Single cell biochemistry to visualize antigen presentation and drug resistance

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Part 1
Antigen Presentation

Presenting antigen presentation in living cells using biophysical techniques

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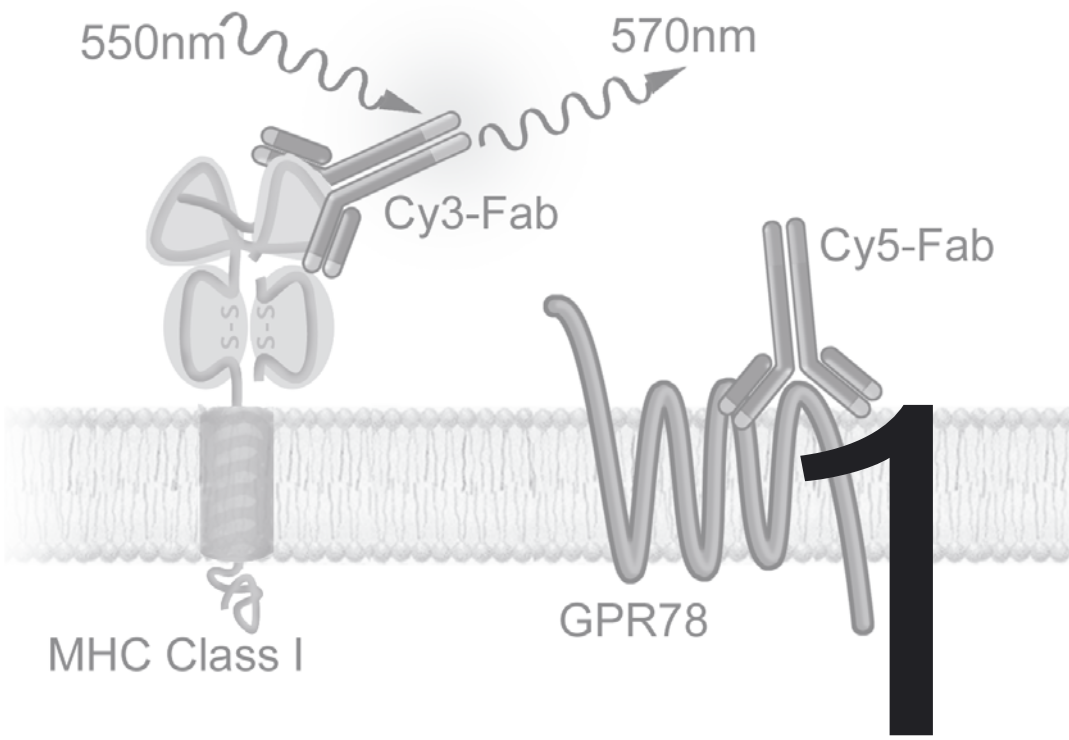

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Presenting antigen presentation in living cells using biophysical techniques

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The combination of genetically encoded fluorescent probes and advanced microscopic techniques has dramatically propelled the understanding of cell biology. Highly complex reactions can now be studied in detail in a relatively cost-effective and easy manner and, perhaps most importantly, in the context of a single living cell. In the past decade, numerous reports have uncovered the localization of key molecules in virtually all cellular processes. However, there remains a need for more accurate determination of genuine protein–protein interactions and quantification of highly dynamic processes, which has resulted in the revival of several biophysical techniques. Recent applications of these techniques have deepened understanding of processes involved in antigen presentation to the immune system.

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Introduction

Antigen presentation forms the basis on which the adaptive immune response is built. In humans, three different protein families facilitate the cell-surface presentation of antigens to cells of the immune system. Each family utilizes a separate antigen presentation pathway that contains distinct steps of antigen processing and loading. Whereas the CD1 molecule presents lipids of both endogenous and exogenous origin, the major histocompatibility complex (MHC) class I and MHC class II molecules present peptides. The MHC class II pathway is only operational in professional antigen presenting cells (APCs). In this pathway, endocytosed material forms the source of the antigens that are presented. By contrast, the MHC class I pathway is functional in all nucleated cells, and continuously samples the intracellular peptide pool derived from degraded old and incorrectly synthesized new proteins.

