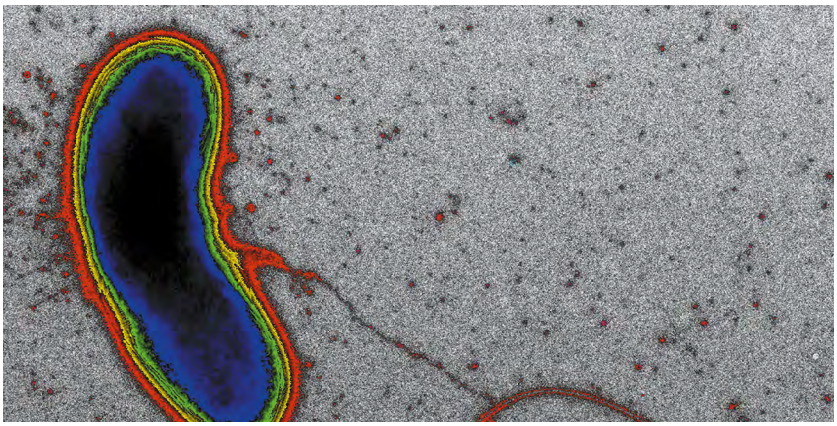

Macromolecular Structures

Scientists in the department work in a large number of biological problems, in particular in the structural and functional characterisation of different molecular machines such as viral structures (Casasnovas, Castón, Martín-Benito, Risco, San Martín and van Raaij), proteins involved in DNA repair (Moreno-Herrero), in the control of the genome stability (Ortega) or in protein homeostasis (Valpuesta).

These studies are carried out using different structural and biophysical techniques, most of them available at the CNB, which include X-ray diffraction, single-molecule techniques (optical and magnetic tweezers) and various spectroscopic techniques. Of special note is the development of microscopy techniques such as atomic force, optical and X-ray microscopy, and particularly cryoelectron microscopy in its distinct variants (single-particle cryoelectron microscopy, cryoelectron tomography and very recently cryocorrelative microscopy), which is supported by the CNB cryoelectron microscopy facility, the first of this kind in Spain. This work is strongly supported by continuous software development in the field of image processing (Carazo and Sánchez Sorzano).

All this has led to the CNB being the Spanish node of the Instruct ERIC network of European structural biology facilities, hosting two of them, the CryoEM CNB-CSIC facility and the Instruct Image Processing Center (I2PC). Technical developments are also pursued in the field of proteomics (Corrales), which resulted in the CNB being chosen to head the Spanish proteomic facilities network (PROTEORED) and its participation in the Human Proteome Project.

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Figure legend: Vibrio anguillarum*, a fish pathogen, imaged by negative staining electron microscopy (Sara Otaegi).*

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Biocomputing unit

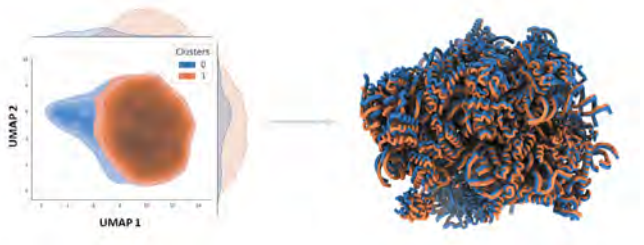
Our group develops and applies image processing algorithms for the analysis of the images of macromolecular structures acquired by an Electron Microscope under cryogenic conditions. The goal is to understand how the structure of a macromolecule and its dynamics captured by the microscope explains its physiological and pathological behavior.

Our image processing algorithms cover the whole pipeline from the microscope to the final structure. We develop algorithms to elucidate this structure when the particles are isolated (Single Particles technique) and when they are in their cellular context (Electron Tomography). Our algorithms are open-source and publicly available under the software package Xmipp. We also develop a workflow engine especially well-suited to image processing in Structural Biology called Scipion that integrates Xmipp and many other software suites also solving the same problem. Scipion provides a traceability, reproducibility and interoperability layer on top of the scientific calculation layer offered by Xmipp and the other software.

We are particularly committed to Open Science and to provide the software tools that allow the FAIR use of the electron microscopy data (FAIR=Findable, Accessible, Interoperable, and Reusable). Once the structure of a macromolecule is solved, our tools allow going further by helping to construct an atomic model and to explore possible ligands that could interact with it.

