Integrative Life Science Doctoral Program

Department of Virology

Medicum, Faculty of Medicine

University of Helsinki

Time-Resolved Förster Resonance Energy Transfer for Rapid Infectious Disease Serodiagnosis

Satu Hepojoki

(née Saraheimo)

ACADEMIC DISSERTATION

To be publicly represented and discussed, with the permission of the Faculty of Medicine of the University of Helsinki, Haartmaninkatu 3, Helsinki, in Lecture Hall 2, on the 15th of October 2016 at 12 noon

Helsinki 2016

Supervisors:

Professor Klaus Hedman, MD, PhD

Medicum

Department of Virology

University of Helsinki and

HUSLAB

Finland

and

Professor Olli Vapalahti, MD, PhD

Medicum

Department of Virology

University of Helsinki and

HUSLAB

Finland

Reviewers:

Doctor Petri Susi, PhD

Department of Virology

University of Turku

Finland

and

Doctor Urpo Lamminmäki, PhD

Department of Biochemistry

University of Turku

Finland

Opponent:

Professor Noël Tordo, DVM, PhD

Pasteur Institute, France

ISBN 978-951-51-2477-7 (paperback)

ISBN 978-951-51-2478-4 (PDF, available at http://ethesis.helsinki.fi)

Printed at Helsinki by Unigrafia Oy

Helsinki 2016

"Uncertainty and mystery are energies of life. Don´t let them scare you unduly, for they keep boredom at bay, and spark creativity"

-R. I. Fitzhenry

to the memory of my mother

CONTENTS

ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred to in the text by their Roman numerals:

I **Saraheimo S.**, Hepojoki J., Nurmi V., Lahtinen A., Hemmilä I., Vaheri A., Vapalahti O., Hedman K. Time-Resolved FRET -Based Approach for Antibody Detection – A New Serodiagnostic Concept. PLoS ONE 2013;8(5):e62739

II **Hepojoki S.**, Nurmi V., Vaheri A., Hedman K. Vapalahti O. ja Hepojoki J. A Protein L -Based Immunodiagnostic Approach Utilizing Time-Resolved Förster Resonance Energy Transfer. PLoS ONE 2014;9(9):e106432

III **Hepojoki S.**, Hepojoki J., Hedman K., Vapalahti O, Vaheri A. Rapid Homogeneous Immunoassay for Serological Diagnosis of Acute Hantavirus Infection. Journal of Clinical Microbiology 2015 Feb;53(2):636-40

IV **Hepojoki S.,** Rusanen J., Hepojoki J., Nurmi V., Lundkvist Å., Hedman K., Vapalahti O. Competitive Homogeneous Immunoassay for Rapid Serodiagnosis of Hantavirus Disease. Journal of Clinical Microbiology 2015 Jul;53(7):2292-7

ABBREVIATIONS

Ab	antibody
AF	Alexa Fluor
BCR	B cell receptor
BSA	bovine serum albumin
CFRET	competitive FRET assay
CRP	C-reactive protein
DNA	deoxyribonucleic acid
DOL	degree of labeling
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
Eu	europium
FA	fluorescence antibody staining assay
Fab	fragment antigen-binding
FRET	Förster resonance energy transfer
GST	glutathione S-transferase
H	heavy
HIV	human immunodeficiency virus
HL	heavy and light
HUSLAB	Helsinki University Central Hospital laboratory diagnostics
IFA	immunofluorescence assay
Ig	immunoglobulin
kDa	kilodalton
L	light
LFRET	Protein L FRET assay
MAb	monoclonal antibody
MALDI-TOF	matrix-assisted laser desorption/ionization time-of-flight
MHC	major histocompatibility complex
Mr	relative molecular mass
MS	mass spectrometry
N	nucleocapsid
NAT	nucleic acid testing

NE	nephropathia epidemica
NGS	next-generation sequencing
PAb	polyclonal antibody
PCR	polymerase chain reaction
POC	point-of-care
PUUV	Puumala virus
PUUV N	Puumala virus nucleocapsid protein
QD	quantum dot
RDT	rapid diagnostic test
RNA	ribonucleic acid
SA	streptavidin
SD	standard deviation
SDS	sodium dodecyl sulfate
S/N	signal-to-noise
spp.	species (plural)
Tb	terbium
TR	time-resolved
TR-FRET	time-resolved FRET
TRF	time-resolved fluorometry
VP1	human parvovirus B19 minor capsid protein
VP1u	the unique region of human parvovirus B19 VP1

ABSTRACT

Novel molecular tools for infectious disease diagnostics are constantly under development to reduce the time between onset of symptoms and diagnosis. Not only is it important to receive appropriate treatment, but also to avoid unnecessary use of antibiotics. The availability of rapid diagnostics is also important when epidemics or pandemics emerge. The purpose of this project was to examine the applicability of Förster resonance energy transfer (FRET) in homogeneous immunoassays, and to develop new diagnostic approaches. FRET has widely been applied in proximity-based assays, such as those measuring antigen-antibody binding. In FRET, energy is transferred between two chromophores, the donor and the acceptor, when in close proximity. Utilizing FRET as detection method for immunoassays enables the development of wash-free (homogeneous) simple workflow assays. In this thesis, of the three FRET-based rapid immunoassays that were set up, two served in clinical diagnosis.

Study I (I) examined the possibility of detecting antibodies by FRET-pair forming fluorophore-labeled antigens, which upon binding to an antibody would induce a FRET signal. This homogeneous immunoassay, designated *FRET-bridge*, was successfully optimized for streptavidin (SA). By combining donor-labeled and acceptor-labeled SAs with anti-SA antibodies, FRET signals were recorded with high signal-to-noise ratios. When molecular determinants behind the FRET signals were examined, most of the FRET activity originated from fairly large immunocomplexes rather than from one IgG and two antigens. At the moment, SA represents the only antigen fully functioning in the FRET-bridge assay. This is most likely due to the multivalent nature of the antigen, which seems beneficial in FRET signal formation.

Next, we introduced another homogeneous immunoassay (II), the *LFRET* assay. Here, a FRET pair forming fluorophore-labeled antigen and fluorophore-labeled protein L induce a signal if bound to the Fab (fragment antigen-binding) region of an immunoglobulin. To demonstrate the usefulness of the assay, SA served as test antigen. The assay was next optimized for virus diagnostics by use of Puumala virus (PUUV) nucleocapsid protein as antigen (III). In all, 211 serum samples underwent examination by the LFRET assay, representing acute (n=61) or past PUUV infection (n=27), and seronegative (n=123) individuals. With a

simple workflow and an assay time of 30 minutes, the LFRET assay, compared to the reference tests, identified acute PUUV infection at 100% specificity and 95% sensitivity.

The fourth study (IV) involved a competitive homogeneous immunoassay for the detection of PUUV antibodies from clinical samples. This assay, *CFRET*, is based on competition between fluorophore-labeled monoclonal antibodies (MAbs) and serum antibodies. Here, a donor-labeled antigen and an acceptor-labeled MAb form the FRET pair. If the clinical sample contains antibodies against the labeled antigen, they compete with the MAb for antigen binding, resulting in FRET signal decrease. Analysis of assay performance included a panel of 329 samples representing acute (n=101) or past (n=42) infection, and negative samples (n=186). The one-step CFRET assay performed at 99% specificity and 100% sensitivity in diagnosis of hantavirus disease compared to the reference tests, and with a rapid assay time of 30 minutes.

