ウェブサイト公開用

（様式10）
（海外特別研究員事業）
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# 海外特別研究員最終報告書

独立行政法人日本学術振興会 理事長　殿

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（氏名は必ず自署すること）

海外特別研究員としての派遣期間を終了しましたので、下記のとおり報告いたします。
なお、下記及び別紙記載の内容については相違ありません。

記

1. 用務地（派遣先国名）用務地：　ウプサラ大学(ウプサラ市)　（国名：　スウェーデン王国　）

2. 研究課題名（和文）※研究課題名は申請時のものと違わないように記載すること。
ウイルス粒子が持つ宿主蛋白質の新規検出法による異種間感染防止ワクチン分子の同定

3. 派遣期間：平成　30年　4月　21日　～　令和　2年　4月　20日

4. 受入機関名及び部局名
Department of Immunology, Genetics and Pathology, Uppsala University

5. 所期の目的の遂行状況及び成果…書式任意　**書式任意（A4判相当3ページ以上、英語で記入も可）**
（研究・調査実施状況及びその成果の発表・関係学会への参加状況等）
（注）「6.研究発表」以降については様式10－別紙1～4に記入の上、併せて提出すること。

次ページより記載。

## PURPOSE AND AIMS

With the emergence of arthropod-borne viruses (arboviruses) and their vaccine resistant variants (1–3), there is a growing demand for new approaches to protect domestic animals from multiple arbovirus infections. As a solution to the problem, I propose a vaccination strategy to promote an immune response against host proteins incorporated in arbovirus particles (FIG. 1). When arboviruses are transferred from arthropod vectors to mammals, vector proteins on the surface of the virion represent immunological non-self for the infected animals and may be targets attacked by immune system. If we find vector proteins incorporated in several types of arboviruses, immunization with those proteins might cross-protect against all those viruses and even vaccine-resistant strains, which have mutated viral proteins, serving as "a universal vaccine".

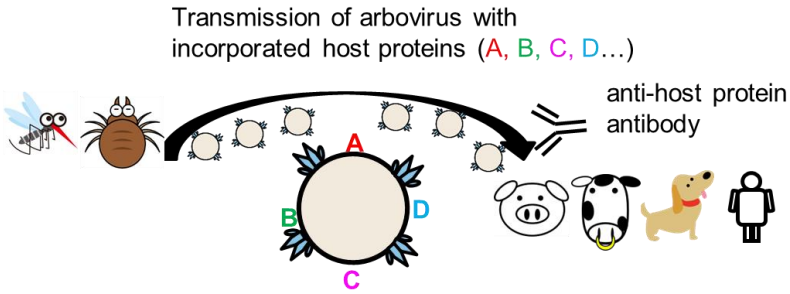


FIGURE 1. Proposed host protein vaccination.
Host proteins have been seen to be incorporated in the envelop of virus particles. Immunization with tick or mosquito proteins incorporated in arbovirus therefore induces production of antibodies specific for virus transmitted from ticks or mosquitoes to animals and humans. These antibodies might protect from infections by arboviruses that express host proteins.

One obstacle to identify tick/mosquito proteins according to my hypothesis is the heterogeneity of the types and amounts of host protein incorporated when the virus assemble. For example, exosome surface markers CD9 and CD81 are present in only 70% and 40% of influenza virus particles (4) (FIG. 2). To our knowledge, this is the only report about host protein heterogeneity in virus particles, pointing to the difficulty of investigating the presence of host proteins in individual virus particles. However, my aim was to identification of a new host protein set which is expressed in 100% of individual virus particles, in order to establish the proposed new class of vaccines.

