

Short Thesis for the degree of doctor of philosophy (PhD)

**Development of sample preparation methods for
characterizing the cell surface pattern of immune
proteins for transmission electron microscopy
studies**

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of immune proteins for transmission electron microscopy studies**

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1. Introduction

1.1. Importance of cell surface protein patterns

The study of the pattern of various proteins, especially cell surface receptors, has long been of great interest in cell and molecular biology, immunology, etc. The reason for this is that the homo- and hetero-association of surface molecules can be associated with several cellular and immune functions. Changes in the conformations of cell surface proteins play a role in signal transduction processes, antigen recognition, and activation processes, among others

1.2 Methods for studying distribution patterns of cell surface proteins

1.2.1 Fluorescence studying methods

The distribution of proteins on the cell surface is mainly studied by immunofluorescence studies, in which, by exploiting the antibody-antigen reaction, different proteins can be specifically labeled with fluorescent dyes.

Thus, the most basic and simplest examination method is fluorescence microscopy imaging, where samples are usually labeled indirectly and the fluorescence can be examined. Taking advantage of the fact that the wavelength of the illuminating light capable of exciting fluorescent dyes and the light emitted by the fluorophores are shifted relative to each other, the labeled molecules can be detected with high sensitivity, excluding the exciting light and the unlabeled details of the sample.

One of a “special” version of fluorescence microscopy is confocal microscopy. The confocal arrangement was published earlier, but the technique only became truly widespread in the 1970s, with the development of lasers. While in the traditional case the microscope illuminates the entire surface of the sample and detects the light coming from the entire surface of the sample, here it is possible to solve the problem that the sample can only be excited at one focal point and only the fluorescent light coming from this focal point is detected from the sample. In this way not only the resolution limit resulting from diffraction, but also the large background of light rays coming from outside the focal point can be reduced. Another great advantage is that the 3D images can be created from different focal planes using 3D reconstruction.

One of the biggest limitations of the technique in studying the pattern of cell surface proteins is the limited lateral resolution. The limit resulting from light diffraction, which is described by Abbe's law, is around 200 nanometers. Thus, fluorescent molecules within these distances cannot be separated from each other and appear as a single light source, a point.

The early confocal laser scanning microscopy (CLSM) was then developed into several advanced versions that are able to overcome the resolution limitations of traditional light microscopes.

Traditional fluorescence microscopes supplemented with other techniques are able to overcome the resolution limitations using various methods. These include among others, photoactivation localization microscopy (PALM), stochastic optical reconstruction microscopy (STORM) or total internal reflection fluorescence microscopy (TRIF).

In this case, we are also able to separate the fluorophores within the 200 nm spacing limits of optical microscopes at 50-100 nm. The disadvantages are limited access to the equipment and need for special dye molecules.

Another widely used super-resolution microscopy is stimulated emission extinction microscopy (STED). Here, the outline of the excited spot of a single-point sample is excited by a special profile STED laser formed with special optics, which causes the wavelength of the photons emitted by the stimulated emission of fluorophores to be different from the wavelength of the photons resulting from spontaneous emission, and thus they can be separated. However, the spontaneously emitting part in the centre of the initially excited few 100 nm spots is only a few times 10 nm. Thus, the theoretical distance that can be covered is approximately 50 nm. The disadvantage is that due to the characteristics of the microscope, the resolution strongly depends on the intensity of the STED laser. This has a negative effect on biological samples, since we cannot use any laser intensity without damaging the sample.

Another technique that can be used to study the proximity relationships of proteins very sensitively which also works on the principle of fluorescence but cannot be classified as a microscope is the Förster-type resonance energy transfer (FRET). In this case, two different fluorescent dyes are used. The two so-called donor and acceptor molecules are specially selected. The emission spectrum of the donor molecule must overlap with the absorption spectrum of the acceptor molecule. In this case, the donor molecule can excite the acceptor molecule with a radiation-free transition if the distance between the molecules does not exceed about 10 nm and the dipole moments of the dye molecules are parallel or nearly parallel to each other. The probability of this transition shows a strong dependence on the distance between the two molecules, which is proportional to the reciprocal of the sixth power of the distance. Thus, this is actually a very sensitive distance measurement option. But its disadvantage is that it can only be used within distances of 10 nm.

1.2.2 Transmission electron microscopy (TEM) investigation technique

Another widely used technique for examining protein patterns is transmission electron microscopy (TEM). TEM microscopy has a much better resolution than the conventional light microscopes (about 1 nm) but the requirements for the sample are very strict. The peculiarity of this microscopy is that it can only be used to a very limited extent for examining biological samples. One complication is the composition of the sample, which is mostly light elements. The electron density of these light atoms so the ability to scatter the electron beam is very low. Thus, biological samples (tissues or cells) provide only very low contrast in transmission electron microscope images. Therefore, for example in anatomical and histological examinations the sample must be “stained” with salts of heavy elements that increase the contrast. Such substances include osmium tetroxide, lead citrate, uranyl acetate. However, these staining methods are not suitable for determining the localizations of specific proteins, as these are not protein-specific staining methods.