Finally, we also have a role in Structural Bioinformatics by integrating genomic, proteomic, and interactomic information onto the solved structure. Our tool in this area is one of the few recommended Interoperability Resources of the European Infrastructure of Life Science Information, ELIXIR. Finally, part of our group uses the image processing tools developed by us to solve specific structures. In this way, we know first-hand the problems encountered in the leading edge of Structural Biology.

Our group also provides access to the whole European scientific community through the Instruct-ERIC Image Processing Center (I2PC) as well as to collaborate in Joint Research Activities in the iNext-Discovery platform. I2PC belongs to the European infrastructure project for Structural Biology and supports the community by having an access program by which users can solve the structures of the macromolecules of their interest, hosting short-term internships, and by a training program through which we give at least 4 courses per year on the technology related to image processing in Electron Microscopy.



Example of Zernike3D continuous heterogeneity analysis. (Left) Contour level representation of the Zernike3D conformational landscape associated with the molecular motions of the Plasmodium falciparum 80S ribosome from EMPIAR-10028. (Right) Synthesis of the two main ribosome states captured by the landscape through the application of the Zernike3D deformation fields.

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Angel Farriols Barbero

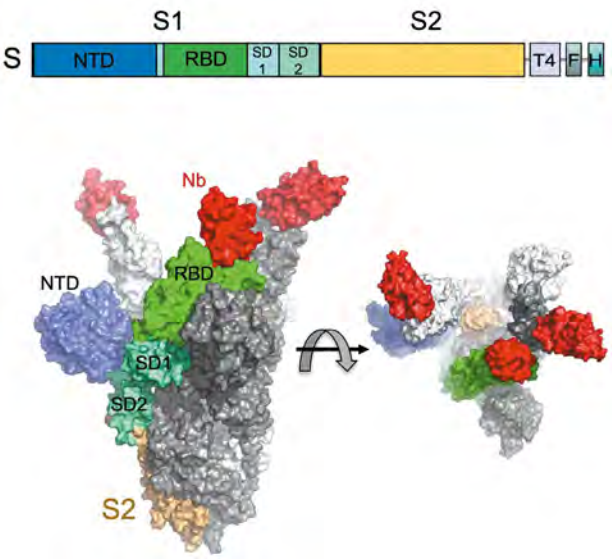
Cell-cell and virus-cell interactions

Our group studies the cell surface molecules that regulate the immune system and virus entry into host cells. We analyse receptor-ligand interactions related to immune processes, as well as virus binding to cells. In addition, we characterise virus neutralisation by humoral immune responses and its correlation with virus cell entry. Our research has provided key observations regarding immune receptor function, and has identified viral epitopes essential for virus infection, some of which are targeted by neutralising antibodies (Abs). Our multidisciplinary research applies structural, biochemical and cell biology approaches.

During the last two years, we have been deeply involved in several collaborative projects related to SARS-CoV-2 detection and COVID-19 prevention with Abs and vaccines. We produced the SARS-CoV-2 envelope spike (S) antigen for serological test development at the CNB-CSIC and at the company Immunostep S.L. Recently, SARS-CoV-2 materials prepared in our laboratory were transferred to WHO for serological tests implementation in Africa.

Most of our recent research work concentrated on the generation of Abs to neutralise SARS-CoV-2. We prepared Abs derived from nanobodies coming out of immunised camels, most of which neutralised the SARS-CoV-2 virus and four of them hindered COVID-19 progression in animals challenged with the virus. The Nbs recognised the S receptor binding domain and bound to different epitopes overlapping with receptor-binding motifs. We generated highly potent neutralising Abs that bound to all SARS-CoV-2 variants except omicron. They are promising candidates for the COVID-19 treatment in infected people and they have been used in a highly sensitive detection test.

Our group also cooperated in the generation of other technologies related to SARS-CoV-2 prevention, such as mouse Abs that neutralise the virus, as well as in the design and characterisation of vaccines that protected animal models from COVID-19 progression.



Structure of the SARS-CoV-2 spike S in complex with a neutralising nanobody (Nb). Top. Scheme of the S protein used to generate the structure. The extracellular S1 with the N-terminal domain (NTD), receptor-binding domain (RBD), subdomains SD1 and SD2, and the S2 region are shown colored. The soluble S contained a T4 trimerization domain (T4), a FLAG peptide (F), and a 6xHis-tag (H) at its C-terminal end. Bottom. Cryo-EM structure of the trimeric S with a Nb bound to its RBD. The S monomers are represented as surfaces, those with the open RBD are shown in white or grey, whereas the monomer with the closed RBD is with the domains colored as in A. The three Nbs bound to the RBD are shown in red. Lateral (left) and frontal (right, from the top) views of the complex..

