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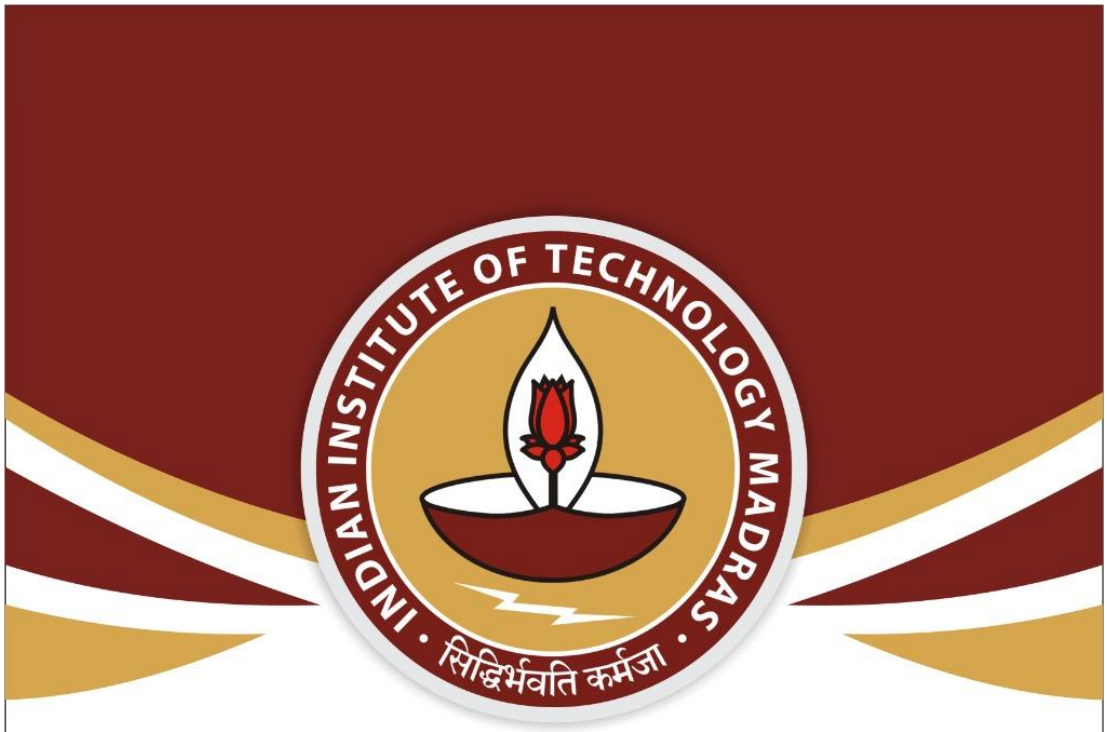

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Recent Advances in Cryogenic Electron Microscopy and Tomography

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Cryogenic electron microscopy (cryoEM) has emerged as a high-resolution tool for structural biology, capable of revealing structural details of biochemically purified macromolecules equivalent to those obtained from X-ray crystallography. Increasing numbers of cryoEM structures of soluble and membrane proteins, as well as RNA, have been reported to resolve waters, ions, and ligands. Rigorous methods have been established to assess their quality and reliability in cryoEM structures. Such quantitative measures at global and local regions are necessary for users in the objective interpretation of chemical mechanisms or guiding drug design for macromolecules on a solid biophysical basis. Cryogenic electron tomography (cryoET) is shown to be useful for purified macromolecules or macromolecules *in situ*. Subtomogram averaging has been advanced to resolve structures to near atomic resolution. In addition. Combination of cryogenic focused ion beam-scanning electron microscopy (cryoFIB-SEM) and cryoET has opened the possibility of revealing subcellular components in cells, organoids, and tissues to capture different snapshots of cellular processes. This emerging technique still requires a lot of refinement in sample preparation, instrument improvement, image processing and 3D visualization. Nevertheless, its potential impact in basic biology discovery and translational biomedicine application can be immense.

