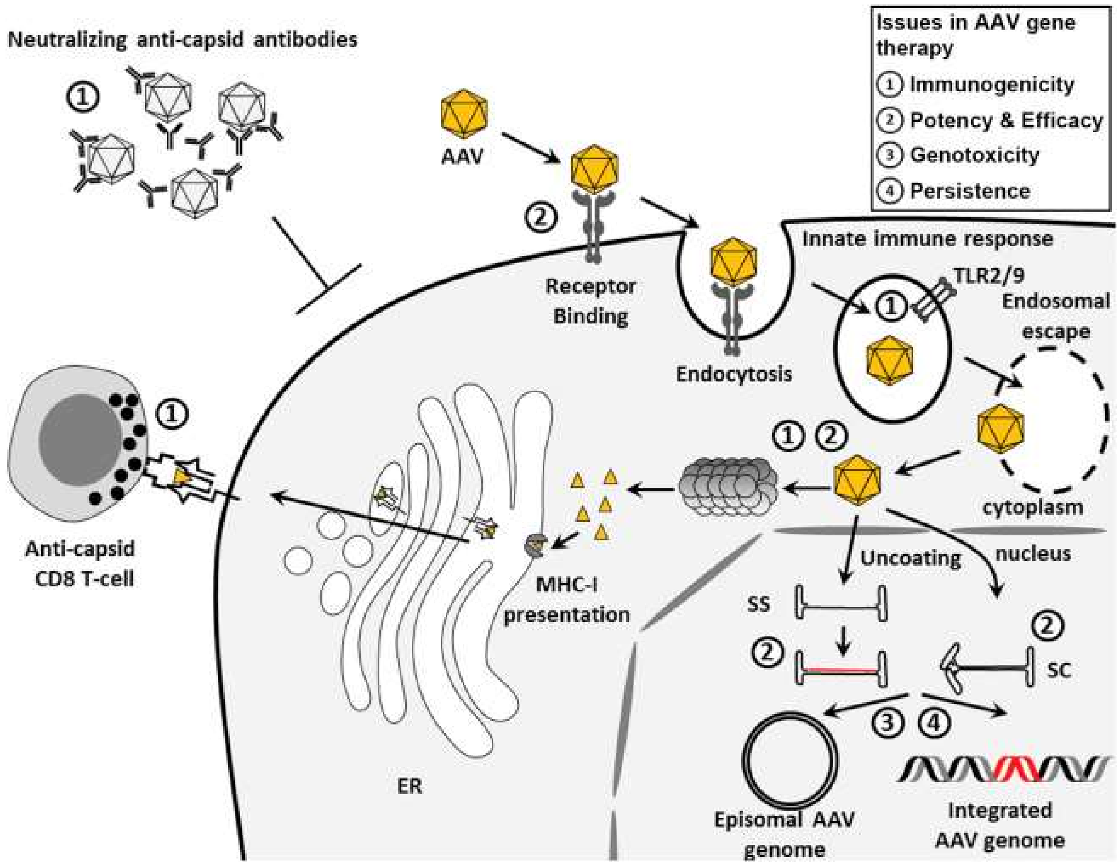
Oncolytic viruses offer several important advantages

- Amplification of the active agent (infectious virus particles) within the tumour. This avoids unnecessary exposure to normal tissues experienced during delivery of traditional stoichiometric chemotherapy and maximises the therapeutic index
- The active cell-killing mechanisms, often independent of programmed death mechanisms, should decrease the emergence of acquired drug resistance
- Lytic death of cancer cells provides a pro-inflammatory microenvironment and the potential for induction of an anticancer vaccine response
- Tumour-selective expression and secretion of encoded anticancer biologics, providing a new realm of potent and cost-effective-targeted therapeutics

Seymour and Fisher
British Journal of Cancer (2016) 114, 357-361 doi:10.1038/bjc.2015.481