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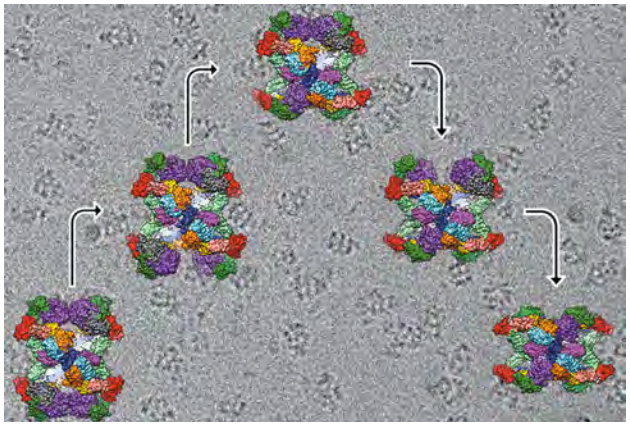
Viral molecular machines

When viruses are viewed as dynamic containers of an infectious genome, their structural, physical, and biochemical analyses become necessary to understand the molecular mechanisms that control their successful life cycle. Information on virus structures at the highest possible resolution is essential for identifying the principles of their structure-function relationship, and could lead to development of antivirals, vaccines, and the advancement of new platforms for virus-based nanotechnology.

Three-dimensional cryogenic electron microscopy (cryo-EM), which has revolutionised structural biology, is central to determining high-resolution structures of many viral assemblies in near-native conditions. We use Cryo-EM to solve near-atomic structures of infectious virions with helical or icosahedral symmetry. State-of-the-art approaches now extend beyond purified symmetric capsids and focus on the asymmetric components as the genome and viral polymerases. Asymmetric structures have important functions in many steps of the virus replication cycle, and many of these will be key targets for the development of new antiviral drugs.

Our group studies several viruses with varying levels of complexity, with focus on a number of double-stranded RNA viruses such as infectious bursal disease virus, the human picobirnavirus, and several fungal viruses (*Saccharomyces cerevisiae* virus L-A, *Penicillium chrysogenum* virus, and Yadonushivirus), as well as single-stranded RNA viruses such as human rhinovirus and rabbit haemorrhagic disease virus. We extended our studies to other macromolecular assemblies such as α_2-macroglobulin, a blood plasma proteinase inhibitor of broad specificity, and encapsulins, bacterial nanocages that naturally confine a functional protein cargo such as an enzyme. Structural analysis is complemented by study of mechanical properties by atomic force microscopy, to examine the relationship between physical properties such as rigidity and mechanical resilience, and virus biological function.

Finally, our research establishes the basis for incorporation of heterologous proteins and/or chemicals into viral capsids, considered as nanocontainers or nanocarriers, of potential use for future biotechnological applications.



Cryo-EM structures of human α_2-macroglobulin functional states. Human plasma α_2-macroglobulin is a 1451-residue protein built of 11 domains. Four protomers associate to a ~720-kDa polyglycosylated complex [$(h\alpha_2M)_4$] with pan-peptidase inhibitory functions that transits between an open native conformation (bottom, left) and a closed induced state (bottom, right), in which endopeptidases are trapped upon cleavage of an accessible bait region. The background shows a cryo-EM image of $(h\alpha_2M)_4$.

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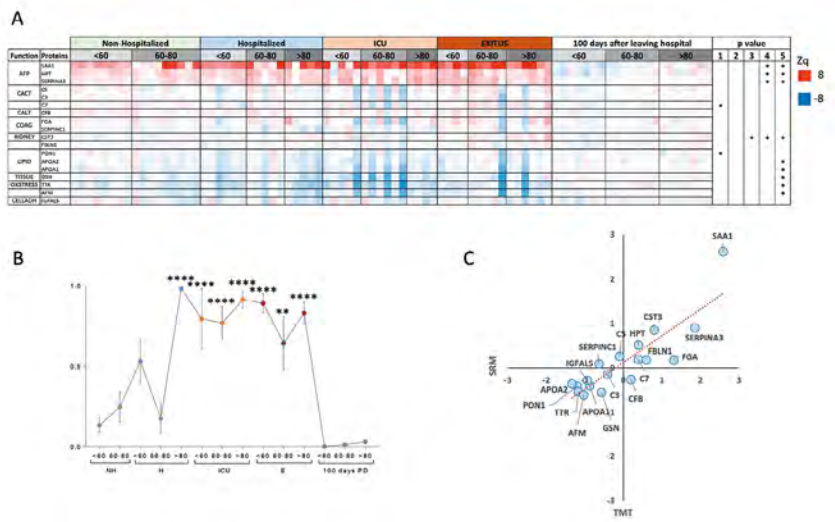
Functional proteomics