Reconstructing dynamic processes in the virus life cycle using cryoelectron microscopy

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Disassembly or uncoating is a key early step in the life cycle of viruses. This step involves the release of the viral genome from packaging proteins or capsid, for downstream replication, translation and formation of progeny viruses. Typically, this step is also associated with puncturing of the host cellular membrane which allows the viral genome to be deposited in the cytosol. Non-enveloped icosahedral viruses have a stable and symmetric capsid, which encapsulates the genome. While stability of the shell is crucial for protecting and transporting the viral genome; dynamic behavior of specific capsid components is also required during cellular membrane penetration and viral genome release in the early stages of entry. We are attempting to establish the molecular pathways of disassembly for non-enveloped viruses using a model system – a T=3 icosahedral insect virus, Flock House Virus (FHV) (1). *In vitro* heat-induced disassembly of infectious FHV particles indicated stepwise alterations in capsid conformation leading to disassembly, which is not evident in non-infectious mutated versions of the same particle (2). Cryoelectron microscopy and single particle reconstructions of disassembling particles indicated conformational alterations including “puffing” of particles triggered by movement of subunit proteins, and major alterations at symmetry axes. Asymmetric reconstructions indicated directional genome release from particles, which suggests structural differences in sequentially identical capsid proteins occupying different positions in the icosahedral asymmetric units of the capsid (2). Further, mutations in the capsid protein and associated alterations in the positioning and behavior of membrane penetrating peptides, leading to reduction in infectivity, highlighted the importance of sequential structural changes for disassembly (3). It is hoped that cryoEM and single particle reconstruction of disassembly intermediates can be utilized to create a molecular roadmap for conformational alterations in viruses during the early stages of host cell interaction and entry.

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Overcoming Preferred Orientation in cryo-EM: Insights into Arp2/3 complex Mediated Actin Nucleation

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The limited orientation of particles in thin ice presents a significant challenge for structure determination using electron cryogenic microscopy (cryo-EM) of diverse macromolecular complexes. This issue leads to an uneven distribution of particle views, resulting in subpar map quality and information loss, manifesting as density smearing corresponding to the missing views. To address this limitation, various sample-specific approaches have been developed, including the use of additives like detergents, specialized support films such as graphene oxide or continuous carbon on holey grids, and adjusting the grid hole size for data collection. However, an alternative solution lies in tilting the microscope stage, offering a universal method to mitigate the “preferred orientation” problem without extensive sample manipulation.

Actin networks often exhibit limited orientation in thin ice films, hindering our understanding of how the key actin filament nucleator, the Arp2/3 complex, initiates new actin filaments. By leveraging the unique biological properties of complexes and tilting the specimen, we successfully overcame this issue and obtained high-resolution reconstructions of different actin architectures nucleated by the Arp2/3 complex. These structures provide crucial insights into the precise conformational changes that the Arp2/3 complex undergoes during activation, elucidating the molecular contacts between the fully activated Arp2/3 complex, the nucleated daughter actin filament, and the mother actin filament.

Structural studies on human orphan receptors

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G-protein–coupled receptors (GPCRs) are a large family of receptors that have many orphans, which are receptors whose signaling reactions are not well known. One of these orphans is GPR158, which is found at high levels in the nervous system and plays a role in processes such as cognition, memory, and mood. However, the structural organization and signaling mechanisms of GPR158 are not well understood. To gain insights into this orphan receptor, we used single-particle cryo–electron microscopy (cryo-EM) to determine the structures of human GPR158 alone and bound to an RGS signaling complex. We found that GPR158 has an unusual dimerization mode that is stabilized by a pair of phospholipids and an extracellular Cache domain, which is a rare ligand-binding domain in GPCRs. The extensive set of interactions at the dimeric interface locks GPR158 in a conformation that is likely to prevent G protein activation. RGS binds to the homodimer at a site that substantially overlaps the surface that binds G proteins, preventing canonical G protein signaling. The binding of a ligand to the extracellular domain may regulate signaling through the RGS complex. Our collaborative study provides insights into the unusual biology of orphan receptors and the formation of GPCR-RGS complexes, where RGS is coupled to GPR158 instead of heterotrimeric G-proteins or arrestins, thereby bringing a paradigm shift in signaling pathways.