In this review, we will follow both peptide presenting pathways in a stepwise manner, and will discuss how biophysical techniques have visualized their functioning in a highly relevant situation—the living cell.

The MHC class I pathway

By sampling protein breakdown products in the form of peptides, the MHC class I pathway facilitates the display of a representation of the intracellular protein content to the immune system. This allows detection of changes to the intracellular protein repertoire, including malignant alterations and foreign intracellular content such as the presence of viral proteins. By way of a complicated cascade of events, degradation products from, for example, viral proteins are intracellularly processed and are presented at the cell surface by so-called MHC class I molecules (for details see **Figure 1**). Viruses have developed many strategies to inhibit the MHC class I pathway, and hence to prevent their own presentation to the immune system. Ironically, as this

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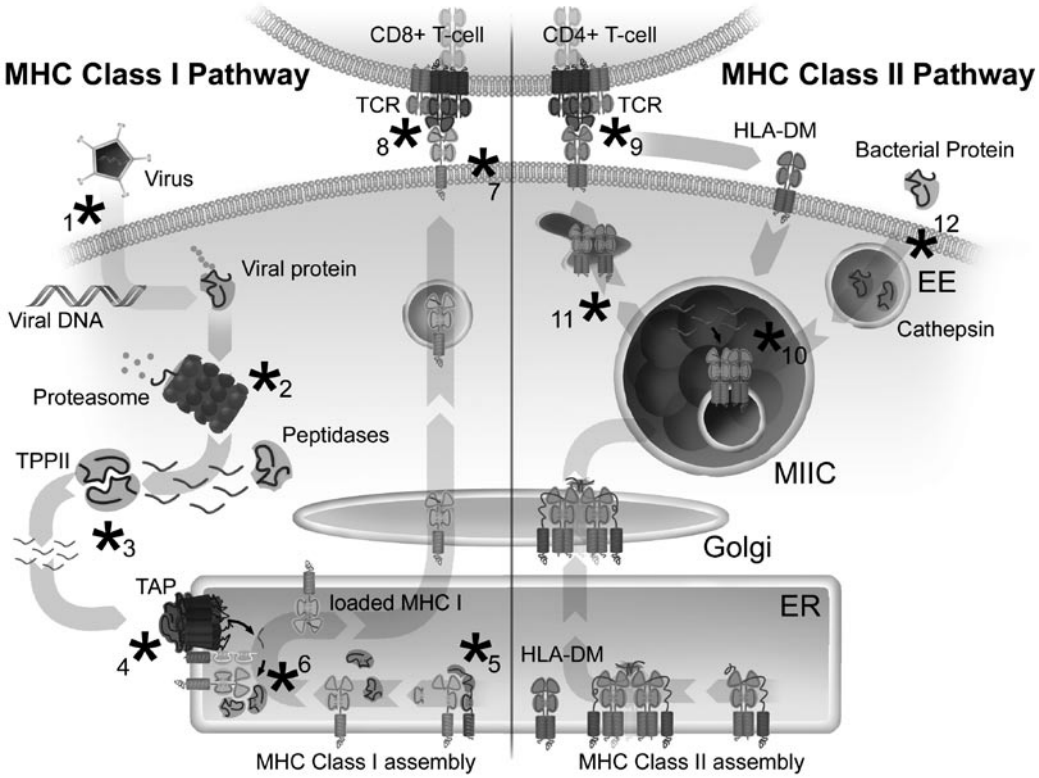