The activity of the Functional Proteomics laboratory of the CNB is focused in two main research areas of interests: the study of mechanisms underlying the progression of liver diseases and COVID-19 using proteomics. We have developed sample processing methods that increase three-fold the coverage of human bile proteome (Ciordia S *et al*, J Proteomics, 2022 and Meth Mol Biol 2022), allow for the study of HLA-presented phosphopeptides (Marcilla M, Meth Mol Biol 2022) and to quantitate one carbon metabolism (OCM) enzymes (Guerrero L *et al*, Meth Mol Biol 2022). The analysis of serum samples from COVID-19 patients revealed a protein panel feasible to assess disease severity (Núñez E *et al*, Biomedicines 2022) and liver proteome and phosphoproteome dynamics shed light on the molecular basis of liver cancer progression (Colyn L *et al*, J Ex Clin Cancer Res 2022) and point to OCM cycle as a functional biomarker for liver disease patients prognosis (Guerrero L. *et al*, J Physiol Biochem 2022; Guerrero L *et al*, Metabolites 2022). We have also identified potential biomarkers of pre-eclampsia in exosomes from afflicted patients (Navajas R *et al*, Clin Proteomics. 2022). Our research is granted by CSIC (2021-2023), MICIN (2022-2026) and CAM (2023-2027). Finally, we have leadership in national and international initiatives such as the Spanish Proteomics Society, European Proteomics Association and the Human Proteome Project.



COVID-19 severity markers monitored by SRM. *(A) Protein abundance changes (Zq) normalised by the average values of the 100 days after leaving hospital patient group. Up-regulated proteins are in red and down-regulated proteins in blue. (B) Prediction of disease severity using a logistic regression model. (C) Protein quantification from TMT and SRM experiments.*

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Ultrastructure of viruses and macromolecular aggregates

The main line of the group is focused on the study of viral ribonucleoproteins (RNPs) that conforms the virus nucleocapsid of some enveloped single stranded RNA viruses. RNPs are macromolecular complexes composed of the genomic RNA bound to multiple monomers of a nucleoprotein and, in some cases, a single copy of the viral polymerase.

In recent years our laboratory has determined the structure of the influenza A RNPs at medium resolution and we have verified that this structure is present in native virions using cryogenic electron tomography. We have also elucidated the structural basis of the transcription process, that is, how RNP produces messenger RNA in influenza A. We are currently extending these studies to the genome replication process of this virus.

Within the framework of research on Covid-19, our laboratory has developed a model for structural and biophysical studies on coronaviruses, using the transmissible gastroenteritis virus (TGEV) as a surrogate, on which we have carried out studies on its biomechanical properties and its resistance to the use of virucidal agents.

On the other hand, our laboratory is also developing a system for the determination of the structure of membrane proteins based on the use of nanodiscs and liposomes by means of cryogenic electron microscopy. In this field, we have solved the structure of two actinoporins, pore-forming proteins, at a resolution of 2 Å. These studies allow us to determine the structure of membrane proteins in the bilayer and to show the interactions of the proteins with lipids.