If we want to specifically label and examine the distribution of proteins by TEM for example the immunogold technique that is a possible solution. In this case, the protein to be examined can be specifically labeled with colloidal-sized gold beads. On the one hand, this only binds to the protein to be examined and on the other hand, the gold as a heavy element so it has a very high electron density, thus providing very good contrast on TEM images. Just as in the case of light microscopes, different proteins can be examined simultaneously with dyes of different spectra, here we can achieve this goal using different sizes of gold beads.

Since transmission electron microscopy is relatively widespread and its resolving power is also outstanding, we chose this technique for the examination and characterization of cell surface protein patterns. The biggest obstacle we encountered, which is one of the strongest conditions for TEM samples. The maximum thickness of the samples cannot exceed approximately 100 nm. This greatly complicates the study of immunogold-labeled cell surface receptors since the thickness of the cells is approximately 10 micrometers which is orders of magnitude larger than the maximum thickness of TEM samples. But our new protocol which used the chemical thinning of the sample, described later, cannot damage the lipid bilayer that surrounds the cell.

However, such a TEM sample preparation procedure was not found in the literature, so prior to our studies, we developed a separate sample preparation procedure that allowed us to examine the distributions and patterns of different proteins on the surface of the cells.

1.2.3 Requirements for the TEM sample

The design of the electron microscope implies that special conditions have to be fulfilled compared to conventional light microscope samples.

- As the electron microscope operates in a vacuum, the sample cannot contain liquid, so we need to ensure that the sample is dried.
- The penetration of the electron beam used in TEM is minimal, so the maximum thickness of the sample is severely limited, which is around 100 nm maximum. Thus, ultra-thin sections of the sample have to be made or other sample thinning procedures have to be applied to biological samples.
- Since the contrast of light organic elements (N₂, O₂, H₂ etc.) in biological samples is low in the TEM, we need to increase the contrast of the sample to be tested with heavy element salts or that we used the immunogold labelling techniques to visualize the proteins to be tested.

2. Aims

1. We wanted to investigate the cell surface pattern of IL-2 and IL-15 receptor molecules. Do they show any particular arrangement between themselves and each other, and if so, what are the sizes and frequencies of these clusters?
2. Improvement of the sample preparation procedure used for transmission electron microscopy of immunogold labelled samples, in order to more accurately describe the analysis of cell surface protein patterns. Using the new sample preparation protocol, to answer the question: what is the clustering of surface-expressed MHC I or MHC II molecules from nanometer scale to micrometer scale compared to a random distribution?
3. We wanted to investigate how the homo- and hetero-association of different cell surface molecules might be affected by the presence of a molecule not previously expressed on the cell surface. HLA II protein is not expressed on BLS cells, but HLA II protein is expressed after transfection and does the presence of HLA II protein affect the proximity relationship between HLA I and HLA II proteins?

3. Materials and methods

3.1 Culturing of cell lines used for the studies

Kit 225 FT7.10 cell line:

Kit 225 FT7.10 (Kyoto University) cells were transfected with full-length IL-15R α , which has an N-terminal FLAG tag. Both cell lines were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin at 37°C in a 5% CO₂ atmosphere. Five hundred pM human recombinant IL-2 was also added to the medium every 48 hours. The medium of FT7.10 cells contained 800 μ g/ml G418 (GIBCO) to suppress the growth of wild-type cells.

JY and BLS-1 lymphoma cell lines:

JY (ATCC, Manassas, VA, USA) and BLS-1 human B-lymphoma cell lines (a gift from Prof. Mark Exley, Harvard Medical School, Boston, USA) were grown to subconfluency, passaged three times weekly in RPMI 1640 medium containing 10% FBS, 2 mM l-glutamine, and 50 μ g/ml gentamicin at 37°C in a 5% CO₂ atmosphere.