Cryo-EM structure of the Sacbrood virus at 3.5 Å

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Honey bees are popularly known as mysterious forest dwellers and are responsible members of the agricultural ecosystem. They not only bestow us with high-quality products such as honey, beeswax, propolis, and honey bee venom but are also central drivers for pollination. Nevertheless, high colonial losses have been reported in different parts of the world for the past few decades. There are many possible reasons for the decline in the bee population, with viruses being a major threat. The first virus identified to infect honeybees is the Sacbrood virus (SBV). The infection results in a 90 – 95% decline in the honey bee population, directly reducing the production of bee products. The decreased bee population also affects crop yield, as about 70% of food production is dependent on pollination by honey bees. In this talk, I will explain our efforts to obtain the high-resolution cryo-EM structure of Sacbrood virus (SBV) at 3.5Å. The structure will help us to understand the arrangement and interaction of capsid protein which will ultimately aid us to develop curative medicines.

Cryo- EM studies of ribosomes from pathogenic protozoa Entamoeba histolytica, reveal unique features of its architecture

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Entamoeba histolytica (Eh), a parasite protozoan, is responsible for amebiasis, an intestinal infection that results in bloody diarrhea and liver abscesses. Amoebiasis is more predominant in tropical areas with poor sanitation conditions, including India. Therefore, amoebiasis puts a huge economic burden on our country. Although ~40% of antibiotics in clinical use target functional centers for protein synthesis machinery, the ribosomes, the current treatment involves only one such drug (paromomycin) for clearing the asymptomatic Eh infection. The currently used drugs have their own side effects, and the drug resistance strains of Eh are also emerging. Moreover, there is no vaccine available to prevent amoebiasis. We determine Eh ribosome's high-resolution cryo-EM structure using cryo-EM single particle reconstruction of the 60S and 80S at 2.8 Å and 3.4 Å resolution, respectively. The structural analysis revealed several unique features such as triple helix structure in 28S rRNA, co-evolution of rRNA segment and r-protein, several Eh specific expansion segments in rRNA, and extension in r-protein. The ribosome also shows helical assemblies. These unique features of Eh ribosome architecture will be presented.

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Knotted proteins: A tale to untangle

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Knotted proteins are a special class of proteins that challenged our understanding of protein folding process. The backbone of these proteins cross over themselves forming a knot , plausibly providing additional mechanical stability against proteasomal degradation and improved ligand binding abilities. In addition, it is not well understood if these proteins needs chaperone assistance to fold back into their knotted structure upon unfolding. In this talk, I will present our recent results on understanding the folding/unfolding process as well as the degradation process of two different knotted proteins with 41 and 52 topologies. Intrinsic tryptophan as well as the near IR fluorescence studies on mi-RFP, a 41 knotted protein show that an unfolded knotted protein doesn't regain its native knotted structure but folds back to a different compact state. In addition, studies using proteases ClpXP and ClpAP show that the knots do not provide any considerable mechanical stability. I will also discuss how SM-AFM could provide additional insights into the folding process of these proteins.

Gold Nanoparticles in the Era of the Resolution Revolution.

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Gold nanoparticles (AuNPs) produce a characteristic and clearly distinguishable image in the electron microscope due to strong electron scattering of the heavy atoms. Water-soluble AuNPs of discrete sizes and controlled reactivity can be attached to biological samples for subsequent visualization under the electron microscope [1]. Direct conjugation of a surface exposed cysteine to the gold core, with the formation of a specific, rigid, and stable covalent bond opens the door to explore the potential of AuNPs in the field of cryo-electron microscopy (cryo-EM) and cryo-electron tomography (cryo-ET). The variety of applications ranges from the use of AuNP-conjugates in a rapid and computationally inexpensive assessment of protein flexibility [2] to tracking a specific small protein in the crowded cell environment through the endocytosis pathway by cryo-ET [3].