1 *The left panel shows high-resolution averages of a pore formed by a protein on lipid nanodiscs, front (top) and side (bottom) views. The right panel shows two details of the high-resolution structure solved using cryogenic electron microscopy, including some amino acid and lipid constituents of the pore structure.*

2 *The left panel presents a cryoelectron tomogram section of a TGEV virion showing the spike proteins (blue arrowheads), the double layer membrane (green arrowheads) and the RNP inside the envelope (red arrowheads). The right panel shows intact virions visualised by Atomic Force microscopy in buffered solution. Blue arrowheads point to putative spike. The colour palette shows the surface height. Scale bar represents 50 nm.*

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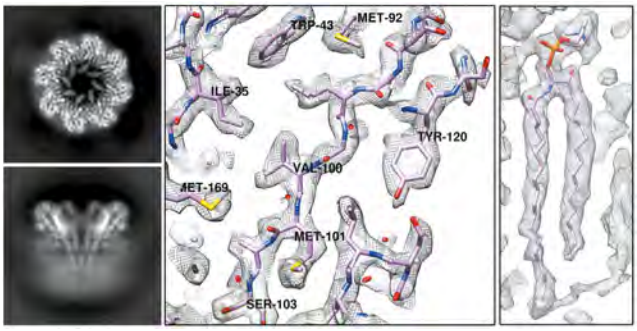

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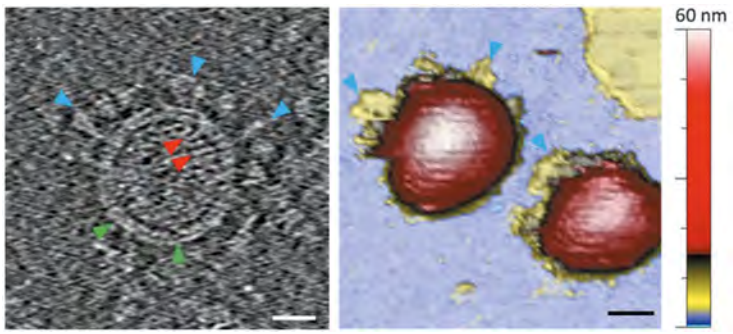

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Molecular biophysics of DNA repair nanomachines

Our group is interested in the development and use of single-molecule techniques to study the mechanical properties of nucleic acids and the mode of action of protein machines involved in the repair, replication and maintenance of DNA structures. We use novel single-molecule approaches based on atomic force microscopy (AFM), optical and magnetic tweezers, and their combination with confocal and TIRF microscopy.

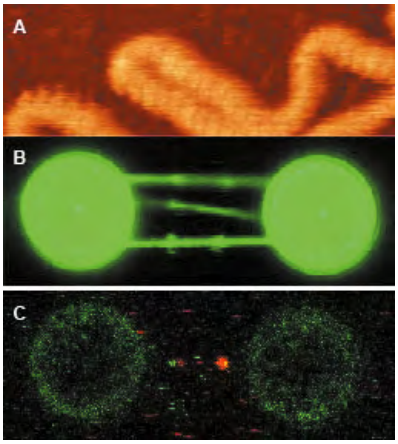
During the last two years, we have continued developing a combined TIRF-AFM system. The combination of high-resolution AFM imaging with single-molecule fluorescence will allow us to correlate morphology with the presence of a particular protein. We had also mastered the use of C-Trap™ from Lumicks, a hybrid system combining optical tweezers and confocal microscopy, which enables manipulating single DNA/RNA molecules while simultaneously imaging the fluorescence between the beads in real time.

Using our expertise in AFM, we showed that NIHCOLE, a lncRNA induced in hepatocellular carcinoma, folds into dsRNA secondary structures and binds the Ku heterodimer, a component of the NHEJ pathway of DSB repair machinery, cooperatively (Unfried et at. 2021). This AFM work involved the development of new methodology to analyse AFM images and to assign sequence regions to folded domains. We have also captured the diffusion of ParB along dsDNA for the first time using the C-trap™ instrument (Balaguer et. al. 2021); and characterised the biochemical activities of the human DNA helicase B (HELB). We showed that HELB is a monomeric protein that binds ssDNA and uses ATP hydrolysis to translocate along ssDNA in the 5'-3' direction. We also showed that HELB translocation is accompanied by the formation of DNA loops (Hormeño *et al*. 2022). Our work required the development of new methods to fabricate long duplex and hybrid single-stranded/double-stranded DNA molecules for single-molecule experiments (Aicart-Ramos *et al*. 2022).