3.2 Investigation of IL-2R α and IL-15R α cell surface receptors on Kit225 FT7.10 cell line

3.2.1 Immunogold labeling of IL-2R α and IL-15R α cell surface receptors

Studying the distribution pattern of IL-2 and IL-15 receptors was performed using the previous sample preparation protocol on the Kit 225 FT7.10 cell line, before the new sample preparation procedure has been developed. In this case, receptors were labeled indirectly, in the case of IL-2R primary antibody was a Fab fragment prepared from the anti-IL-2R α 7G7/B6 (IG2a) antibody purified from mouse hybridoma supernatant, and in the case of IL-15 the primary antibody was the anti-IL-15R α 7A424 (IgG) whole antibody purified from mouse hybridoma supernatant. In the colocalization studies, the secondary labels were polyclonal anti-mouse antibodies bound to 15 and 30 nm gold beads. (Aurogamig G15 was loaded with polyclonal antibodies against the heavy and light chains of anti-mouse IgG, and Aurogamig G30 with polyclonal antibodies against the Fc part of anti-mouse IgG, Amersham Pharmacia). In the sequential labeling, the IL2 receptor of the cells was first labeled with a Fab fragment prepared from the anti-IL-2R α 7G7/B6 (IG2a) antibody, followed by washing with Aurogamig G15. This was followed by repeated washing, and the unused binding sites of the polyclonal antibodies coating the Aurogamig G15 gold beads were blocked with an excess of non-specific Fab fragments (not binding to cell surface proteins). In the next step, the IL-15 receptor was

labeled with the anti-IL-15R α 7A424 (IgG) whole antibody, following the washing by PBS the labeling of the Fc part of the second antibody was performed with Aurogamig G 30. After another wash with PBS, the samples were fixed in 1% formaldehyde/PBS solution for 30 minutes and then in sodium cacodylate buffer (0.1 M, pH: 7.2) containing 2% glutaraldehyde overnight.

3.2.2 Sample preparation of the immunogold labelled IL-2R α and IL-15R α receptors

In the first step, the TEM sample carrier grids were coated with a "formvar" membrane, a thin plastic membrane having few times 10 nm thickness to hold the cells, the surface of which was treated with poly-L lysine to allow the cells to adhere to the membrane.

Then, a suspension of immunogold-labeled cells was dropped onto the surface. Then, the sample was dehydrated with an ascending series of alcohol (10%, 30%, 50%, 70%, 95%).

In the previous studies, the immunogold-labeled sample was also dehydrated with an ascending series of alcohol and then dried in open air. A sufficiently thin area was created on the outer edge of the collapsed cells, which was visible for TEM.

3.3 Investigation of homo- and hetero-association of HLA I and HLA II using a new sample preparation procedure

3.3.1 Labeling of HLA I and HLA II cell surface receptors using immunogold conjugation kit

Immunogold labelling is traditionally indirect labelling, i.e., labelling first with a specific primary antibody, then with a specific secondary antibody, in this case gold spheres conjugated to a specific secondary antibody. The disadvantage of this protocol is that several washing steps have to be applied, which can be particularly detrimental in the case of immunogold labelling. In the case of direct labelling, 1-2 washes after labelling are sufficient. Washing is technically achieved by draining the liquid from the cells settled by centrifugation, thus removing unbound antibodies. Centrifugation, however, has an increased disadvantage due to the relatively large mass of the immunogold gold spheres, making it six times more likely that antibodies already bound will be removed from the sample.

Prior to direct labelling, we need to conjugate the gold spheres with our own primary specific antibody. For this purpose we used the conjugation kit commercialized by Abcam (Cambridge, UK). The conjugation protocol, which includes several steps, is available on the Abcam website in PDF format (<https://www.abcam.com/en-gb/products/conjugation-kits/gold-conjugation-kit-10-nm-20-od-ab201808#tab=support>).

3.3.2 TEM sample preparation of immunogold-labeled HLA I, HLA II receptors on JY, BLS-1 and tDQ6-BLS-1 cell lines

In our previous experiments, the TEM sample preparation procedure had three significant shortcomings that reduced the statistical accuracy of our results:

1. The biggest problem was the size of the area that could be examined, because after drying only a very limited ring at the edge of the cell became thin enough for TEM analysis (~100 nm).
2. The other problem was that after the sample was labeled in suspension, the transmission electron microscope provided a projection image of the sample. This way, the image showed not only immunogold spheres present on one cell membrane, but also those on both membranes of the cell. Thus, in reality, we can examine the receptor distribution projected on the two membranes (lower and upper), which can falsify our results.
3. The shape and morphology of the cells change as a result of drying in open air, which can affect the results.

Therefore, we developed a new sample preparation protocol that overcomes the shortcomings of the previous method.

The essence of the new procedure is to create a kind of sandwich structure, which can be thinned so that only the upper lipid bilayer of the cells, labeled with immunogold, remains, which can then be examined using TEM.

The steps of the protocol, briefly summarized in points, are as follows:

- The sample (cells) labeled in suspension with immunogold are prepared on a TEM sample carrier grid previously coated with a collodion membrane treated with poly-L lysine
- then dehydrated with an ascending ethanol series (10%, 30%, 50%, 70%, 95%)
- the alcohol is replaced with carbon dioxide by rinsing several times, then carbon dioxide is present in the liquid phase, and the sample is heated to a critical temperature (31.5°C)
- then the sample is dried at a critical point, avoiding morphological changes after drying
- coated with a thin layer of carbon, a few times 10 nm, using a vacuum evaporator, which mechanically fixes the cells to the grid TEM carrier
- using amyl acetate, we dissolve the collodion membrane under the sample

- we chemically dissolve the cell components from under the sample, leaving the upper labeled lipid bilayer fixed with the carbon membrane
- after washing, the sample is suitable for transmission electron microscopy examination.