Figure 1. Schematic overview of the major histocompatibility complex (MHC) class I and II antigen presentation pathways. (**Left**) MHC class I molecules are assembled in the endoplasmic reticulum (ER), with the help of the chaperones calnexin, calreticulin and ERp57, after which they dock onto the ER-resident peptide transporter associated with antigen processing (TAP). This docking is facilitated by the specialized chaperone tapasin. TAP pumps antigenic peptides into the ER lumen. These peptides are of cellular or viral origin, and are produced in the cytosol or in the nucleus by the proteasome, tripeptidyl peptidase II (TPPII), and other peptidases. Once in the ER, peptides can bind to MHC class I molecules, which are subsequently released from the TAP–tapasin loading complex. MHC class I–peptide complexes can then leave the ER for transport to the plasma membrane. Here they can be inspected by the T-cell receptor of CD8+ T cells. Examples of processes studied using biophysical techniques that are discussed in this review include: (1) viral entry, (2) proteasome compartmentalization, (3) TPPII and peptidase activity, (4) TAP activity, (5) MHC class I assembly, (6) peptide loading, (7) plasma membrane behaviour and (8) interaction of the T-cell receptor with peptide-loaded MHC class I molecules.
(**Right**) MHC class II molecules are assembled as dimers in the ER. This assembly process is supported by the specialized chaperone invariant chain, which in addition occupies the peptide-binding groove. Three of these MHC class II–invariant chain complexes form a nonameric complex that is transported to the MHC class II containing compartment (MIIC). Here the invariant chain is degraded by cathepsins and other proteases until only the part that occupies the peptide-binding groove is left. This is called class II-associated invariant chain peptide (CLIP). In these compartments, MHC class II molecules also encounter antigenic peptide fragments derived from proteins that have been degraded in the endocytic route. CLIP is then exchanged for one of these fragments with help of the chaperone HLA-DM, and the peptide-loaded MHC class II molecules are transported to the plasma membrane for presentation of their cargo (peptides) to the immune system. Examples of processes studied using biophysical techniques that are discussed in this review include: (9) interaction of the T-cell receptor with peptide-loaded MHC class II molecules, (10) peptide loading, (11) cellular transport and (12) immune evasion.

pathway is active in almost all cells, some viruses have even evolved to exploit these molecules as co-receptors for cell entry (1).

Viral entry

To demonstrate that the interaction between a cell surface protein, glucose regulated protein 78 (GRP78), and MHC class I molecules is essential for coxsackievirus internalization, Triantafilou *et al.* (1) made use of a physical phenomenon known as fluorescence resonance energy transfer (FRET). FRET is the radiationless transfer of energy from an excited donor fluorophore to a suitable acceptor fluorophore — a physical process that depends on spectral overlap and proper dipole alignment of the two fluorophores. The occurrence of FRET is characterized by a decrease in donor emission and by simultaneously sensitized (increased) acceptor emission. Importantly, FRET is extremely sensitive to the distance between the fluorophores (its efficiency decays proportional to the sixth power of distance). Also, the characteristic half-maximum distance (Förster radius) is in the same order of size as most cellular proteins. FRET will therefore only occur when two proteins are, on average, no more than one molecule in distance apart, which usually means that they interact directly (**Figure 2**). However, if they are present at very high concentrations, FRET will occur by simple collision. FRET offers an important advantage over fluorescence co-localization studies that reveal whether two proteins are in close vicinity (>200 nm), but do not indicate if they really interact.

To detect FRET, Triantafilou *et al.* (1) used a technique called acceptor photobleaching, in which bleaching of the acceptor molecules abrogates energy transfer from the donor molecules. This results in increased donor emission. After the Cy5-labeled GRP78 molecules were bleached, Triantafilou *et al.* detected an increase in fluorescence from the Cy3-labeled MHC class I molecules, which demonstrates a genuine interaction between these molecules. In a more recent study, Martin-Fernandez *et al.* (2) also used FRET techniques to characterize the entry and the disassembly of adenovirus particles in living cells.

Proteasomal degradation

Viral infection of cells is detected by the immune system as soon as the viral products contribute to the intracellular protein pool and subsequently join the