1 *(A) High-resolution AFM image where the double-helix structure of the DNA molecule can be observed. (B) Confocal image (C-trap) where three DNA molecules held by two optically trapped microspheres are visualised. (C) Confocal image (C-trap) where the binding between a protein and a DNA molecule trapped using 2 optical traps is observed.*

2 *AFM image of lncRNA molecules.*

1



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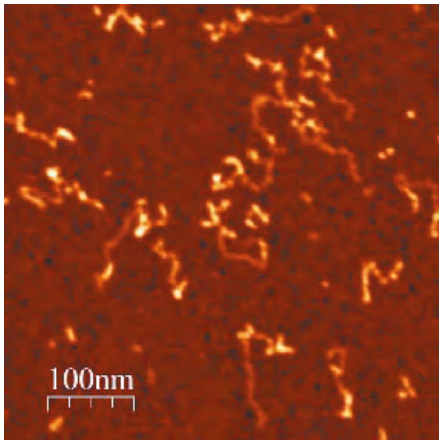

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Structural biology of bacteriophage and virus proteins

Correct recognition of the bacterial cell wall is of crucial importance to the life cycle of a bacteriophage, both in deciding which bacterium to infect as in lysing the host after phage multiplication. We perform high-resolution structural studies on the proteins involved in these processes, using X-ray crystallography, preferably in complex with their receptors or substrates.

Tailed bacteriophages bind to their host cell receptors via specialised spike or fibre proteins. In the last two years, we have solved structures of the fibre protein gp20 of *Salmonella* phage epsilon15 in complex with fragments of its lipopolysaccharide receptor and of the Staphylococcus phage S24-1 fibre protein orf16 in complex with a teichoic acid analogue. In an international collaboration, we also analysed structures of antibody Fab fragments in complex with teichoic acid (Figure 1; Di Carluccio *et al*, 2022), identifying important teichoic acid recognising features.

To lyse their host cells, bacteriophages produce endolysins that digest the bacterial peptidoglycan layer. In the last two years, among others, we have solved structures of the Pseudomonas phage JG004 endolysin Pae87 in complex with a peptidoglycan fragment. We identified a previously unknown intra-domain substrate binding domain likely to be common to a whole subfamily of monodomain endolysins (Figure 2; Vázquez *et al*, 2022). Other endolysins have separate cell wall binding domains, while Pae87 has incorporated a cell-binding subdomain in its enzymatic domain.

Knowledge of the structures of bacteriophage receptor-binding and endolysin proteins may lead to different applications. Modification of the bacteriophage fibre receptor binding specificities may lead to improved detection of specific bacteria and to mutant phages with improved host ranges. A better understanding of endolysin structure, stability and specificity may similarly lead to better elimination of pathogenic or otherwise unwanted bacteria, and to mutant endolysins with a different or wider range of target bacteria.

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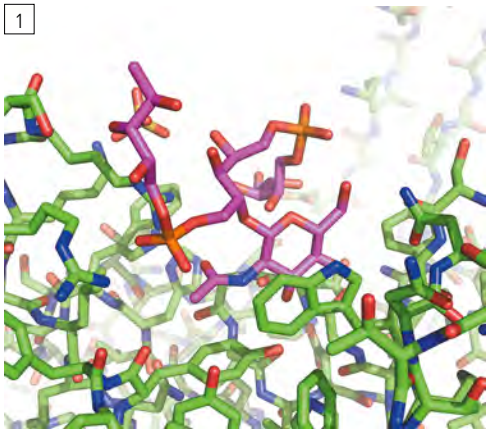

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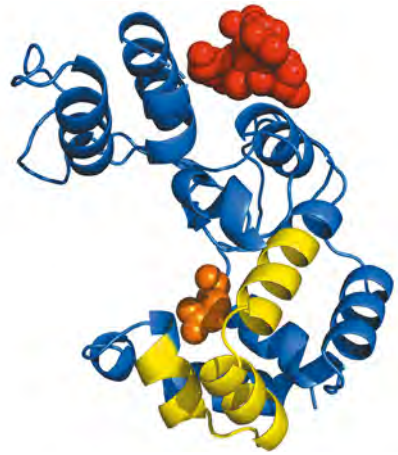

1 *Detail of a crystal structure of a teichoic acid fragment (pink backbone) bound to a Staphylococcus antibody Fab domain (green backbone).*

2 *Crystal structure of the Pseudomonas phage JG004 endolysin Pae87 (in blue and yellow). In orange, a buffer molecule in the active site is shown; in red a peptidoglycan fragment is bound to the opposite side of the protein.*

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Cell structure laboratory

The Cell Structure Laboratory makes use of state-of-the-art imaging techniques to study virus-host interactions. Together with cell biology, virology, biochemistry and proteomics, we use light microscopy, transmission electron microscopy (TEM) and correlative light and electron microscopy (CLEM) to unveil new targets for antiviral treatments.