The three rapid diagnostic approaches introduced herein represent simplicity, and show that diagnostics need not be time-consuming. Although the assays were optimized for accurate diagnosis of acute infection, both assays also recognized life-long immunity, albeit with lower sensitivity. By optimization, the assays could be developed towards more sensitive detection of past infection as well. The LFRET and CFRET assays thus represent innovative tools for rapid antibody detection, and their potential in serodiagnosis of diverse microbial infections and possibly even in detection of auto- and anti-allergen antibodies calls for further exploration.

TIIVISTELMÄ

Tartuntatautien diagnostiikkaan kehitetään jatkuvasti uusia pikatestejä, jotta diagnoosi saataisiin mahdollisimman nopeasti oireiden ilmaannuttua. Nopea diagnoosi varmistaa sen, että oikea hoito voidaan aloittaa mahdollisimman pian, ja samalla vältytään turhilta antibiooteilta. Diagnostiset pikatestit ovat myös tärkeitä epidemioiden tai pandemioiden hallinnassa. Tämän projektin tarkoituksena oli hyödyntää Förster resonance energy transfer (FRET) ilmiötä homogeenisten vasta-ainetestien kehittämisessä. Tutkimuksen tuloksena pystytettiin kolme erityyppistä vasta-ainetestiä, joista kahta sovellettiin kliiniseen diagnostiikkaan.

Ensimmäisessä osajulkaisussa (I) tutkittiin, voisiko vasta-aineita tunnistaa sellaisten fluorofori-leimattujen antigeenien avulla, jotka yhteen liittyessään muodostaisivat FRET-parin. Tutkimusasetelma perustui oletukseen, jonka mukaan näiden fluorofori-leimattujen antigeenien sitoutuessa samaan vasta-aineeseen, muodostuu FRET-signaali. Tämä testi, *FRET-bridge*, kehitettiin alun perin streptavidiini (SA) -malliantigeenille. FRET-signaali muodostui, kun luovuttaja- ja vastaanottajafluoroforileimatut SA:t yhdistyivät SA-vasta-aineiden toimesta. Työssä tutkittiin muodostuvia FRET-signaaleja molekyylitasolla ja huomattiin, että suurin osa signaaleista oli peräisin isoista immuunikomplekseista, eikä niinkään kahdesta antigeenista ja yhdestä vasta-aineesta. Toistaiseksi SA on ainoa antigeeni, joka tässä testissä toimii kunnolla. Tämä johtuu todennäköisesti SA:n multivalentista luonteesta, mikä vaikuttaisi edistävän FRET-signaalin muodostusta.

Toisessa ja kolmannessa osajulkaisussa (II, III) tutkittiin homogeenisen vasta-ainetestin soveltuvuutta tartuntatautien diagnostiikkaan. Kyseinen testi, nimeltään LFRET, perustuu fluoroforileimatun antigeenin sekä fluoroforileimatun proteiini L:n interaktioon niiden sitoutuessa saman vasta-aineen Fab (fragment antigen-binding) -osaan. Myös tämä testi optimoitiin käyttäen SA:ia, jonka jälkeen ensimmäisenä virusantigeenina käytettiin Puumala-viruksen (PUUV) nukleokapsidiproteiinia. Työssä testattiin kaikkiaan 211 seeruminäytettä, jotka edustivat akuuttia vaihetta (n=61), vanhaa immuniteettia (n=27) ja PUUV:lle negatiivisia näytteitä (n=123). LFRET-testi tunnisti akuuttia myyräkuumetta 100% tarkkuudella ja 95% herkkyydellä verrattuna referenssitestien tuloksiin.

Neljännessä osajulkaisussa (IV) pystytettiin kilpaileva homogeeninen vasta-ainetesti nimeltään CFRET. Tämä testi perustuu kilpailuun antigeeniin sitoutumisesta fluoroforileimatun monoklonaalisen vasta-aineen (MAb) ja seerumin vasta-aineiden välillä. Tässä testissä MAb sekä antigeeni muodostavat FRET-parin. Jos seeruminäyte sisältää vasta-aineita, jotka sitovat kyseistä antigeenia, syntyy kilpailutilanne vasta-aineiden välille, mikä puolestaan johtaa FRET-signaalin laskuun. Työssä testattiin yhteensä 329 näytettä, jotka edustivat akuuttia vaihetta (n=101), vanhaa immuniteettia (n=42) ja PUUV:lle negatiivisia näytteitä (n=186). CFRET tunnisti akuuttia myyräkuumetta 99% tarkkuudella ja 100% herkkyydellä verrattuna referenssitestien tuloksiin.

Edellä kuvatut kolme pikatestiä ovat yksinkertaisia ja ne todistavat, että diagnostisen testin ei tarvitse olla aikaavievä. Vaikka sekä LFRET- että CFRET-testi kehitettiin tunnistamaan akuuttia infektiota, molemmat testit tunnistivat myös vanhaa immuniteettia, joskaan ei yhtä herkästi kuin akuuttia infektiota. Hienosäädön myötä on mahdollista, että kyseistä ominaisuutta voitaisiin parantaa entistä herkemmäksi. LFRET- ja CFRET-testit tarjoavat uraauurtavia mahdollisuuksia vasta-aineiden pikadiagnostiikkaan, ja niiden soveltuvuutta useiden eri taudinaiheuttajien diagnostiikkaan sekä auto- että anti-allergeeni vasta-aineiden tunnistamiseen tulisi tutkia lisää.

FOREWORD

Infectious diseases have greatly influenced the history of mankind. One of the most lethal pandemics took place in the 14th century, the Black Death (the plague, caused by *Yersinia pestis*), resulting in the death of an estimated 75 to 200 million people. Another devastating example of deadly pandemics was the Spanish flu caused by the influenza A virus in the 1918, causing 20 to 40 million deaths. Both pandemics occurred in an era when no proper diagnostics or cure existed.

Today, a variety of infectious diseases are ”under control” due to national vaccination programs. However, the emergence and re-emergence of pathogens, most often viruses, is continuous. Globalization, urbanization, environmental changes, and increased travel facilitate the rapid spread of highly contagious pathogens. The role of diagnostics in the battle against novel and re-emerging diseases is crucial. In fact, the development of diagnostics is one of the first reactions to any novel pathogen.

The first part of the literature review focuses on diagnostics with emphasis on immunology and serodiagnostics. After review of the basics of established diagnostics, ranging from nucleic-acid detection to immunoassays, comes presentation of the concept of rapid diagnostic tests (RDTs). The general reason for RDTs is to shorten the time for accurate diagnosis, and also to reduce labor and possibly even assay costs. In addition, such tests can be utilized in resource-limited conditions such as field laboratories and in developing countries at the point of care.