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Evolution of Single Particle CryoEM- from Blobs to True Atomic Resolution

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Determining the three-dimensional structures of macromolecules is important to understand their functions and role in human health and disease. Single particle cryoEM has evolved significantly, emerging as a powerful method for direct visualization and 3D reconstruction of macromolecules and their complexes at near atomic-level resolution. A notable milestone was achieved recently with the determination of the apoferritin structure at true atomic resolution. In this presentation, I will delve into the latest technological advancements across the entire single-particle workflow, which have played a pivotal role in driving and expanding the resolution revolution, thereby establishing cryoEM as a mainstream technique in structural biology.

Development and Applications of Microcrystal Electron Diffraction (MicroED)

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A common barrier to high-resolution structure determination is the growth of large well-ordered crystals. Electron diffraction is capable of producing diffraction data from crystals that are orders of magnitude smaller than those needed for conventional X-ray crystallographic experiments. The cryo-EM technique of microcrystal electron diffraction, or MicroED allows the collection of high-resolution diffraction data from extremely small nano and microcrystals that resist other structure determination techniques. The method has been used to solve high-resolution structures from a variety of diverse of targets and is becoming a powerful tool for macromolecular and chemical crystallography. In this presentation, MicroED methods will be described along with representative applications of the method to biomolecular crystals, organic crystals, and materials. Additionally, current work in our lab, which is focused on improving MicroED methodology and extending this technique to new samples will be presented.

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Unraveling the structural basis of reaction specificity in PLP-dependent decarboxylases

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Pyridoxal-5′-phosphate (PLP) is a ubiquitous cofactor of over 238 distinct enzyme reactions including decarboxylation, transamination, racemization, elimination and substitution in amino acids, amines or oxoacid substrates. The basic chemistry of the multiple mechanistic steps involving the cofactor and the enzyme is universal and well-described. However, the structural basis of the exquisite reaction specificity within a highly conserved protein fold and active site pocket is poorly understood owing to the absence of structures of two key transient reaction intermediates, namely, the Dunathan and the quinonoid intermediates. In this work, an archaeal Group II PLP-dependent Tyrosine Decarboxylase (MjDC) was employed as the model system to capture multiple snapshots of the catalytic process using *in-crystallo* enzyme complexes with the cognate intermediates. The crystal structures reveal that a subtle rearrangement of a conserved Arg residue in concert with a water-mediated interaction with the carboxylate of the Dunathan intermediate appears to directly stabilize the alignment and facilitate the release of CO_2 to yield the quinonoid. Moreover, the conformational switch of a dynamic catalytic loop from an open-to-closed form controls a conserved network of hydrogen bond interactions between catalytic residues to protonate the quinonoid to complete the reaction cycle. Our results provide a structural framework to elucidate mechanistic roles of residues that govern reaction specificity and catalysis in PLP-dependent decarboxylation.

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A tale of two kinases: Unraveling the mechanistic differences between IKK1 and IKK2 using CryoEM and other tools

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Phosphorylation of proteins, catalyzed by protein kinases, ushers novel physico chemical properties to them. In many occasions they are considered crucial drug targets. Needless to mention, many protein kinases are important signaling molecules. There are more than 500 proteins kinases coded in our genome and about a third of our proteome are phosphorylated. Regulation and roles of protein kinases follow complex, context dependent mechanism.

Inhibitor of kappaB Kinases (IKKs) are the key regulators of the NF-kappaB pathways. Despite high sequence similarity and similar domain organizations, they function very differently. IKK2/β show a rapid activation kinetics in response to a plethora of extracellular cues including, but not limited to, pathogenic and inflammatory signals to activate the canonical NF-κB pathway to regulate innate immune response. IKK1/α, on the other hand, show a slow and sustained activation profile that in turn activate the non-canonical/alternative NF-κB pathway involved in the developmental and adaptive immune responses.