The pandemic caused by SARS-CoV-2 made evident that we count with very few virus-fighting drugs. The current list of priority diseases of the World Health Organization includes pathologies caused by three coronaviruses and by three bunyaviruses.

During the last two years we have studied the mechanism of action of several compounds that inhibit bunyavirus growth. We have also discovered how Plitidepsin blocks SARS-CoV-2 infection in cultured cells. Plitidepsin is a potent anti-SARS-CoV-2 antiviral that targets a cell protein used by the virus and is currently being studied in clinical trials (phase III) to treat Covid-19. We have also validated four more repurposed drugs from our library in cells infected with two different coronaviruses. One of the compounds is a promising candidate to be used in the therapeutic treatments for SARS-CoV-2 and possibly other respiratory viruses. This compound is currently being tested *in vivo* with one of the animal models for Covid-19.

With our studies on the cell biology of viral infections, we have discovered the role of the lipid transfer protein (LTP) NPC1 in early, post-entry steps of reovirus infection. In addition, CLEM studies helped to discover a new pathway for bunyavirus propagation that bypasses the secretory pathway of the cell. The role of several LTPs, lipid flows and mitochondrial proteins in bunyavirus infection has been also studied in detail. This knowledge will be crucial to characterise promising compounds that interfere with host factors whose implication in key biological processes can be applied to design pan-antiviral strategies.

1 *Live-cell imaging and CLEM of bunyavirus-infected cells uncovers a new mechanism for virus propagation. Scale bars, 20 µm; 200 nm (image on the right).*

2 *TEM of cells infected with SARS-CoV-2 in the absence (image on top) and presence (bottom image) of Plitidepsin. The viral replication organelle (asterisks) is not assembled in cells treated with the drug. N, nucleus. P, plasma membrane. Scale bars, 1 µm.*

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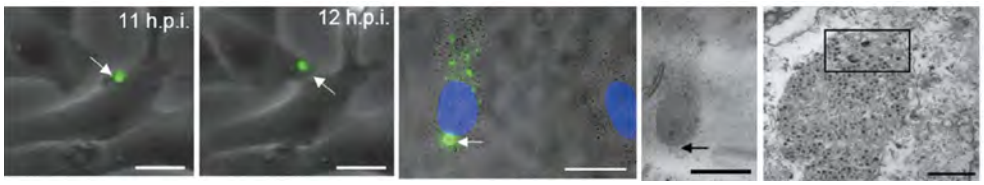

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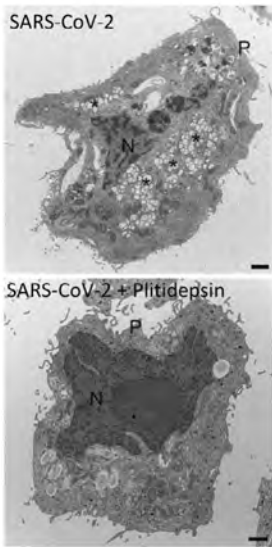

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Structural and physical determinants of complex virus assembly

We investigate the principles governing complex virus assembly. Our main model system is adenovirus, an icosahedral virus with a capsid composed of more than 10 different proteins. Adenoviruses are pathogens, but they can be engineered as therapeutic tools. Understanding adenovirus assembly poses many challenges due to the virion complexity. Although several hundred adenovirus types are described, most of our knowledge focuses mainly on HAdV-C5. From the 2021-2022 period, we highlight our work elucidating two new adenovirus structures.

Enteric human adenoviruses are one of the main global causes of viral diarrhoea, but knowledge on their biology lags behind that of adenoviruses with respiratory or ocular tropisms. We solved the structure of an enteric adenovirus, HAdV-F41. We focused on relating its structural features with survival to the deleterious conditions found in the gastrointestinal tract. We described differences in the capsid surface that may account for resistance to neutralisation by intestinal defensins, as well as partially ordered structural elements that could have a role in enhancing the stability of the HAdV-F41 capsid.