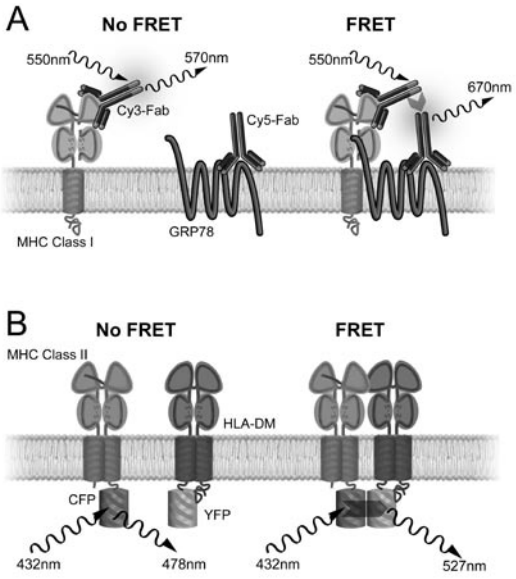

Figure 2. Fluorescence resonance energy transfer (FRET). (**A**) A schematic that demonstrates the principle of FRET. Exciting Cy3 that is linked to a MHC class I bound Fab fragment (Cy3–Fab) at 550 nm results in emission at 570 nm, unless energy is transferred to a GRP78-bound Fab fragment that is labelled with Cy5 (Cy5–Fab). This phenomenon only occurs when MHC class I and GRP78 physically interact, and results in increased Cy5 emission (at 670 nm). (**B**) A similar protocol is followed for green fluorescent protein (GFP)-modified proteins. MHC class II molecules tagged with the blue variant of GFP (cyan fluorescent protein; CFP) can transfer energy to HLA-DM tagged with the yellow variant of GFP (yellow fluorescent protein; YFP), but only when they are within 5 nm distance (i.e. when MHC class II molecules and HLA-DM are directly interacting). As a result, the observed emission spectrum after excitation with light at 432 nm will shift from 478 nm (emission of CFP) to 527 nm (emission of YFP).

household degradation pathway by way of the proteasome. This megadalton large proteolytic complex is present in both the cytosol and the nucleus. The presence of proteasomes in the latter location is rather remarkable. Given the size of the complex, it is hard to imagine that it can be transported through nuclear pores. To test whether both compartments show exchange of particular proteins, two variants of a bleaching technique can be applied: fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP). By bleaching fluorescent molecules in a region of interest within a living cell and by measuring changes of fluorescence levels in the same (FRAP) or a distant (FLIP) area, one can deter-

mine the exchange rate between these two compartments (**Figure 3**). Reits *et al*. (3) used these techniques to demonstrate that the nucleus and cytoplasm harbor spatially separated pools of proteasomes that do not efficiently exchange.

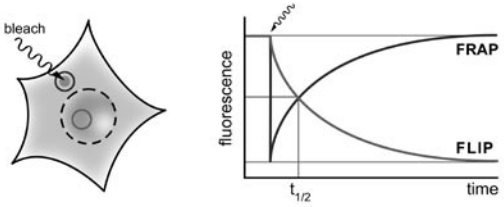

Figure 3. Fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP). By bleaching fluorescent molecules in a region of interest in a living cell (marked by the arrow) and measuring changes of fluorescence levels in the same area (FRAP) or in a distant area (FLIP), the exchange rate of fluorescent molecules between two compartments can be determined. Shown on the right is a theoretical example of a FRAP (blue line) and FLIP (red line) experiment, in which the fluorescence is measured in the marked circles. From these curves, the $t_{½}$ (half-time of recovery) can be deduced – a measure of process dynamics. The timescale for FRAP and FLIP do not necessarily have to be the same, and are dependent on the experimental setup.

Peptidases

The proteasome cleaves substrates into smaller peptides, which then become the targets of various cytosolic peptidases for further processing into single amino acids. A minor percentage of these peptides is rescued from degradation however, and sampled by the MHC class I pathway. To determine the average half-life of peptides *in vivo* and to calculate the fraction sampled by the MHC class I pathway, Reits *et al*. (4) microinjected chemically synthesized peptides that contain both a fluorescent group and a quencher moiety (so-called internally quenched peptides) into living cells. When intact, energy of the excited fluorophore is absorbed by the quencher group, analogous to the quenching of the donor observed in FRET, albeit without fluorescence emission by the acceptor molecule. Yet, cleavage by peptidases will spatially separate the quencher from the fluorophore, allowing fluorescence to be detected (**Figure 4**). Using this approach, the authors showed that the majority of peptides are lost for antigen presentation owing to the remarkable cellular capacity to recycle peptides back to single amino acids. 9-mer peptides have an *in vivo* half-life of around 5 s, with ~1 amino acid removed every 1–2 s. More recently, the same approach was used to show that individual classes of peptidases exhibit subspecialization for defined lengths of substrates. Tripeptidyl peptidase II (TPPII), for example, cleaves proteasome substrates that are longer than 15 amino acids into peptides that are highly relevant for presentation by MHC class I molecules (5).