The last section of the literature review deals with luminescence and applications based on this phenomenon. Luminescence is light emitted by a substance, and can be produced in several ways. This section also provides a detailed description of a photoluminescent phenomenon called Förster resonance energy transfer (FRET). FRET has proven specifically useful in proximity-based assays such as those detecting antibody-antigen interaction.

LITERATURE REVIEW

Diagnosis of microbial infections

The vast majority of methods for microbiological diagnostics rely on the following procedures: microbial culture, morphological identification of the pathogen, detection of nucleic acids, or detection of antigens or specific antibodies. Bacteria are often cultivated on selective plates followed by their morphological and biochemical identification. Numerous methods exist for detection of the microbial genome, which are based on the specific knowledge of the nucleic acid composition. Rapid methods often rely on detection of the surface antigen(s) of a pathogen, whereas serodiagnostic methods measure specific antibodies developed against a pathogen. The choice of the method used depends much on the patient´s symptoms, medical history, travel history, and especially on characteristics of the suspected pathogen. The first task is to distinguish whether the pathogen is a bacterium, a virus, a fungus, or a parasite. One of the routine actions is to take a blood sample from the patient. In the case of a bacterial infection, the white blood cell count may be elevated. Another indication of a bacterial infection may be the abundance of blood C-reactive protein (CRP) (5,50). This literature review focuses on methods for bacterial and viral diagnostics, with emphasis on serodiagnostics and viral infections.

Immunity and infectious diseases

In human beings, the immune system is divided into innate and adaptive systems. The innate immune system plays the central role in generic responses to pathogens, whereas the adaptive immune system is responsible for more specific immune responses such as the production of antibodies against a given pathogen and immunological memory. Innate immunity is normally short-lived, whereas the adaptive immune state may last for a lifetime. The innate immune system has several functions. These include activation of the complement system by the pathogen or its fragments, which may directly kill the pathogen or activates other immune mediators to the site of infection, or both. White blood cells (leukocytes), such as natural killer cells, macrophages, and dendritic cells, play a major role in killing the pathogen and evoking adaptive immune responses.

The innate immune system activates the adaptive immune system through a process called antigen presentation. In the early stage of infection, macrophage and dendritic cells of the innate immune system recognize the foreign material, engulf it and present peptide fragments of the invader on the cell surface as part of the machinery called the major histocompatibility complex (MHC). This process is called “antigen presentation.” Peptides are recognized by the T cells of the adaptive immune system, which initiates the process for differentiation into active defence cells and production of inflammatory mediators. This process includes the activation and maturation of antibody-producing B cells. Differentiated B cells called plasma cells produce antibodies against foreign material, such as a virus, an action that helps to eliminate the pathogen. A fraction of these mature B cells also remain as memory cells to develop immunological memory (95).

Immunoglobulins

Immunoglobulin (Ig) is a multimeric glycoprotein produced by the plasma cells of the adaptive system. Immunoglobulins are produced in soluble form, which are secreted from the cell, and also in membrane-bound forms attached to the cell surface. The membrane-bound Igs are referred to as B cell receptors (BCRs), whereas the soluble forms are called antibodies (95).

Depictions of the basic structure of human IgGs are often Y-shaped, even though, according to the structural studies, the structure is actually somewhere between a T- and Y-shape (Figure 1) (123). This includes four polypeptide chains in which two identical heavy (H) and light (L) chains are linked by disulphide bonds. These L chains exist in two forms: kappa (κ) and lambda (λ). The heavy and light chains are synthesized separately and thereafter united to form two H and L chain (HL chain) pairs, both being able to bind one antigen molecule. Thus, given the bivalent nature of immunoglobulin, one Y-shaped immunoglobulin can simultaneously bind two antigens (92).

The Y-shaped immunoglobulin molecule, roughly 150 kDa in size, can be divided into several fragments. For example, an immunoglobulin treated with papain yields three fragments: two Fab fragments and one Fc (fragment crystallizable) fragment. The papain cleavage site is located in the hinge-region. The Fc fragment contains domains of the H chains, whereas the Fab fragment contains domains of both H and L chains, and mediates the antigen recognition. The Fab

regions include both variable (V) and constant (C) domains, whereas the Fc region includes only C domains. The antigen-binding sites of an immunoglobulin locate at the tips of the Fab fragments, which consist of variable H- and L-chain domains. These sites are highly specific for each antigen (88,110). The Fc region interacts with cell surface receptors called Fc receptors, and also with the complement system, thus activating the adaptive immune system (95).

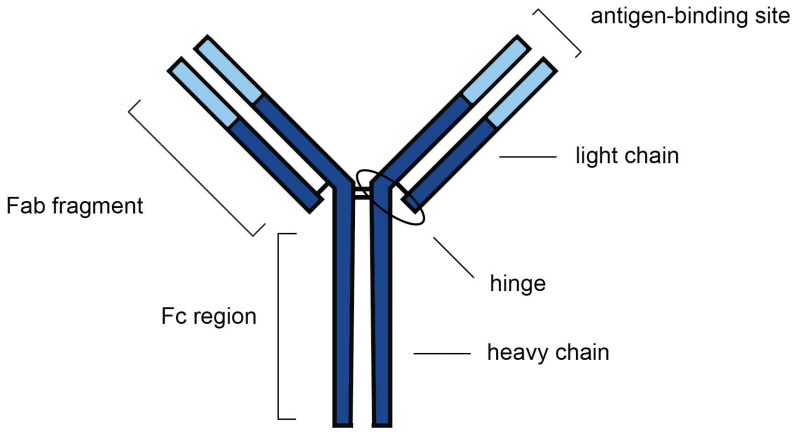

Figure 1. **Schematic structure of an immunoglobulin molecule.** Variable regions of the H and L chain are in light blue.

Based on differences in the structure of the H chain, immunoglobulins are divided into five classes: IgG, IgM, IgA, IgE, and IgD. The amount of the basic Y structure of Ig varies between the five classes. IgG, IgE, and IgD exist as monomers, whereas IgA exists usually as a monomer (in serum) or dimer (in secretions), and IgM mostly as a pentamer (4,110). In serum, the predominant immunoglobulins are those of the IgG class, whereas the IgM-class immunoglobulins are abundant only during infections. The IgA-class immunoglobulins are found in external sections such as tears, milk, saliva, and in the gut, and they play a role in mucosal immunity. The-IgE class immunoglobulins are mainly associated with allergies, whereas the function of IgD is to stimulate B cells and antimicrobial actions through interactions with basophils and mast cells (20,92).

The IgG and IgA classes are further divided into subclasses that vary in their H-chain physico-chemical, antigenic, and biologic properties. The IgG subclasses

include IgG1-IgG4, whereas the IgA class has only two subclasses: IgA1 and IgA2. The four IgG subclasses differ in their C domain, particularly in the hinge regions and upper CH2 domains, and are highly conserved. These regions are responsible for effector functions of antibodies, since they contain binding sites to both IgG-Fc receptors (FcγR) and C1q (complement). Hence, the effector functions of IgG subclasses differ in terms of triggering FcγR-expressing cells and complement activation. Each subclass also exhibits a unique profile regarding antigen binding, immune-complex formation, half-life, and placental transportation.