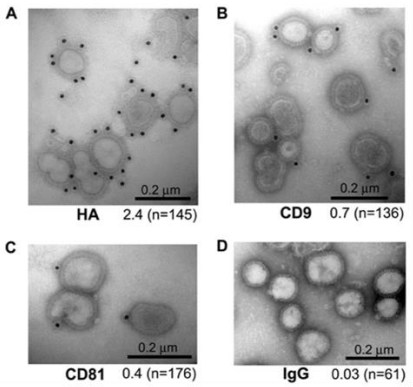


FIGURE 2. Heterogeneity of host protein expression between individual virus particles.
CD9 and CD81 were found to be incorporated on the surface of influenza virions derived from monkey cell lines (B and C). The proteins were found to be expressed on 70 and 40% of viral particles, respectively, while all particles expressed virus protein HA (A). Figure adapted from Figure 5. in Shaw et al., 2008.

To overcome this obstacle, conventional technologies needed to be complemented by high-sensitivity, multi-parameter, and high-throughput protein analysis at individual virus level.
The approach for host protein detection that I used has taken advantage of technologies developed in my host lab for transfer of protein information to DNA in order to create stable reporters that can be amplified and quantified via microscopy, flow cytometry, or using real-time PCR and sequencing. This family of methods, representing variants of in situ proximity ligation assay (isPLA) concept (5,6), can for instance serve to visualize proximity between two or more proteins over distances estimated to less than 40 nm (5) (FIG. 3). This dimension is similar to the sizes of exosomes and small virus particles. When using PLA for detecting proteins immobilized on solid phases, 10 ~ 100 times lower concentrations of the proteins were detected, compared to sandwich ELISAs (7).

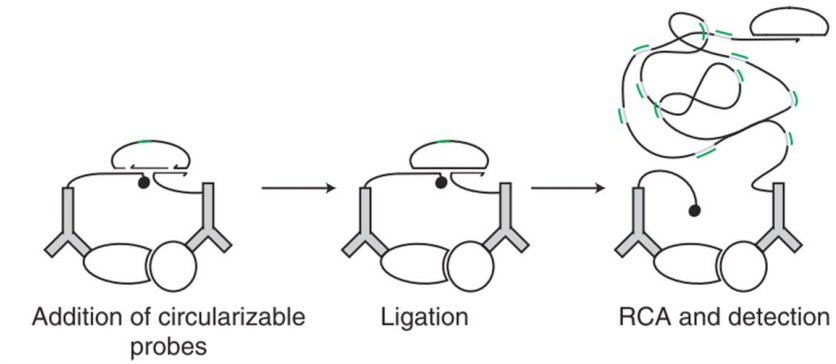


FIGURE 3. In situ proximity ligation assay (PLA) with rolling circle amplification (RCA). If two proximity probes bind close to each other, then subsequently added linear connector oligonucleotides are guided to form a circular structure covalently joined by enzymatic DNA ligation. After ligation, RCA is initiated using one of the proximity probes as a primer. The RCA product is detected through hybridization of fluorescence-labeled oligonucleotides (detection oligo) complementary to a tag sequence in the RCA product (green). The Figure is adapted from Figure 1a. in Söderberg et al., 2006.

Based on a unique set of protein detection methods established in my host lab according to the PLA principle, I aimed to adapt and further develop methods to analyze the heterogeneous presence of host proteins in individual virus particles and to enrich the virus "subsets" defined by the host protein expression profiles. These new technologies helped identify candidate molecules for host protein vaccination against infections with a variety of enveloped arboviruses.
My research program has the following aims:

I. Screening for host protein on the surface of arbovirus particles at bulk level by mass spectrometry and at single-virus level by proximity binding assay (PBA).