Transporter associated with antigen processing

Peptides sampled by the MHC class I pathway are captured by the transporter associated with antigen processing (TAP), a transmembrane ATP-binding cassettetransporter that pumps cytosolic peptides into the lumen of the endoplasmic reticulum (ER). The functioning of this transporter was elegantly studied using FRAP (6). The Brownian motion of particles in solution was first described by Einstein (7), who indicated that diffusion was proportional to the inverse third of the mass ($D \sim M^{-⅓}$). This was later modified for transmembrane proteins, in which diffusion is proportional to the viscosity of the lipid bi-layer and is related to the radius of the transmembrane segment (for details see (8)). When TAP transports a peptide, it probably changes the diameter of the transmembrane pore, which results in a reduced lateral mobility of the complex in the ER membrane. This can be measured using FRAP on green fluorescent protein (GFP)-tagged TAP. From their experiments, Reits *et al*. (8) were able to characterize TAP activity in living cells, which correlates with the size of the intracellular

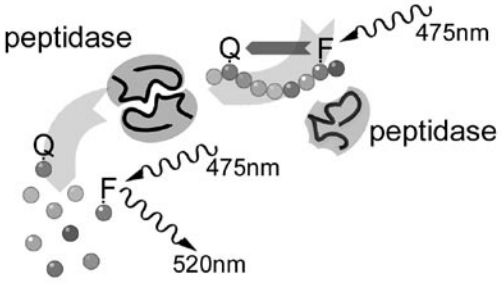

Figure 4. Internally quenched peptides. Peptidase activity can be measured in living cells using, so-called, internally quenched peptides. These chemically synthesized peptides contain both a fluorescent group (F) and a quencher moiety (Q), and do not emit fluorescence when excited with 475 nm light when the peptide is intact. Upon microinjection and cleavage by peptidases, the quencher will be spatially separated from fluorophore, and fluorescence-emitted light of 520 nm can be detected.

peptide pool. They concluded that the major sources of peptides under steady state conditions are newly synthesized rather than old proteins.Thus, besides determination of protein compartmentalization, FRAP is a valuable tool to study process dynamics and is used in this study as a biochemical tool to visualize enzyme activity in living cells.

Antigen loading

In addition to pumping peptides, TAP acts as a scaffold during antigen loading onto class I molecules, a process in which the ER chaperone tapasin also plays an essential role. Tapasin forms a bridge between TAP and newly synthesized MHC class I molecules that are waiting for peptides to dock. Again, using FRAP, Marguet *et al.* (9) showed that empty MHC class I molecules adapted the relatively slow lateral mobility of the TAP transporter, whereas binding of peptides resulted in release from this loading complex. This was measured as an increase in mobility. Both tapasin and unassembled MHC class I molecules are retained in the ER and are excluded from ER exit sites, yet each in a different manner. All tapasin molecules are located in dynamic clusters, whereas unassembled MHC class I molecules are excluded from clusters, as determined by FRET measurements (10,11). Using FRET and polarized fluorescence measurements, the same group showed that peptide-loaded MHC class I molecules cluster at ER exit sites for transport through the Golgi to the plasma membrane (12,13).

Antigen presentation

Once the antigen-loaded MHC class I molecules arrive at the plasma membrane, they are accessible for inspection by $CD8^+$ T cells. Using fluorescently tagged Fab fragments and FRET measurements, Catipovic *et al.* (14) demonstrated that peptide-loaded MHC class I molecules attached to the cell surface adopt a more compact structure than their empty counterparts. The interaction between MHC molecules and T-cell receptors, and the subsequent recruitment of co-receptors, has also been studied using FRET measurements. Lee and Kranz (15) visualized the recruitment of CD8 to the MHC class I bound T-cell receptor, whereas Zal *et al.* (16) studied the interaction between the CD4 co-receptor and the MHC class II-bound T-cell receptor. Interestingly, this last step of antigen presentation is highly similar for both pathways, although the source of the presented antigen and its processing is completely different.