In addition, the subclasses vary in their abundance in serum, IgG1 being the most abundant, followed by IgG2, IgG3, and IgG4 in decreasing order (131). Of the IgA subclasses, IgA1 is more abundant in serum, whereas IgA2 predominates in secretions such as milk and saliva (26). The L chain types (κ and λ) can also serve to classify immunoglobulins, and hence the subclasses can be further divided into IgG1-4, κ and IgG1-4, λ (92). Immunoglobulins can also be classified according to their V gene segments. Both heavy and light chains have their own V segments (V_H and V_L, respectively), and the V_H and V_L segments can be further divided into families based on sequence and structural similarity (110).

Immunoglobulin binding molecules

The target molecules that immunoglobulins bind are designated as antigens. The soluble immunoglobulins (i.e. antibodies) can recognize almost any type of biological material as an antigen, including hormones, lipids, sugars, carbohydrates, phospholipids, nucleic acids, and proteins. The parts of an antigen that immunoglobulins recognize are called epitopes. One antigen can contain several epitopes, but each Fab arm of an immunoglobulin binds only to a single epitope. Epitopes formed by several adjacent amino acid residues are called linear epitopes. These epitopes are often inaccessible within the native form of the antigen, and become accessible when the antigen is denatured. Conformation epitopes, in contrast, form in areas where the residues are in juxtaposition, not due to their proximity in the amino acid sequence, but due to the protein folding. This antigen-antibody binding can be highly specific, but cross-reactions may occur, for example, if two antigens show marked similarity in their structure (2). Microbial proteins, or respective recombinant expression

products, serve in diagnostics as antigens to detect microbe-specific antibodies (102). The interaction of antigen and antibody is noncovalent and reversible (2).

Several immunoglobulin-binding proteins commonly serve to immobilize, detect, or purify immunoglobulins. Most were originally identified in bacteria, and thereafter produced as recombinant proteins. These microbial superantigens function to activate T-cells, and their properties differ in terms of the binding site in Ig and their ability to bind different Ig subclasses. The most commonly used are protein G, protein A, protein A/G, and protein L. Protein A is derived from *Staphylococcus aureus* (43,83), and has a binding site in the Fc region of an immunoglobulin, whereas Protein G originates from group C and G Streptococcal bacteria, and binds both Fc and Fab regions (36,117). Protein A and G both show strong affinity towards human IgG-class Igs. A chimeric form (protein A/G) also combining the IgG-binding domains of both Protein A and Protein G has been generated (30). Protein L derives from *Finegoldia magna* and has a binding site in the Fab region of an Ig. Protein L binds the human κ L chain without interfering with antigen recognition (3), and is capable of binding all Ig classes (IgG, IgM, IgA, IgE, and IgD) (24). Of the four major $V_L\kappa$ (I-VI) subgroups, only those from $V_L\kappa$II were unable to bind protein L (97,98,137). Additionally, protein L binds to single-chain variable fragments (scFv) and to Fab fragments bearing κ L chains. Hence, protein L binds to a wider range of Ig classes and subclasses than do the other Ig-binding proteins described here (11,97).

Immunoassays

Immunoassays in general rely on the ability of an antibody to identify and bind a specific macromolecule that is referred to as an antigen. Depending on the assay type, the analyte detected can be either an antibody or an antigen. Solomon Berson and Rosalyn Yalow introduced the first immunoassay techniques in the 1950s and 1960s (8,139). During the first decades, the immunoassays were mostly performed by means of radioactive labels. Since these labels were not considered the safest choice, scientists started developing novel approaches based on other reporter techniques (78).

The first descriptions of immunoassays using enzyme labels appeared in the 1970s, and are referred to as enzyme-immunoassay (EIA) or enzyme-linked immunosorbent assay (ELISA) (32,128). Ever since, ELISA-based applications

have played an important role in experimental research, veterinary medicine, clinical laboratories, and agriculture (33,78). Other clinically important immunoassays include IFA, immunoblotting, and immunochromatography. Basic characteristics of these assays are in Table 1 (41,63,86,101).

Immunoassays in general can be classified into heterogeneous and homogeneous, and competitive or non-competitive. Heterogeneous assays include various steps such as incubations and washings, whereas homogeneous assays are wash-free, and thus easier and more rapid to perform. In a competitive format, labeled and non-labeled analytes compete in binding of an antibody or antigen, and the signal detected inversely correlates with the amount of the analyte. In the non-competitive formats, such as in many ELISAs, the signal measured is proportional to the amount of analyte (23).

Table 1. Clinically important immunoassays. The basic characteristics of IFA, immunoblotting, immunochromatography, and ELISA with examples of sample material, assay time, and main benefits of each assay.

Immunoassay	Specimen	Assay time	Benefits
IFA	serum, tissue	2-3 h	staining pattern visible in the cell (morphological identification), quantitative
Immunoblotting	serum, tissue	5-8 h	sensitive, simultaneous detecion of antibodies against several antigens
Immunochromatography	serum, blood, urine	5-15 min	rapid (point-of-care), simple
ELISA	serum,	3-4h	quantitative, sensitive, high throughput format

Bacterial infections

Gram staining is a method classifying bacteria into either Gram-positive or Gram-negative; it often serves as the initial diagnostic method. Gram staining differentiates bacterial species into two groups by the composition of their cell walls. Microscopy, with or without staining, enables the identification of several bacterial infections, but morphological identification is always accompanied by other diagnostic procedures (9,15). By culturing the bacteria, specific features of the bacterial species, such as growth temperature, culture media, susceptibility to antibiotics, metabolic properties, and oxygen requirement (aerobic vs. anaerobic) emerge that are useful in identification. After primary classification as Gram-

positive or -negative, bacterial identification normally utilizes enzymatic and other assays (5).

Antigen detection, although uncommon in bacterial diagnostics, also serves as a method for identification of certain bacterial infections. For example, several of the rapid tests available for group A streptococcal infection are based on antigen detection (79,121). Another important and increasingly popular method in specific detection of bacterial types is nucleic acid detection, such as the polymerase chain reaction (PCR) (115). Serological diagnosis of bacterial infections is relatively rare, but is commonly employed in certain cases, when the culture method is not optimal for diagnostics. Serological approaches are applied in diagnosis of, for example, *Borrelia* spp. (21,100).

Viral infections

The most common methods in virus diagnostics include detection of the virus itself by virus culture or electron microscopy, detection of antibodies developed against the virus in the host, and detection of fragments of the virus such as the viral genome or proteins (antigens). Growing the virus in cell culture allows specific features such as the host-cell specificity and morphological changes caused in the infected cell to be identifiable. The role of viral culture is, however, diminishing. Antigen detection is particularly useful for viruses that are difficult to cultivate, or that grow slowly. The advantages of antigen detection include rapid assay time and sample handling, since the virus needs not to be viable in the sample. Fluorescence antibody staining (FA), EIA, and immunoperoxidase staining (IP) are common methods of viral antigen detection (70,122).