3.4 Transmission electron microscopic examinations

Our measurements were performed using a Jeol 2000FX (Akishima, Tokyo, Japan) transmission electron microscope using an accelerating voltage of 200 kV. Biological samples are typically examined using an accelerating voltage of 80-100 kV, for two reasons:

First, because the mechanical strength of biological samples is low, and the high-energy electron beam would destroy the samples and second because the atomic number of the elements in biological samples is low, so using a high accelerating voltage would provide small contrast.

In our case, however, we are not examining cellular components, but immunogold labeling particles, which label cell surface proteins and which have a very high atomic number, and therefore high electron density. As a result, the gold atom deflects the electrons to a large extent, and this deflection provides the contrast of the transmission electron microscope. The low mechanical strength of the sample is not a problem since the carbon layer evaporated onto the sample during the sample preparation protocol provides sufficient strength, and thus the application of an accelerating voltage of up to 200 kV does not cause damage to the sample.

The TEM images were photographed on erasable and reusable “image plates” and developed with the Ditabis® (Pforzheim, Germany) “imaging scanner” which provides a significantly better-quality image than a traditional photographic plate, and the great advantage is that the TEM images are obtained directly in digital format.

3.5 Determining the position (coordinates) of immunogold particles

In the first step, we cropped the parts of interest from the digitized images. Then, using the “ClickOnGold” software previously developed by our working group, we found the coordinates of the centers of the black disks imaged by TEM on the immunogold particles. The software then saved these coordinates for later evaluation.

3.6 Determination of pair and cross pair correlation functions (PCF and PCCF) used for evaluation

The PCF and PCCF functions were determined using the "Gold" program developed by the Institute of Molecular Genetics of the Czech Academy of Sciences. The software can be easily used to determine PCF and PCCF functions of immunogold labeled samples.

The values of the functions and their corresponding 95% confidence intervals are determined by the software using Monte Carlo simulation.

3.7 Interpreting PCF and PCCF to describe point patterns

The pair correlation and pair cross correlation functions are the so-called second degree point distribution descriptors. The term second-degree refers to the fact that the function has two independent variables which in our case are the Descartes coordinates of the spots (x,y) in the TEM images as described in the previous chapter. However, the PCF and PFFC function can provide information not only about whether or not it differs from a random point pattern but also about the properties of any clusters that may exist. The function we use is the so-called pair correlation and cross correlation function (PCF, PCCF), which can be derived from Ripley's pair correlation function.

There are actually 3 types of point patterns: random, regular and clustered. In the random pattern, there is no correlation between the individual points (cell surface receptors), in the regular case the points are distributed in some regular network as if a repulsive force were acting between them, and in the clustered case they are arranged in clusters, i.e., some attractive interaction can be attributed to them.

The following PCFF and PCCF dishes can be assigned to these cases:

- Random PCF=1
- regular PCF<1
- clustered PCF>1

In addition to the function values associated with each distance interval, the "Gold" software also determines the upper and lower 95% confidence intervals.

Thus, if the function value is less than the lower confidence interval for that interval, the point pattern is regular, if it is between the lower and upper intervals it is random and if it exceeds the upper confidence interval, the point pattern is clustered. The size of the intervals gives an estimate of, for example, the sizes of possible clusters, and the magnitude of the function gives an estimate of the number of clusters of a given size.

4. Results

4.1 Examination of the proximity relationships of IL-2R α and IL-15R α cell surface proteins in a Kit 225 FT7.10 T-lymphocyte cell line

IL-2 and IL-15 often elicit similar cellular responses, but they also have opposing effects on the life and death of T lymphocytes. The divergence of the signaling pathways must occur

at the plasma membrane, where the cytokines interact with their receptors. Therefore, understanding the molecular details of receptor organization is crucial. As a model system, we examined the cell surface organization and co-localization of IL-2R α and IL-15R α in Kit225 FT7.10 human T-lymphoma cells using TEM. The captured images were analyzed with our image processing application, and then the pair-correlation and cross-pair-correlation functions were determined using Monte Carlo simulation. The proteins in question were labeled with immunogold spheres of different sizes (15, 30 nm).

We labeled with primary anti-IL-2R α 7G7/B6 and primary anti-IL-15R α 7A424, as well as secondary antibodies of goat anti-mouse IgG conjugated to gold beads (IL2 receptor: 15 nm and IL15 receptor: 30 nm).