The MHC class II pathway

MHC class II molecules present antigens from extracellular origins that are degraded by way of the endocytic route on the surface of so-called professional APCs. Correct folding of these molecules in the ER requires the invariant chain, a dedicated chaperone that stabilizes the empty MHC class II molecules by occupying its peptide-binding groove (for details see **Figure 1**). In addition, the invariant chain targets the MHC class II complex to the MHC class II containing compartments (MIICs), which are late endosomal and/or lysosomal vesicles that have a multi-vesicular or multi-lamellar architecture. These vesicles form the environment in which MHC class II molecules meet potential antigens, such as bacterial-derived peptides, and acquire fragments from them.

Antigen loading

When in the MIIC, the invariant chain is degraded by lysosomal proteases until only the part remains that occupies the peptide-binding groove of MHC class II molecules. This is known as the class II-associated invariant chain peptide (CLIP) fragment. The crucial exchange of this CLIP fragment for an antigenic peptide is facilitated by a second dedicated chaperone, Human Leukocyte Antigen (HLA)-DM. As the size of the average MIIC compartment is smaller than the wavelength of visible light, conventional colocalization studies did not suffice to study the interaction between HLA-DM and MHC class II molecules. We therefore applied confocal laser scanning microscopy (CLSM) FRET measurements (17), which enabled us to visualize the interaction between MHC class II molecules and HLA-DM in the MIIC of living cells (**Figure 2b**; (18)). By reconstituting the MHC class II pathway with fluorescently tagged molecules, we were able to demonstrate that the interaction between HLA-DM and MHC class II molecules in native MIIC is pH independent, and that it only occurs on the internal structures, and not at the limiting membrane (18). The internal membranes of the MIIC thus form a MHC class II loading microdomain in which the CLIP fragment is exchanged for an antigenic peptide.

Transport to the plasma membrane

After successful antigen loading, the MHC class II molecules traffic to the cell surface. The transport pathways involved in this have been quite thoroughly

studied by use of a wide variety of techniques. Key regulators in this pathway are the small GTPases from the Rab family, most notably Rab5 and Rab7, which control the activity of microtubule-based motor proteins. These proteins are socalled molecular switches that cycle between an inactive cytosolic- and an active membrane-associated state. Their cycling has been successfully studied with FRAP, in which the membrane-bound fraction was bleached and recovery of fluorescence was quantified (19,20). In addition, Galperin and Sorkin (21) used FRET measurements to visualize the interaction between the Rab5 protein and its effector (which only binds to the active form of the Rab protein).

Immune evasion

Intracellular bacteria, such as *Mycobacterium tuberculosis* and *Salmonella*, affect the endocytic pathway and multivesicular body formation. By doing this they form a phagosome that does not fuse with lysosomes (22). By manipulation of the small GTPases Rab5 and Rab7, these bacteria manage to diverge from the normal endocytic pathway to create their own cellular compartment (the phagosome) in which they survive and replicate. Here, they are usually unrecognized by the immune system. Manipulation of the endocytic pathway starts immediately after bacteria have entered into cells by phagocytosis. This is a complex process that was elegantly studied using FRET by Hoppe and Swanson (23). Importantly, the creation of a phagosome allows these bacteria to evade the immune system; for example, our FRET measurements show that HLA-DM and MHC class II molecules do not interact on the membrane of the phagosome, thus preventing antigen loading (18). These bacteria can locally evade the immune system by disruption of formation of the required internal MHC class II loading microdomains, a conclusion that could not have been drawn without the use of advanced biophysical techniques.

Conclusions

The two antigen presentation pathways discussed in this review are prime examples of multi-step and highly dynamic cellular processes. To unravel their details, sensitive microscopic measurements are required that have high temporal and spatial resolution in single living cells. In this review, we have discussed several techniques that satisfy these requirements, and recent advances in both microscopic techniques and fluorescent probes further extend the array of possibilities. For example, the discovery of photoactivatable fluorescent proteins (24,25) provides new ways in which to study cellular trafficking and compartmentalization. Likewise, many of the advanced microscopic techniques described in this review are becoming commercially available for use by cell biologists and microbiologists, rather than by biophysicists only. Taken together, this promises the onset of many more studies that apply biophysical techniques to gain further insight into antigen presentation as well as into many other processes, all in living cells.

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