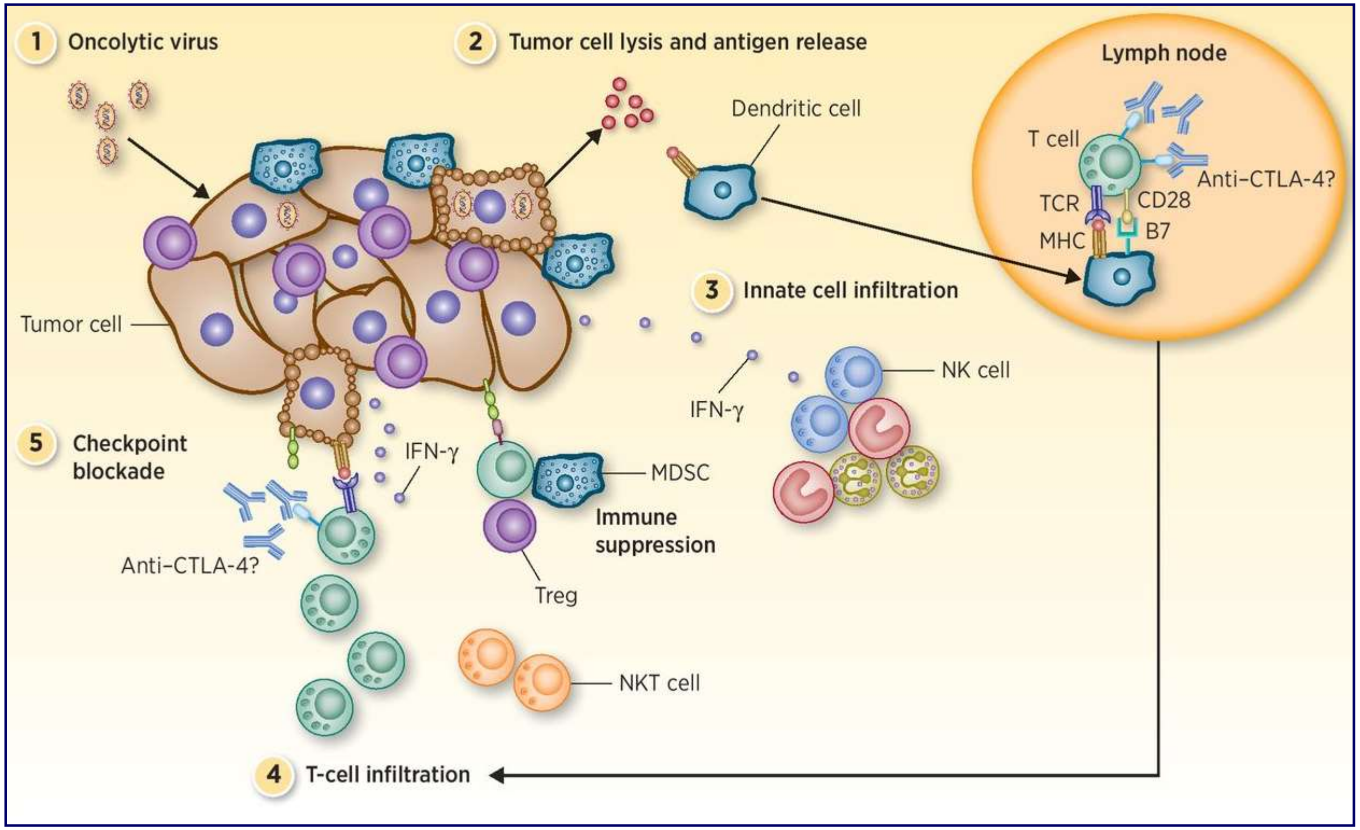
BJC British Journal of Cancer
Multidisciplinary Journal of Cancer Research