It is however not precisely known why these two kinases behave so differently. We employ CryoEM in addition to biochemical, cellular, mass spectrometric and X-ray crystallographic analyses to understand the differences between these two kinases. We found that they employ very different mechanism to assert substrate specificity. We are currently trying to understand the structural basis for the nuances of the function of these kinases and their regulation. Their molecular weight (~80kDa) and intrinsic conformational flexibilities make the problem non-trivial. Difficulties in performing such studies will be discussed.

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Cryo-EM analysis of epitope targeting by broadly neutralizing antibodies

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Structural elucidation of surface epitopes on pathogenic enveloped viruses can provide insights into how these viruses are recognized by neutralizing antibodies. In the case of Human Immunodeficiency virus (HIV)-1's envelope glycoprotein (Env), very little information is known in terms of how broadly neutralizing antibodies (bnAbs) against the Env trimer overcome its inherent sequence, structural and antigenic variability to bind across different strains of HIV-1. In this study, single particle cryo-electron microscopy in combination with structural mass-spectroscopy has been used to analyze the binding characteristics of an apex-targeting bnAb against HIV-1's Env. High resolution structures against divergent Env isolates have shed light on how the bnAb likely achieves breadth and potency by binding in a highly focused fashion to its 3D epitope. Our results demonstrate that antibody binding can be influenced by both protein and glycan units, with glycans playing an important role in antigen recognition.

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Signal induced antibiotic biosynthesis in Streptomyces

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Streptomycetes are a prolific source of specialized secondary metabolite (SSM), including antibiotics and drugs used in veterinary, agriculture, and human medicine. These SSMs are produced by a biosynthetic gene cluster (BGC) encoding an assembly-line of mega-enzymes with multiple domains which are under a tight control of transcription regulators (TRs). TRs respond to the inter- and intra-cellular signals as well as the cues originating from the environment and play a central role in coupling these signals to the physiological response of SSM biosynthesis providing survival advantage to the producer organism. Thus, major bacterial signaling systems use a receptor domain to sense the stimuli along with an output domain responsible for transcription regulation through DNA-binding. Biosynthesis of SSMs is heavily regulated by TRs as it diverts the cell's resources towards the production of these expendable compounds, which also have clinical applications. These TRs have evolved to relay information across specific signals and target genes, thus providing a rich source of unique mechanisms to explore towards enhanced SSM production. Moreover, understanding this mechanism aids in activating the silent BGCs (not expressed under laboratory conditions) that has tremendous novel SSM discovery potential, which are addressing the rapid escalation in antimicrobial resistance.

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Tracing the cytoskeletal blueprint in vitro

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The size and shape of all eukaryotic cells are regulated by the cytoskeleton, which is made up of actin, microtubules, and intermediate filaments. These filaments are bound by multiple proteins which regulate their dynamics and mediate the transport of molecular cargo within cells. Studying the cytoskeletal system in living cells is challenging due to its indispensable and complex nature. In our studies, we took a bottom-up approach involving *in vitro* reconstitution and cryo-electron microscopy (cryo-EM) to investigate various aspects of filament organisation. This method allowed us to identify important characteristics of microtubules and associated proteins in neuronal cells and parasite systems. In this talk, I will outline the unique mechanism by which the essential protein Microtubule-associated protein 7 (MAP7) stabilises the microtubule lattice in neurons. I will also discuss the significant challenges in the determination of the three-dimensional structure of microtubules that were overcome to uncover this mechanism. Additionally, I will show how key differences between the dynamics of the malarial parasite and mammalian microtubules can be explained using a combination of three-dimensional structures and molecular dynamics simulations. This is valuable in understanding the cytoskeletal variations observed in cellular forms of the parasite as well as for comparison with its mammalian counterparts with implications in drug binding and inhibitor studies.

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