Although clustering of IL-2R α and IL-15R α on a scale of a few hundred nanometers has been observed by confocal microscopy and Near-Field Scanning Optical Microscopy (SNOM), direct analysis of the distribution of the clusters has been lacking so far. Using immunogold labeling, however, we were able to directly observe the domains of IL-2R α and IL-15R α . At this hierarchical level, we also observed clustering of IL-15R α and IL-2R α in the plasma membrane of FT7.10 cells. Our TEM experiments confirmed the co-localization of membrane domains containing IL-2R α and IL-15R α . After analyzing the PCF and PCCF values, which confirm their homo- and hetero-associations, we found that the clustering of IL-2 (15 nm gold particles) is clearly visible in the intervals of 10-20 nm, 40-50 nm and 70-80 nm, i.e., the PCF functions in these intervals significantly exceed the value of 1 corresponding to the random distribution. The clustering of IL-15 receptors (30 nm gold particles) can be placed in the intervals of 20-30 nm and 50-60 nm. The PCCF values indicate the colocalization of IL-2 and IL-15 receptors. Thus, we can observe the colocalization of two receptors in intervals of 40-80 nm and 140-170 nm, respectively.

4.2 Further development of transmission electron microscopy sample preparation for the study of cell surface protein distribution

The most important goal of our experiments was to describe the correlation and cross-correlation of the distribution of cell surface proteins with the most accurate statistics. Mathematically, this can be achieved if there are as many “events” in the given sample as possible, which in our case is the number of immunogold spheres in individual TEM images. This can be achieved by either increasing the surface density of the labeling molecules or the size of the area to be examined. In the first case, our options are relatively limited. However, when labeling cell surface proteins with gold spheres, the possibility of the spheres detaching

during washing (centrifugation) of the sample can be a problem. Thus, one of the most effective methods to reduce this possibility is to reduce the number of washes and use direct labeling instead of indirect labeling.

TEM samples must meet several conditions. One of the most important conditions concerns the thickness of the sample, i.e., it should not exceed 80-100 nm. In general, the biological sample is embedded in epoxy resin, and then ultrathin sections are made with an ultramicrotome, i.e., the sample is sliced. In our case, this is not applicable, because we want to examine the surface of the samples previously labeled with immunogold, so after slicing the labeled lipid bilayer would not form a continuous surface, i.e., the requirement of examining the largest possible area of the sample would not be met.

Previously, the basis for the preparation of the sample examined in our working group was that the labeled sample in the cell suspension was dropped onto the surface of the TEM sample carrier and dried in open air. Thus, the band formed on a thin outer contour of the cell was already transparent for the transmission electron microscope.

Thus, the cell components located in the middle of the cells are opaque to TEM, so one of the disadvantages of the method is that it loses a large amount of area that can be examined. Another disadvantage is that after the sample dries, the projection image does not show the gold spheres located on one lipid bilayer, but rather two lipid bilayers formed after the two layers dry together. Therefore, we do not see the distribution of the immunogold gold spheres on the cell surface, but the image of the two surfaces projected on each other. Another problem is that after drying in open air, the morphology of the cell changes and thus the protein distribution on the cell surface may change.

Therefore, we developed a complex sample preparation method that forms a kind of sandwich structure. We used chemical etching with an aqueous solution of sodium hydroxide with a concentration of 100 g/l to thin the sample, and we chose critical point drying for drying. The biggest challenge was how to physically fix the cells to the support surface during the treatments.

Thus, we created a kind of sandwich structure, which we handled:

- The cells directly labeled in suspension were dropped onto a TEM carrier grid previously coated with a collodion membrane and treated with poly-L-lysine
- dehydrated with an ascending ethanol series (10%, 30%, 50%, 70%, 95%)
- the alcohol was replaced with carbon dioxide by rinsing several times, at which point carbon dioxide is present in the liquid phase, and the sample was heated to a critical temperature (31.5°C)

- then the sample was dried at a critical point, which preserves the morphological properties of the cells
- the sample was coated with a thin, several times 10 nm layer of carbon using a vacuum evaporator
- the collodion membrane providing the temporary support function was dissolved using filter paper soaked in amyl acetate
- the sample components were chemically dissolved from the cells in 100 g/l, retaining the upper lipid bilayer fixed by the carbon membrane with immunogold particles.

Due to the carbon membrane, the immunogold marker gold spheres are fixed and provide sufficient stability to withstand acceleration voltages of up to 200 kV instead of the 80-100 kV TEM acceleration speed conventionally used for biological samples.

4.3 Investigation of changes in the proximity affinity of cell surface proteins with the HLA DQ protein Bare Lymphocyte Syndrome (BLS) in a cell line using transmission electron microscopy

Our experiments are designed to test the hypothesis that the cell surface expression (and hence homo- and hetero-association) of HLA class I and II molecules may influence their distributions on the nanometer and tens of nanometer scales, respectively.

