Clathrin mediated endocytosis - Methods and Protocols
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Clathrin is one of the interesting "*moonlighting proteins*" which perform multiple functions relevant to biochemical or biophysical aspects. It can be considered the master regulator of vesicular trafficking being the main player of the Clathrin-Mediated Endocytosis (CME).

The eighteen chapters of this book unveil different aspects of CME with detailed description of methods and protocols that can be reproduced or adapted according to the different needs of our studies but with special attention to neurotransmission.

Chapters 1 and 2 focus on rat brains and on the protocol for isolating Clathrin-Coated Vesicles (CCVs) by using differential centrifugation coupled with density gradient centrifugation (the article is supported by a scheme that makes the methodology easier to follow), and the isolation and purification of intact synaptosomes from brain tissue to examine the molecular mechanisms of neurotransmission. The schematic representation of the protocol and the picture showing the correct layer containing the synaptosomes after density gradient centrifugation are also very useful, too.

Chapter 3 describes how to study endocytosis during the cell cycle with minimal perturbations. Protocols usually include steps that involve serum-starvation, dominant-negative mutant overexpression, or mitotic arrest produced by chemicals to synchronize cells which induce a blockage of CME during mitosis not observed in unperturbed, dividing cells, as the authors suggest. Here, a protocol rich in images explains how to measure endocytosis in primary and normal cells cultured with minimal experimental perturbations.

The following Chapter, written by S. Boulant (Heidelberg, Germany), is really interesting because describes an advanced technology to investigate the role of membrane tension in regulating Clathrin-dependent endocytosis while performing live fluorescence microscopy. The possibility to follow *in vivo* this aspect and to monitor its contribution to the Clathrin Coated Pit (CCP) formation is very informative and of immediate impact.

Chapter 5 describes an ELISA-based, high-throughput screening method used to identify small molecules that inhibit the interactions of Clathrin terminal domain with endocytic proteins.

Neurotransmission is the topic of Chapter 6. Here the authors from the Marine Biological Laboratory of Woods Hole (MA, USA) and The Feinstein Institute for Medical Research (Manhasset, NY, USA) describe the methodology for perturbing CME at the lamprey giant reticulospinal synapse. Lampreys represent wonderful models for the identification of the mechanisms (protein-protein interactions) regulating CME in presynaptic nerve terminals and in many other different cell types.

The following chapter presents an interesting methodology useful to monitor when endocytosis is triggered and to predetermine what's inside the internalized vesicle. This method is called Hot-Wired CME and allows to control the timing of endocytosis initiation and the content of the vesicle through live cell imaging.

Real-time endocytosis measurements at the central nerve terminals is the topic of Chapter 8. The technique presented here requires a phase-sensitive detector or "lock-in amplifier" implemented in hardware or software during patch-clamp recordings. It can be applied to many secretory cells, to the large synapse of the mammalian auditory central nervous system named calyx of Held and also in mouse pancreatic β-cells. Informative figures enrich this chapter.

Drosophila melanogaster is the animal model described in Chapter 9 where the consequences of the loss-of-function mutations on endocytosis in the photoreceptors of living fruit flies is obtained by recording electroretinograms. The generation of genetic mosaicism is obtained by means of FLP/FRT mitotic recombination. As the authors explain "this system flippase (FLP)-mediated recombination takes place between two flippase recognition target (FRT) sites positioned at identical loci and in the same orientation on homologous chromosomes".

Chapter 10 deals with the disassembly of Clathrin lattice by the coordinated recruitment of auxilin and Hsc70. The methodology is here presented in a very detailed way which is certainly appreciated by scientists interested in studying this final step of endocytosis. In my opinion this is the best chapter of the book.

Phosphatidylinositol-(4,5)-bisphosphate (PI(4,5)P2) is required for Clathrin coated pits assembly: the balance of its synthesis in the bulk plasma membrane, as well as its local turnover within CCPs, controls multiple stages of CCVs formation. Chapter 11 describes an experimental procedure for the detection of PI(4,5)P2 spatial distribution and the semi-quantification of PI(4,5)P2 levels in the plasma membrane using fluorescence microscopy while Chapter 12 describes a methodology to follow adaptor-induced Clathrin assembly in real-time using fluorescence microscopy.

The following chapters describe the use of different microscopic techniques useful to image different stages of CME: TIRF microscopy (total internal reflection fluorescence) for the analysis of CCP assembly (Chapter 13); STED microscopy to image endocytic events at the plasma membrane of living cells (Chapter 14); single molecule resolution using TIRF to study the assembly of clathrin-coated vesicles (Chapter 15); electron microscopy technique that enables 3D visualization of unstained, fully hydrated cellular structures at molecular resolution to detail the structure of the mammalian synapse (Chapter 16); live cell fluorescence microscopy to study the CME in yeast (Chapter 17); the use of FM dyes in realtime to monitor CME in primary neuronal culture (Chapter 18).

All the eighteen chapters contain a note section full of tricks and suggestions to help scientists to obtain reproducible results.

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