The virus can also be identified by nucleic acid detection (or testing, NAT), a highly sensitive diagnostic method. NAT relies on amplification of the viral DNA or RNA, such as by PCR, much as in bacterial diagnostics. PCR use requires prior knowledge of the pathogen´s genome, and the design of specific primers(86). In addition to PCR, in clinical virology, serology (i.e. detection of antibodies) is common (122).

Serodiagnosis

Serodiagnosis of viral infections is classically based on the detection of immunoglobulin G (IgG)- and immunoglobulin M (IgM)-class antibodies. Serological approaches are especially useful for viruses difficult to culture, such

as the human immunodeficiency virus (HIV) or the Epstein-Barr virus (EBV) (5,25,39). The disadvantage of serology is the usual lag between onset of acute symptoms of infection and antibody formation, making it a rather slow method. In most cases, an acute infection is diagnosed by the presence of IgM or rising IgG titers between the acute and convalescent phase of infection (Figure 2) (5). In certain infections such as rubella, and conditions such as pregnancy, IgM detection is insufficiently accurate, and serodiagnosis should be based not solely on IgM detection. If such an infection is suspected, performance of serodiagnosis can be based on IgG avidity (functional affinity) (60,106). IgG avidity refers to the overall strength of the antigen-antibody interaction, the strength of which matures over time, meaning that low IgG avidity is an indicator of primary infection, whereas high IgG avidity suggests past infection (48,77,104). IgG avidity has been applicable to the diagnosis of rubella, PUUV, hepatitis virus, human herpes viruses, HIV, and parvovirus B19, to name a few (19,31,42,49,104,124).

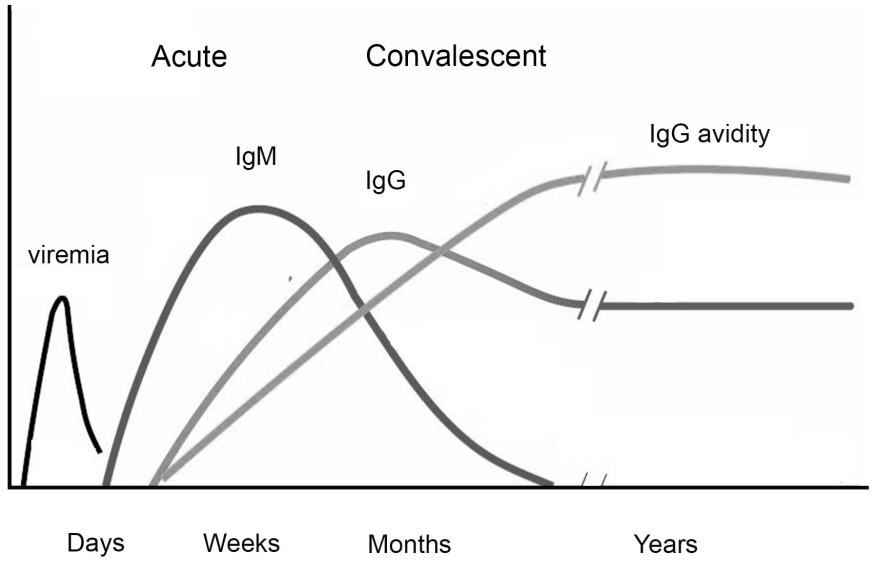

Figure 2. Infection- and antibody-response kinetics. At the onset of primary infection, the IgM response is usually formed within days of the exposure, after which the IgG molecules begin to form. IgG avidity matures during the first 6 months. Modified with the permission of the creater of the figure, Aftab Jasir (66).

Usually past-infection antibodies (IgG) are detectable even years after infection, and many viral infections provide life-long immunity (95). Unfortunately, some

viruses, ones such as influenza A, show such high genetic variation that acquired immunity against a certain virus strain does not necessarily protect against other strains (14,105). The presence of IgG-class antibodies, and the lack of IgM, is usually an indication of past infection. In some cases, the early IgG response is against epitopes or antigens differing from those involved in the late IgG response (67).

Diagnosis of Puumala virus infection

Puumala virus (PUUV, genus *Hantavirus*, family *Bunyaviridae*), a zoonotic virus carried by chronically infected bank voles (*Myodes glareolus*), is endemic in Finland. Human infection by PUUV is most commonly acquired via inhalation of aerosolized rodent excreta and manifests as a disease called nephropathia epidemica (NE) (35,130).

Typical symptoms of NE include abrupt fever, followed by headache, nausea, vomiting, abdominal pain, and signs of renal insufficiency. Somnolence and visual disturbances as well as pulmonary, cardiac, and central nervous system symptoms are also common (73,113,114,130). In acute-phase PUUV infection, the IgG antibodies are mainly targeted to the viral nucleocapsid (N) protein, whereas in its later stages, IgG antibodies against the glycoproteins Gc and Gn become more prominent (130). The N protein exists as monomeric, dimeric, and trimeric forms; the first two are transient, and the trimeric is the most stable form (93). Laboratory diagnosis of PUUV infection is based on detection of immunoglobulins (IgM and IgG), i.e. serodiagnostics. Antibody detection is commonly based on EIA, immunochromatography (IgM), immunoblotting using recombinant PUUV N as antigen, or an immunofluorescence assay (IFA) based on acetone-fixed infected cells (49,63,67).

Next-generation diagnostics

In addition to the classical methods, a number of new diagnostic tools exist. These new technologies offer great advantages, such as rapid assay time, multiplexing (simultaneous detection of several pathogens in a single assay), portable devices (enabling use in resource-limited settings), automation, and one-step workflows—advantages that the classical methods lack (15).

The platform design of microarrays, for example, enables high-throughput screening and multiplexing (16,81). Microarrays have been applied to a variety of

analytes. DNA microarray-based commercial products are available, for example, for diagnosis of sepsis (125) or of human herpesviruses(64). As an example, one commercial DNA-based microarray simultaneously identifies 50 different bacterial species or taxons as well as one antibiotic-resistance gene. The procedure is simple: after extraction of the DNA from blood culture, followed by (multiplex) PCR amplification, the products are hybridized on a microarray, and the results detected with specific software (125). Other examples of popular microarray applications include those detecting peptides and proteins (65,132).

Another example of a modern diagnostic method is mass spectrometry (MS). MALDI-TOF (Matrix-Assisted Laser Desorption Ionization Time-Of-Flight) -based MS methods have proven useful specifically for identifying difficult-to-cultivate bacteria (10). The method itself is not new, but the applications for microbial diagnostics are (17,87). Mass spectrometry allows for assessment of the chemical composition of a sample by detection of the mass-to-charge ratio of a bioanalyte. The resultant mass-spectral fingerprint of sample is thereafter compared to a database of known pathogens. The MS-based applications provide interesting alternatives for conventional diagnostics, with certain benefits such as rapid assay time, cost-efficiency, and the ability to analyze all pathogens present in a sample during a single assay (87). This method, however, relies on an up-to-date protein-fragment database that requires constant updating; this makes the user dependent on the manufacturer.