To this end, three cell lines were analyzed: the BLS-1 cell line, which does not express HLA II proteins but does express endogenous class I proteins; the tDQ6-BLS-1 cell line, which expresses both transfected HLA DQ (class II) and endogenous HLA class I molecules; and the JY cell line, which endogenously expresses both HLA I and HLA II molecules at appropriate levels. We examined homo- and hetero-associations of HLA class I, HLA class II cell surface molecules.

Since the results of previous FRET studies indicated an altered cell surface distribution pattern of HLA proteins [58], we aimed to investigate the membrane topology of HLA I and HLA II molecules on a sub-micrometer scale by TEM. To visualize the different molecules per cluster, we performed TEM on the immunogold labeled sample, which provides information on the molecular patterns between a few 100 nm and one micrometer scale.

Here, we directly measured the distribution of HLA I and HLA II clusters at this hierarchical level in the plasma membrane of three different cell lines (JY, tDQ6-BLS-1, BLS-1) using immunogold labeling. Both HLA I and HLA II molecules were labelled with antibodies conjugated to gold beads of different sizes, 10, 20 or 30 nm.

Analysis of PCF shows that the distribution of HLA I molecules in JY cells in the 10-20 nm and 200 nm distance range differs significantly from the values assumed from the random distribution. HLA II molecules also showed a non-random distribution on JY cells in the 10-20, 30-150 and 190-210 nm range. In BLS-1 cells, HLA I molecules showed a non-random distribution in the 10-20 nm, 30-120 nm, and to a small extent in the 210-350 nm distance range according to the PCF function. BLS-1 cells do not express HLA II molecules on the cell surface, so no PCF analysis was performed here. In tDQ6-BLS-1 cells, the expression of HLA I molecules showed a non-random distribution in the 10-210 nm range, and newly synthesized HLA II (HLA-DQ) molecules appeared on the cell surface, forming clusters, in the 10-200 nm and 400-600 nm range. The *de novo* synthesized HLA-DQ molecules clearly showed non-random characteristics at this hierarchical level.

We also wanted to investigate whether HLA I and HLA II cell surface molecules form clusters around each other, i.e., whether some kind of cross-correlation between these molecules on the cell surface can be detected. Therefore, we double immunolabeled JY and tDQ6-BLS-1 cells with antibodies against HLA I and HLA II molecules conjugated to 10 and 20 nm gold spheres, respectively.

In the control JY cell line, PCCF of HLA I and HLA II molecules showed slight association between HLA I and HLA II molecules at approximately 25, 75 and 175 nm. Note that some of the peaks barely exceed the 95% significance level, so interpretation of these results should be treated with caution.

In contrast to JY cells, PCCFs of HLA I and HLA II molecules from tDQ6-BLS-1 cells showed no measurable differences between HLA I and HLA II molecules compared to random (Figure 26(F) panel), suggesting that *de novo* synthesized HLA DQ molecules in these cells do not mix with HLA I clusters.

5. Discussion

The organization of cell surface membrane proteins at different levels in space and time has long been a well-known and widely researched topic.

The most common microscopic methods for examining cell surface protein structures are various optical microscopes and, within this, fluorescence microscopes. The great advantage of traditional fluorescence microscopy is its easy accessibility and relative simplicity. The greatest disadvantage of traditional light microscopes is the maximum resolution or diffraction limit, which, according to Abbe's law, is proportional to the wavelength of the emission light. Thus, the minimum resolution distance achievable is approximately 200 nm.

However, in recent times, there has been great progress in the development of various super-resolution optical microscopes, which are able to circumvent the diffraction limit of traditional microscopes through various nonlinear optical principles. The disadvantage is that these devices are difficult to access or they must meet special requirements, such as the samples or the special dyes that can be used.

A separate class is Förster resonance energy transfer (FRET), which can be used to study protein proximity in the nanometer range. The conditions for this energy transfer are that the distance between fluorescent molecules should not exceed 10 nm, and on the other hand, the dipole orientations of the molecules should match as closely as possible. Another disadvantage is that only molecules in the proximity range of 1 to 10 nm can be studied with it.

That is why we chose transmission electron microscopy (TEM). TEM is a relatively widespread device that can be used from the mm range to the nm range. TEM can be used to examine receptor patterns if the specific label is so-called immunogold, which is a colloidal gold sphere (a few times 10 nm), which provides perfect contrast in TEM images.

Our working group has previously conducted studies to characterize the molecular patterns on the cell surface of immunogold-labeled samples using transmission electron microscopy. However, by using the pair correlation function (PCF) or pair cross correlation function (PCCF) instead of the previously used Poisson function, we were able to provide a significantly more accurate description of the receptor patterns. With the help of the functions, we were able to estimate the possible existence of clusters, the size of the clusters, and their number.