Emerging issues in AAV-mediated *in vivo* gene therapy



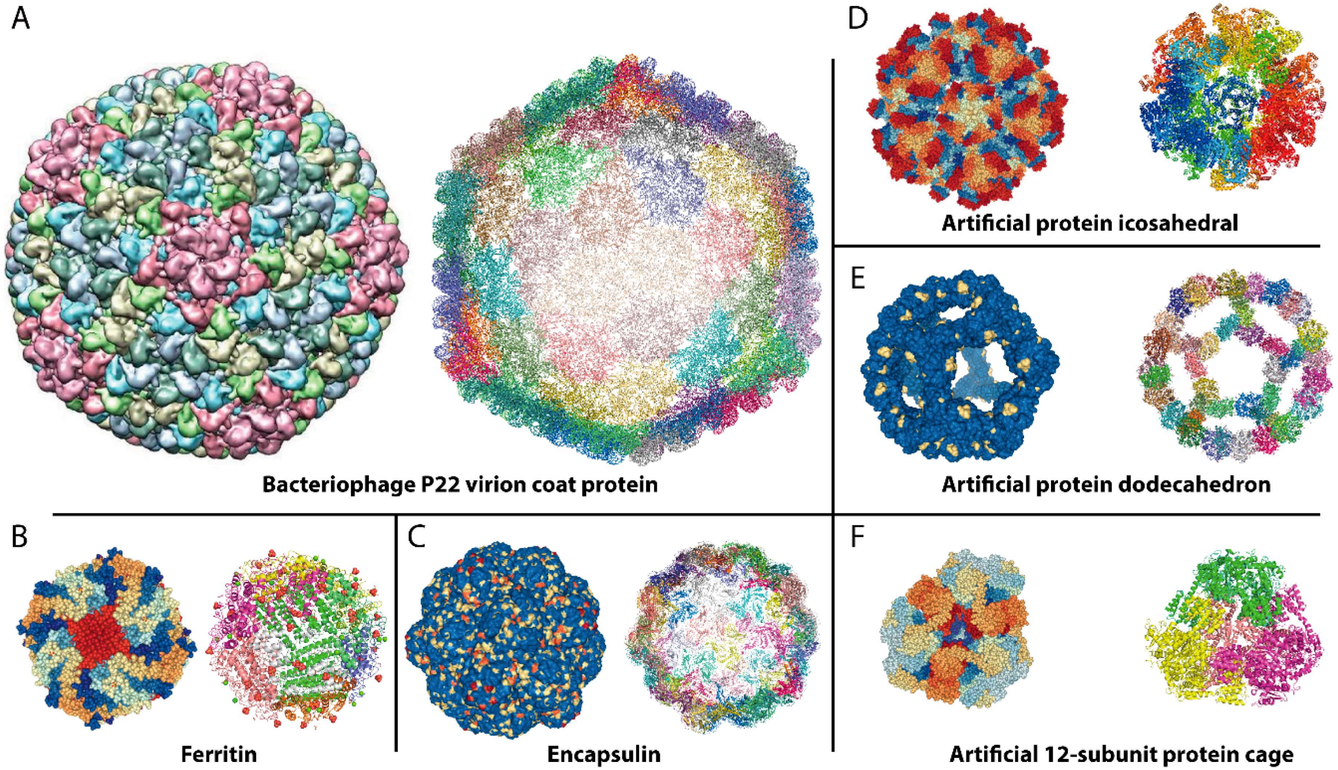
- In recent years, the number of clinical trials in which adeno-associated virus (AAV) vectors have been used for *in vivo* gene transfer has steadily increased
- The excellent safety profile, together with the high efficiency of transduction of a broad range of target tissues, has established AAV vectors as the platform of choice for *in vivo* gene therapy
- Successful application of the AAV technology has also been achieved in the clinic for a variety of conditions, including coagulation disorders, inherited blindness, and neurodegenerative diseases, among others
- Clinical translation of novel and effective “therapeutic products” is, however, a long process that involves several cycles of iterations from bench to bedside that are required to address issues encountered during drug development

Combined oncolytic virus and checkpoint blockade therapy



1: The delivery of oncolytic virus leads to specific infection and replication within the tumor. **2:** Some of the infected tumor cells undergo cell lysis and release antigen. Antigen-presenting cells, such as dendritic cells, are activated and migrate to lymph nodes to present antigen to T cells, where the administration of anti–CTLA-4 mAb may increase the amplitude of T-cell activation. **3:** Concurrently, innate cells, including natural killer (NK) and natural killer T (NKT) cells, are recruited to the inflammatory tumor bed that may further contribute to antitumor immune responses, through secretion of cytokines such as IFNγ. **4:** The activated T cells traffic to the tumor site and react against cancer cells through recognition of MHC/peptide complexes **5:** Administration of CTLA-4 antibody may further contribute to increasing T-cell activity at the tumor site. MDSC, myeloid-derived suppressor cell; TCR, T-cell receptor.

Examples of natural and non-natural protein compartments



(**A**), Bacteriophage P22, (PDB: 2XYZ); (**B**), Ferritin, (PDB: 6A4U); (**C**), Encapsulins, (PDB: 4PT2); (**D**), Artificial protein icosahedral, (PDB: 5KP9); (**E**), Artificial protein dodecahedron, (PDB: 5IM5); (**F**), Artificial 12-subunit protein cage, (PDB: 3VDX).

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