Employment of next-generation sequencing (NGS) in infectious disease diagnostics is expanding. The traditional PCRs are based on specific primers, and hence are able to identify only the targeted microbe; in many cases the method is unable to discriminate between genotypes, since the primers are targeted on conserved genomic areas. Moreover, several tests need to be conducted if the first etiological hypothesis proves incorrect. The advantages of applying NGS in clinical diagnostics is that it does not require specific primers, and only one assay provides information on all pathogenic DNA or RNA present in the sample. Further development is required in terms of sample preparation, availability of the sequencer in central laboratories, of bioinformatics, and of costs, before NGS could become routine practice in diagnostics (76). Scientists anticipate that NGS technology will prove particularly useful in identifying novel pathogens (6).

Rapid diagnostics

Rapid and accurate treatment calls for rapid and specific diagnostics, which is often useful to prevent misuse of antibiotics. Over the years, researchers have developed rapid diagnostic tests (RDTs) for a variety of disease conditions. The motivation to develop such tests is to shorten the turnaround time, as well as the ability to use these in resource-limited settings such as in the developing world at the point of care (29,47,61,85,103). Rapid diagnostics also plays an important role during epidemics and pandemics. One estimation is that availability of RDTs during recent West African Ebola epidemics would have reduced the scale of the epidemics by over a third, if primary tests (RDTs) had been used in combination with a confirmatory PCR (99).

Many RDTs are also point-of-care (POC) tests. Among several ways to define the term POC test is as ”a medical test conducted at or near the site of patient care” (29). The first POC test was developed in 1962 for measuring blood glucose (22). The next breakthrough in this field was the development of a rapid pregnancy test in 1977 (1). During the past decades, POC-test availability has been expanding rapidly, also for infectious disease diagnostics. POC testing has demonstrated effective sampling and diagnostic testing in resource-limited settings. Simple POC assays require only minimal personnel training, but interpretation of test results requires a health-care professional, who may or may not be on site. The characteristics of an ideal POC assay include accuracy (sensitivity and specificity), heat-stable reagents, portability, minimal user-training requirements, rapidity (assay time of 1-2 hours), cost efficiency, and suitability for a number of clinical specimens. In resource-limited locations, diagnostic assays should require little or no power input, and minimal training requirements for both operation and interpretation of test results. Any need for cold-chain (storage of samples or reagents) should also be minimal (15). Several rapid assays are available for infectious diseases, including HIV, influenza A and B, group-A streptococcal infection, syphilis, and malaria. Many of these tests, however, suffer from poor sensitivity and specificity (15,103,122).

Luminescence

Luminescence is light emitted from certain materials at relatively low temperatures, such as room temperature. In luminescence, an energy source triggers a sequence of events, in which an electron enters into an excited state and dissipates its energy by emitting light at certain (usually lower) wavelengths. Luminescence can be formed in several ways, depending upon its energy source (127). Luminescence types are described in Table 2, but it should be noted that several classification patterns exist in the literature, of which this table represents only one (126).

Table 2. Luminence types and their energy sources.

Type of Luminescence	Energy source
Chemiluminescence	Chemical reaction
Electroluminescence	Electric field
Cathodoluminescence	Cathode rays
Triboluminescence	Frictional and electrostatic forces
Bioluminescence	*In vivo* biochemical reaction
Sonoluminescence	Ultrasound
Photoluminescence	Absorption of light (photons)
Fluorescence	
Phosphorescence	
Radioluminescence	Ionizing radiation
Thermoluminescence	Heating after prior storage of energy

Photoluminescence is the form of luminescence in which radiation serves as the energy source, and absorption of photons produces emission of light (127). One specific type of photoluminescence, fluorescence, has been employed in a variety of immunodiagnostic tools. One luminescence-based phenomenon is called Förster resonance energy transfer (FRET).

FRET

Förster Resonance Energy Transfer (FRET) is a process of non-radiative energy transfer between two chromophores, the donor and the acceptor. A German scientist, Theodor Förster, was the first to describe the phenomenon back in the 1940s (40). In FRET, the acceptor molecule can either be fluorescent or non-fluorescent, with a spectral overlap of the donor and acceptor chromophores required (Fig 3). Another requirement for FRET is close proximity of the two chromophores, typically in the range of 15 to 60 Å. Moreover, the dipole-dipole angles of the donor and acceptor should not be perpendicular to each other (75). The theoretical intensity of FRET between a given donor-acceptor pair can be estimated by the Förster distance, R_0, which represents the distance between the donor and acceptor, at which the energy transfer is 50% efficient (40,75). The Förster distance can be calculated by the following equation:

$$R_0 = 0.211[\kappa^2 n^{-4} Q_D J(\lambda)]^{-1/6}$$

where κ^2 is a factor describing the dipole orientation (usually assumed to be 2/3), n is the refractive index of the medium (typically ¼ for aquetious solutions), Q_D is the quantum yield of the donor in the absence of the acceptor, and $J(\lambda)$ is the spectral overlap of the donor-acceptor pair (75).

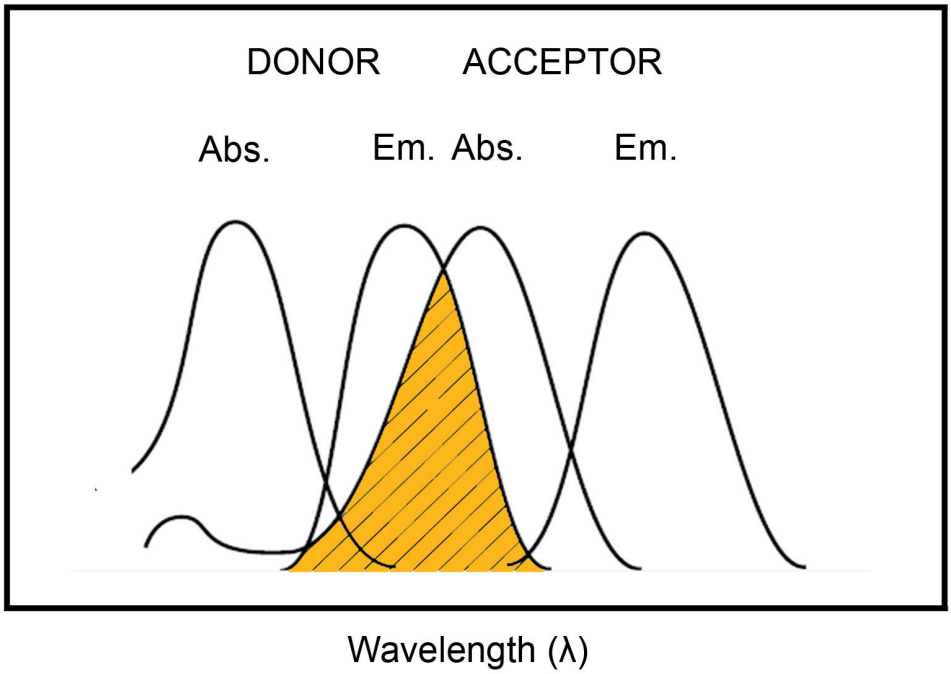

Figure 3. **Schematic illustration of the overlapping spectra of the donor-emission and acceptor-absorption wavelengths.** The overlapping spectra are in orange. Abs, absorbance; Em, emission. Modified with the permission from the publisher. (Valeur B, Berberan-Santos MN editors. Molecular Fluorescence: Principles and Applications. second ed.: Wiley-VCH Verlag GmbH & Co.; 2013, page 113.)