We wanted to investigate the distribution of IL-2 and IL-15 cell surface receptors expressed on the Kit225 FT7.10 cell line using the new PCF function. According to our results, IL-2 receptors form clusters in the 10-20 nm, 40-50 nm and 70-80 nm intervals, and IL-15 receptors form clusters in the 20-30 nm and 50-60 nm intervals according to the obtained PCF values. We also examined the cross-correlations of IL-2 and IL-15 receptors using the PCCF, i.e., whether there is any cross-correlation between the two cell surface proteins. The obtained results showed a cross-correlation of IL-2 and IL-15 receptors in the 40-80 nm and 140-170 nm intervals, respectively. In summary, our experimental results indicate that the distribution of IL-2 and IL-15 cell surface receptors is not random, and that the two receptors cross-correlate with each other on the cell surface. IL-2 and IL-15 are key players in the activity and function of T cells. These cytokines exert their biological activity through receptor complexes sharing the signal-transducing β and γ_c subunits, and therefore induce similar cellular responses in many cases. However, they may also have quite different contributions to T-cell coordinated

immunity. A possible mechanism that may explain the difference in signaling of IL-2 and IL-15 is based on the unique α -chains of the two cytokines. Therefore, the study of the cell surface organization and interactions of IL-2R α and IL-15R α may contribute to the understanding of the function of the two cytokines.

During the investigation, however, we encountered the problem that one of the disadvantages of the sample preparation used so far is that the area to be examined is very limited. In our first experiments, the TEM sample preparation was briefly as follows: The labeled cells were dried on a TEM sample carrier by alcohol dehydration. Thus, a thin edge of the cell became thin enough to become transparent for the transmission electron microscope.

To eliminate these errors, we developed a new sample preparation procedure. The developed protocol is a kind of sandwich structure. The labeled and critical-point dried cells are physically fixed to the surface of the sample carrier, the so-called TEM grid (a 3 mm diameter gold grid), with a carbon film of a few times 10 nm, from which the cell components were removed by chemical etching. On the samples prepared in this way, the surface area to be examined became several times that used previously, more precisely, the upper lipid bilayer of the entire cell, labeled with immunogold, can be illuminated for the microscope. By applying our new sample preparation procedure, we can achieve greater statistical accuracy in the description of the pattern (homo- and hetero-associations) of the proteins labeled with immunogold on the cell surface in subsequent studies.

During the development of the new TEM method, I joined the research conducted by our working group, where they investigated the effect of the appearance of a previously unexpressed protein on the cell surface on the distribution of other cell surface proteins associated with it. For these studies, we chose the Bare lymphocyte syndrome (BLS) cell line (BLS-1), which, due to a rare genetic mutation, does not express human leukocyte antigen class II (HLA II) at all on the cell surface.

BLS-1 cells were transfected with DQA1*0102/pCIneo and DQB1*0602/pCIneo vectors. As a result of the transfection, the HLA DQ molecule was expressed on the cell surface.

We were curious about the effect of the newly expressed molecule on the cell surface distribution of other surrounding proteins, and we were also curious about whether we could detect differences compared to a third, control cell line (JY). Therefore, we labeled the HLA I, HLA II molecules on the BLS, transfected-BLS and JY cell lines with specific antibodies conjugated with immunogold, with double labeling.

We concluded that the newly expressed (HLA II) DQ molecules showed a high correlation on the transfected tDQ6-BLS-1 cell line, i.e., they were expressed in clusters

associated with themselves, but did not mix with the HLA I molecules and their clusters. This behavior was not observed in the JY cell line, where HLA I clusters, HLA II clusters, and clusters containing HLA I and HLA II molecules were also observed.

6. New scientific results of the dissertation

1. In our working group, the analysis of the pattern of cell surface receptors, including the examination of immunogold-labeled samples with a transmission electron microscope, has previously played a prominent role. But the evaluation process was a difficult and long process. Furthermore, the applied mathematical statistical analysis was not suitable for a sufficiently detailed characterization of any existing patterns. Thus, with the software we developed, I was able to detect the images (spots) of the immunogold-labeled colloidal gold spheres on the TEM images taken from the samples and save their coordinates for later analysis. I was able to analyze the data obtained in this way later using software developed by the Czech Academy of Sciences, which used a much more efficient statistical function.

This allowed me to adapt two new functions to my research, the pair correlation and pair cross correlation functions, which enabled me to estimate the size and number of possible clusters of cell surface proteins. I applied it to characterize the clusters of IL-2 and IL-15 receptors, where previous research by our working group could only detect the presence of clusters in the Kit225 FT7.10 cell line.