II. Investigation of host protein expression in individual arbovirus particles
   - This required development of new approaches to study proteins in a multiplex manner at the level of individual virus particles by exosome PLA (exoPLA) or isPLA

III. Investigation of infective titers of arbovirus subsets
   - This required development of new approaches to sort virus subsets expressing both virus protein(s) and host protein(s) of interest

**MATERIALS**

Tick-borne encephalitis virus (TBEV) was used as a model of arboviruses. I produced TBEV

subviral particles (SVP), established by my collaborator Dr, Yoshii (8), from human cell lines, 293T cells. TBEV-incorporated human proteins were screened by PBA (9). The PBA assay was conducted by Vesicode, a spin-out company from the host lab (https://www.vesicode.com/). To detect human proteins in the TBEV-SVP by exoPLA and isPLA, I used a number of antibodies for human proteins, custom oligos and enzymes all of which were commercially available. To establish novel means to sort virus subsets, I used commercial secondary antibodies and custom oligos.

## RESULTS & FUTURE WORK

### Aim I. Screening for host protein on the surface of TBEV-SVP

I screened more than one hundred exosome marker proteins on the TBEV SVP membrane with PBA, a method established in the host lab and now being commercialized by the spin-out company Vesicode. We found tens of proteins present in the SVP and we used the bioinformatics techniques tSNE and Phenograph to visualize the single-virus expression profiles (FIG. 4). Informatics analyses of the PBA data revealed that TBEV-VLP, secreted from 293T cells, were composed of unique subsets (green, blue and orange dots in Fig4) as compared to EVs from mock-transfected 293T cells. This unpublished data reveals heterogeneous presence of host proteins in TBEV virions.

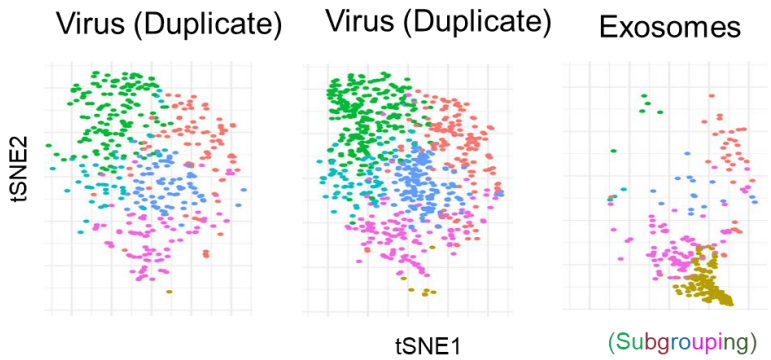


FIGURE 4. Experimental flow of proposal research.
tSNE projections of expression profiles of exosome markers at single-virus levels. Single dots indicate single-virus and colors indicates subgroups defined by Phenograph.

Unlike the original plan, I decided not to carry out a comprehensive screening of host proteins by mass spectrometry, because mass spectrometry generates only bulk data of host protein incorporation, while PBA data provided sufficient resolution regarding host protein incorporation to study about single-virus heterogeneity.

### Aim II. Investigation of host protein expression in individual TBEV-SVP

I observed human protein incorporation into the membrane of TBEV-VLP by isPLA (5) (Fig5 left), a method that possesses the capacity to visualize protein proximity at the single particle and single-molecule levels by regular fluorescence microscopy or flow cytometry. I detected co-occurrence of TBEV-E protein and several host proteins, such as CD9, CD81, integrin b1 (CD29) and CD59, at single-virus resolution on the surfaces of purified TBEV-SVP (Fig5 middle). These data were published in a peer-reviewed journal (10).

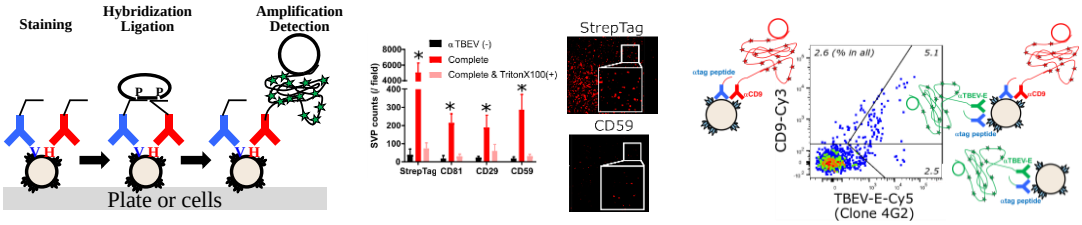


Figure 5. (Left) Schematic illustration of isPLA. DNA oligonucleotides conjugated to antibodies for virus (V) and host proteins (H) guide the formation of DNA circles and serve as rolling-circle amplification (RCA) primers. (Middle) TBEV-incorporated host protein detection by isPLA. Single fluorescent dots indicate single-virions expressing both TBEV-E and each of host proteins. (Right) 2-color isPLA with readout via conventional flow cytometry.