Molecular processes behind the absorption and emission of light in FRET are illustrated in the Figure 4 Jablonski diagram. As the donor absorbs energy, it moves from the singlet ground state (S_0) to the higher level of the first excited singlet state (S_1), The molecule rapidly returns to the lowest energy level of S_1 (vibrational relaxation), and if in close-enough proximity to an appropriate acceptor, the donor transfers part of its excitation energy to the acceptor via dipole-dipole coupling (75,126).

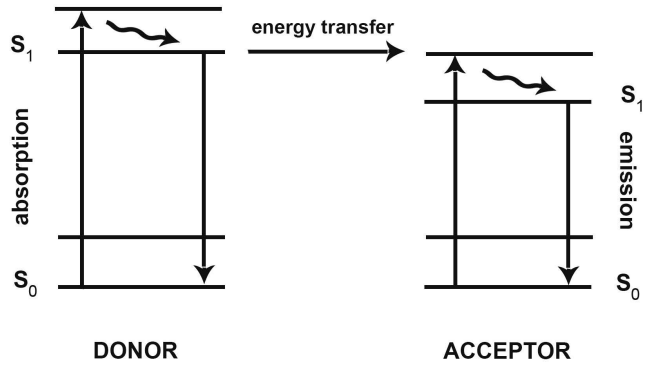

Figure 4. Jablonski diagram illustrating energy states and transitions between donor and acceptor molecules. Horizontal arrows illustrate energy state transitions due to absorption or emission of photons. Wavy arrows illustrate vibrational relaxation.

Time-Resolved FRET

Biological samples such as serum are prone to autofluorescence, which limits the use of conventional fluorophores. Time-resolved detection of FRET efficiently minimizes the sample-derived autofluorescence. Time-resolved FRET relies on two important properties of the donor fluorophore: long emission half-life and a large Stokes shift. Stokes shift is the gap between the maximum of the first absorption band and emission spectra of the same electronic transition, measured as wavelengths or frequency units. For example, in FRET, the donor absorbs a photon, enters an excited state and transfers its excitation energy to the acceptor. When the acceptor emits light at lower energy than the original absorption, the energy difference is called the Stokes shift (75). TR-FRET utilizes rare-earth elements, ones with exceptionally long emission half-lives in their cryptic or chelated forms, to enable time-resolved detection. These unique rare earth elements, such as europium (Eu) or terbium (Tb), are called lanthanides, and they typically serve as donors for acceptors such as Alexa Fluor or cyanine dyes such as Cy5. The correct term for the light emitted by lanthanides would be luminescence or photoluminescence, however, this light is commonly referred to as time-resolved fluorescence (46). This thesis uses the term “fluorescence”.

The applicability of lanthanide chelates as “reporters” (also known as “labels”) for time-resolved fluorometry (TRF) was already attracting interest in the 1970s

(118), with much progress and research on this topic resulting (28,51,54,74,94,119). An ideal reporter should fulfill certain requirements such as small size, stability, hydrophilic nature, and high luminescent intensity. To fulfil these requirements, lanthanide ions must be combined with an organic chromophore (chelate). The chelate absorbs the excitation energy and transfers it to the lanthanide ion, which then moves into an excited state. The chelate structures include a reactive group used for coupling the reporter to biomolecules, and this also shields the lanthanide ion from the quenching effects of water (51,55,112). Figure 5 illustrates the structure of an Eu chelate (W1024) with isothiocyanate activation chemistry. Luminescent Eu chelates are commonly used as donors for TR-FRET-based applications. The spectral properties of Eu chelates make them good candidates as donors, since they exhibit a large Stokes shift with no overlap between the excitation (at around 340) and emission (at around 615 nm) wavelengths. In addition, the emission wavelength of Eu is above the autofluorescence originating from biological samples (usually 400–600 nm) (44,54).

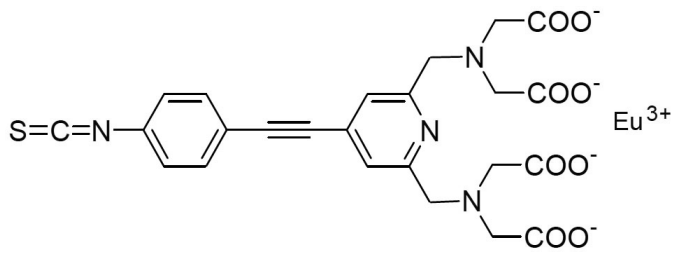

Figure 5. The structure of an Eu chelate (W1024) with isothiocyanate activation chemistry. Modified with permission of BN products & Services Ab Oy.

FRET-based applications

FRET has been widely applied in biomedical research in proximity-based assays. Here the term “proximity” refers to the characteristics of FRET, because FRET requires the close proximity of the donor and acceptor for sufficient energy transfer. Examples of FRET-based applications include those studying protein-protein interactions, DNA-hybridization, ligand-receptor binding, and antigen-antibody binding (7,12,59,62,69,82,90,135,136,138).

Many of the applications studying antigen-antibody binding by FRET rely on time-resolved detection. Four basic configurations for such assays are commonly used depending on whether the assay is performed on a solid-phase (heterogeneous, multistep) or a solution-phase format (homogeneous, separation-free), and whether the assay is competitive or non-competitive (45). One of the first homogeneous immunoassays utilizing TR-FRET was established in the 1980s for the detection of thyroxin (53). FRET as a detection method also enables multiplexing by use of a variety of distinct label pairs (71,72,135). Such applications have been reported with either distinct donors (116) or a variety of acceptors in the same dual-label assay (71). Quantum dots (QDs) offer an interesting tool for applications utilizing FRET for multiplexing. These are small nanocrystals with unique photophysical properties, and can serve as FRET donors or acceptors. The high extinction coefficients, narrow emission bands, broad excitation and absorption spectra, and enhanced photostability make them superior to traditional organic fluorophores. The fluorescence emission of QDs is size-dependent, which makes them tunable. In addition, QDs appear particularly efficient in sensitivity, and due to their broad emission spectra, they also enable multiplexing at even up to six distinct wavelengths (18,59,96,140).

.

AIMS OF THIS STUDY

Aims of the present study were to explore the possibilities of TR-FRET for rapid serodiagnosis of infectious diseases.

More specifically the aims were

- to determine whether a bivalent IgG molecule can act as a “FRET bridge”
- to determine the applicability of TR-FRET in homogeneous immunoassays
- to evaluate the clinical performance of these homogenous TR-FRET assays with hantavirus disease as the model

MATERIALS AND METHODS

Ethics statement

All serum samples for this study were originally sent to Helsinki University Central Hospital Laboratory Diagnostics (HUSLAB, Department of Virology and immunology), and studied under research permits § 32, 14 June 2013, and 553/E6/2001.