2. The sample preparation procedure used in our previous transmission electron microscopy measurements had several shortcomings. The area that could be examined was small, which significantly limited statistical accuracy. Furthermore, morphological changes resulting from drying could have influenced the results obtained from the pattern of cell surface proteins. The images obtained did not show immunogold gold spheres representing the location of proteins present on a lipid bilayer, but rather two layers stacked on top of each other, which could distort the results.

Due to the disadvantages of the previously used sample preparation method, I developed a new transmission electron microscopy sample preparation method that was able to eliminate the disadvantages of the previous method.

3. With the help of the developed software and the newly adapted functions, I joined the ongoing research in our working group, where we were looking for an answer to the question of how the pattern of a cell surface molecule changes when a previously unexpressed molecule appears. I was curious about how the pattern of a previously

expressed molecule changes on a nanometer scale, what pattern the newly expressed molecule shows, and what the proximity relationship of the two molecules is, where the two examined cell surface molecules were HLA I and HLA II. In our model system, it is not expressed on the Bare Lymphocyte Syndrome (BLS) cell line due to a rare gene mutation. However, after transfection, the HLA II protein appeared on the cell surface. As a control cell line, we used the JY cell line, where both cell surface proteins appear. Examining the different cell lines with the new method, I obtained the result that on the transfected BLS cell line, both HLA I and HLA II molecules form similar clusters, but in contrast to the JY cell line, the HLA I and HLA II clusters do not mix here, and the hetero-association of the two proteins is not significant.

7. Summary

1. Using our developed evaluation software and a new software for describing the pattern of cell surface receptors (planar dot pattern), we mapped the homo- and hetero-associations of molecules expressed on the cell surface of Interleukin-2 (IL-2R α) and Interleukin-15 (IL-15R α) on the KIT 225 FT7.10 cell line. The given molecules were visualized by transmission electron microscopy (TEM) using the immunogold labeling technique. After evaluating several TEM images, we concluded that the distribution of the pattern of IL-2R α and IL15R α expressed on the cell surface significantly deviates from the random distribution, which indicates homo- and hetero-associations of the examined receptors.

2. In our first experiments, several problems arose in the TEM sample preparation procedure, mainly the limitation of the size of the area to be examined and thus the inaccuracy of the pattern description. To this end, we developed a new TEM sample preparation protocol that eliminated the previous shortcomings. Using the new procedure, we examined the pattern of major histocompatibility complex (HLA) type I and II cell surface proteins expressed on the human JY cell line. As a result of our study, we could draw conclusions that the examined receptors are displayed on the cell surface in clusters of several different sizes.

3. Using the TEM sample preparation protocol we developed, we joined the studies already underway in our working group, the aim of which is to determine how the appearance of the previously unexpressed major histocompatibility complex type II (HLA II) after the transfection of Bare Lymphocyte Syndrome (BLS-1) and this transfected cell line (tDQ6-BLS-1) the newly expressed HLA II molecules affect the distribution of the previously expressed major histocompatibility complex type I (HLA I), and what their proximity relationships are to

each other. We compared our results with the results obtained on the JY cell line, where both molecules were present. According to our results, on the one hand, the HLA I and HLA II molecules showed a similar pattern on both the JY and tDQ6-BLS-1 cell lines, but on the JY cells the HLA I and HLA II clusters are partially mixed, while on the tDQ6-BLS-1 cell line they are segregated. It is conceivable that a longer time (up to weeks) is required for the formation of mixed clusters containing both pre-existing HLA I molecules and *de novo* synthesized DQ molecules on transfected tDQ6-BLS-1 cells.



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List of publications related to the dissertation

1. **Kormos, J.**, Daróczy, L., Szöllősi, J., Mátyus, L., Jenei, A.: Novel transmission electron microscopic method to quantify protein clustering on the cell surface.
Curr. Protoc. 4 (5), 1-14, 2024.
DOI: <http://dx.doi.org/10.1002/cpz1.1045>
IF: 2.2
2. **Kormos, J.**, Veres, A. J., Imre, L., Mátyus, L., Benkő, S., Szöllősi, J., Jenei, A.: HLA DQ protein changes the cell surface distribution pattern of HLA proteins as monitored by Förster resonance energy transfer and high-resolution electron microscopy.
Cytom. Part A. 103 (12), 978-991, 2023.
DOI: <http://dx.doi.org/10.1002/cyto.a.24787>
IF: 2.5
3. Jenei, A., **Kormos, J.**, Szentesi, G., Veres, A. J., Varga, S., Dóczy-Bodnár, A., Damjanovich, S., Mátyus, L.: Non-Random Distribution of Interleukin Receptors on the Cell Surface.
Chemphyschem. 10 (9-10), 1577-1585, 2009.
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