ExoPLA can be applied to simultaneously validate the presence of three surface proteins in individual exosomes or virions (6). Using this technique, I demonstrated that 75% of TBEV SVP expressed human CD9 (FIG. 5 right), however non-specific RCA products were often observed in technological and biological negative controls. Thus, I discontinued exoPLA assays.

isPLA with appropriate DNA sequence design allow researchers to detect nucleic acid and proteins in multiplex, while maintaining detection specificity. The host laboratory and I have developed an approach for multiplex isPLA using a strand displacement mechanism to facilitate higher multiplex detection than detector number installed in fluorescence microscopy (Björkesten et al., in press). The combination of this multiplex technique and image processing will allow researchers to detect multiple targets, such as viral and host proteins in virions, through regular fluorescence microscopy at low cost.

**Aim III. Investigation of infective titers of arbovirus subsets**

Infectivity characterization of the viral subset identified by PBA and multiplex isPLA analyses (Aim I and II) will help us examine which subset markers can be vaccine candidate molecules. Such subsets can be isolated with some difficulty and considerable losses by repeated immunoprecipitation (IP) reactions (11). It is also technically possible to sort virus particles reacted with exoPLA using a regular cell sorter, as shown in FIG. 5 right. However, this approach may not be suitable for biosafety laboratory that are not equipped with cell sorters. To overcome this, I have established tools using combinations of pairs of affinity reagents for preparative proximity reactions. No intellectual property protection has been secured so far for this technology, so it is not possible to describe our approach in greater detail. In preliminary model experiments using DNA oligonucleotides as both probes and targets, I have reached 30% capture efficiency and greater than 90% purity. In experiments using protein complexes, 10% capturing efficiency was observed, but the method will be further improved for routine applications with virions for my research and protein complexes for other researchers.

After establishing this novel IP technique for the simple model experiments, such as using TBEV-SVP with and without a tag peptide, I will then sort viral subsets identified in Aim II from infectious virus. The isolated virus subsets will be subjected to *in vitro* infection experiments. If the infectivity differs between the subset, host proteins expressed in the most highly infective subset will be considered priority vaccine targets.

**SCIENTIFIC DELIVERABLES**

**(1) Publication**

Ikebuchi, R., Isaac, A.W., Yoshii, K., Doulabi, E.M., Löf, L., Azimi, A., Chen, L., Fredolini, C., Gallini, R., Landegren, U., et al. (2020), in press. Human proteins incorporated into tick-borne encephalitis virus revealed by in situ proximity ligation. Biochem. Biophys. Res. Commun.

**(2) Conference**

12th International workshop on approaches to single cell analysis (Uppsala, Sweden)、Single virus analysis of host protein expression in tick-borne encephalitis virus, Poster presentation, (March 4-5th, 2019)

第 770 回　獣医学研究談話会 (北海道大学; Sapporo, Japan)、T 細胞の機能と遊走に関するシングルセル解析、宿主蛋白質のシングルウイルス解析　～Method を基準にラボと研究テーマを決める～, Oral presentation, (April 16th, 2019)

MIRAI: Moving together toward a sustainable future (Stockholm and Uppsala, Sweden)、Proximity assays for protein dimer detection and purification with enhanced antibody specificity, Poster presentation, (November 12-14th, 2019)

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Björkesten et al is now in press in Nucleic Acids Research