We also solved the first high-resolution structure of an adenovirus infecting a non-mammalian host: lizard adenovirus LAdV-2. LAdV-2 virions carry on their surface a protein called LH3 that does not exist in human adenoviruses. Instead, human adenoviruses have a protein called IX, with a very different fold (even mostly disordered in HAdV-F41). They also produce another protein with certain sequence similarity to LH3, E1B-55K, which is not part of the viral particle, but alters the cell division cycle. Our analysis showed that the LH3 gene was probably captured from a bacterial gene, incorporated as capsid decoration in reptilian adenoviruses, and later on duplicated to become protein IX and the non-structural, oncoprotein E1B 55K in mammalian adenoviruses. This work provided information on the evolution of complex viruses, and protein function exaptation across evolution.

Structures of HAdV-C5 (left), the most studied adenovirus and used as reference in the field; the enteric adenovirus HAdV-F41 (centre); and the reptilian adenovirus LAdV-2 (right). Proteins IX and LH3 are depicted in gold.

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Structure and function of molecular chaperones

We use different various biophysical techniques, mostly cryoelectron microscopy (cryoEM), to study the structure and function of different macromolecular complexes, in particular those formed by molecular chaperones, a group of proteins involved in cell homeostasis through two opposite functions, protein folding and degradation. These two cellular processes are carried out through the transient formation of complexes between different chaperones and cochaperones, acting like an assembly line and making the process a more efficient one. Our main goal is the structural characterisation, at the highest possible resolution of some of these complexes, using as a main tool state-of-the-art cryoEM and image processing techniques. We also aim to study from a structural point of view the implication of different chaperones in the regulation of complex cellular events as the immune synapse. For that we are implementing correlative approaches to locate and resolve molecular events in a native cellular context.

We are also interested in the quantitative study of molecular events at the membrane interface, focusing on the role of the membrane physical properties in the functional outcome of molecular interactions. Our toolbox majorly includes advanced optical microscopy and single molecule optical spectroscopy techniques (such as single-molecule tracking and Fluorescence Correlation Spectroscopy).

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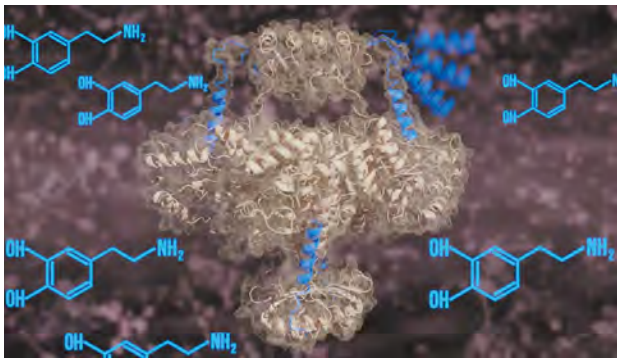

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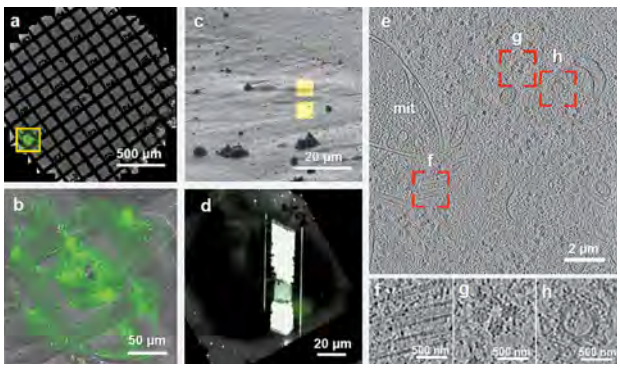

1 Artistic image of the tetrameric enzyme tyrosine hydroxylase with the 3D reconstruction obtained by cryoelectron microscopy (envelope) in which the atomic model has been fitted. The blue *α*-helices are a flexible part which can block the active site depending on the level of dopamine (its chemical formula drawn in the background) present in the solution

2 Cryocorrelative light and electron microscopy (CLEM) workflow for lamellae preparation of Jurkat cells expressing GFP-EB3 protein. (a) overlay of bright field (gray) and maximum intensity projection of cryoconfocal microscopy stack of images of Jurkat cells attached to the grid. (b) higher magnification of the area yellow squared in (a). (c) Focused Ion Beam image of the area in (b) where the milling pattern positioned using the confocal fluorescence information is shown in yellow. (d) cryo-TEM image of the area shown in (c) overlayed with a single slice of a confocal stack showing EB3 protein signal. (e) cryoelectron tomogram section selected based on the confocal imaging information; mit, mitochondrion. (f-h), higher detail of the areas squared in (e);(f) microtubules; (g) ribosomes and (h) a coated vesicle.

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