Antigens and antibodies

Antigens and antibodies are indicated in Table 3, in which PAb refers to polyclonal antibody and MAb to monoclonal antibody. PUUV-N represents an immunodominant antigen in PUUV infection, and for our experiments was expressed by use of the baculovirus system in insect cells (129). VP1u-GST is an antigen including the unique region of human parvovirus B19 minor capsid protein (VP1), expressed in *E. coli* as a GST-fusion protein (89). The antigenic region we targeted was the GST.

Fab fragments from an anti-streptavidin (anti-SA) MAb, when generated and purified, served as a control in the FRET-bridge assay. Briefly, the MAb was fragmented with papain, and the reaction was carried out at 37°C for 4 h. Thereafter, the papain was inactivated with iodoacetamide. To remove any remaining intact IgGs and to separate the cleavage products (Fab and Fc), we used Gammabind™ Plus Sepharose™ (GE Healthcare), and the resulting supernatant containing the Fab fragments was concentrated with Amicon Ultra 10 kDa centrifugal filter units (Millipore). A more detailed protocol for this procedure appears elsewhere (109).

Table 3. **Antigens and antibodies for the experiments, including suppliers.**

Product name	Supplier
PUUV-N	Reagena Ltd (129)
Anti-PUUV N MAb (clone 3H9)	Åke Lundkvist, University of Uppsala (84)
Anti-PUUV N MAb (clone 1C12)	Åke Lundkvist, University of Uppsala (84)
Pierce™ Recombinant protein L	Thermo Fischer Scientific
Europium chelate-labeled streptavidin	PerkinElmer
Alexa Fluor 647-labeled streptavidin	Thermo Fischer Scientific
Anti-SA MAb (mouse IgG2, clone S3E11)	Thermo Fischer Scientific
Anti-SA PAb	Abcam Ltd
Anti-SA PAb	Springbioscience Inc
Anti-GST MAb	Abcam Ltd
VP1u-GST	An in-house product (89)

Fluorophore-labeling reactions

The antigens PUUV-N and VP1u-GST, and the protein L were labeled with Europium-chelate (Eu) by the QuickAIIAssay Eu-chelate protein-labeling kit (BN Products & Services Oy). The VP1u-GST, the protein L, and the anti-PUUV MAbs were labeled by the Alexa Fluor 647 (AF647) protein-labeling kit (Invitrogen). All labeling reactions were according to manufacturers´ instructions.

Clinical samples

All serum samples were originally sent to HUSLAB for analysis of their PUUV antibodies. While setting up the LFRET assay, we used a panel of 58 archival samples, and the assay performance was further evaluated by 153 coded samples, which we examined blindly. The parameters of the CFRET were optimized by use of 128 retrospective samples, and further evaluated by 201 coded samples.

TR-FRET assays

FRET bridge

The protocol, as described (109), was as follows: the labeled antigens and the antibody were combined at a reaction volume of 20 µl and dispensed by pipette onto a microwell plate. The results were thereafter detected with a Wallac Victor2 fluorometer (PerkinElmer) by excitation at 320 nm followed by a delay of 70 µs before recording the fluorescence. The fluorescent counts were recorded for 100 µs with 615 nm (Eu) and 665 nm (AF647) emission filters and normalized following the equation: $AF647_N$ = AF647–k*Eu, in which $AF647_N$ means normalized AF647 fluorescent counts, AF647 means unnormalized A647 counts (at 655 nm), k means Eu emission at 665 nm/Eu emission at 615 nm, and Eu means Eu fluorescent counts (at 615 nm). The assay principle is illustrated in Figure 6.

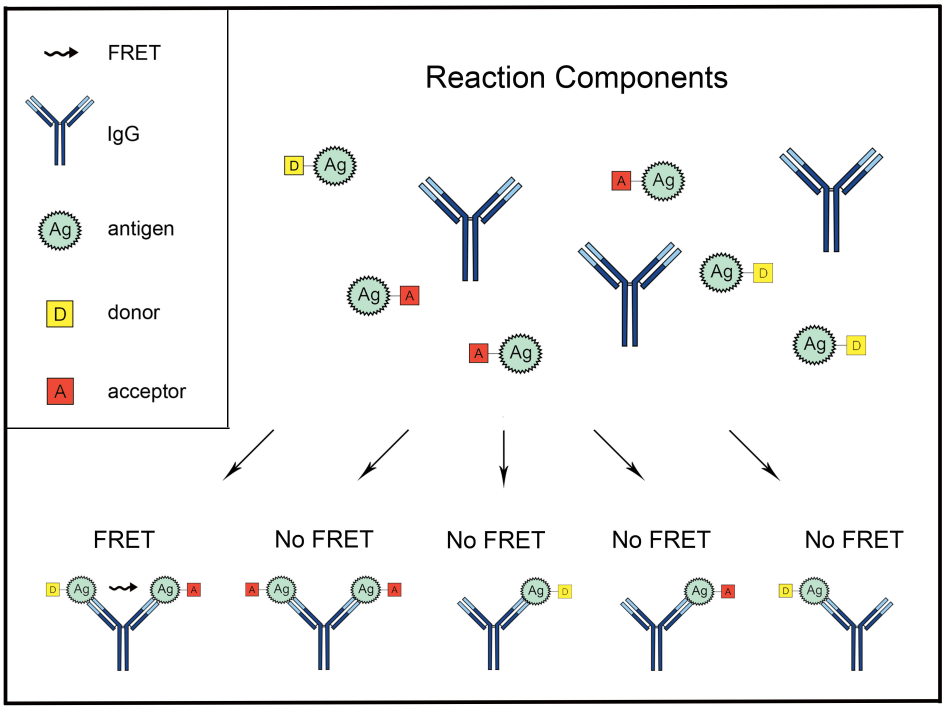

Figure 6. The FRET-bridge assay principle. A variety of molecule combinations form after the combination of the reaction components. FRET signal forms when donor-labeled and acceptor-labeled antigens are simultaneously bound to an IgG.

LFRET

Initially the protocol was this: labeled protein L was mixed with the antibody, followed by 15 min incubation at 37°C, and thereafter mixed with labeled antigen at a reaction volume of 20 µl. This mixture was dispensed onto a microwell plate, and the results were detected with a Wallac Victor2 fluorometer (PerkinElmer). The protocol was slightly modified when adapted to clinical samples. The serum samples were IgG-depleted by diluting them 1/10 in GullSORB (Meridian Bioscience Inc.), followed by centrifugation at a relative centrifugal force (RCF) of 16 000 for 10 s. The remaining supernatant was further diluted to obtain a final dilution (on plate) of 1/100. Two sample preparations were studied: one with and one without IgG depletion. At first, labeled protein L was combined with the antigen, and 15 µl of the mixture was dispensed onto a microwell plate followed by addition of 5 µl of serum dilution. The results were detectable with a Wallac Victor2 fluorometer after 30 min at +37 °C. The measurement parameters were the same as described above for FRET bridge. More details are available (56,57) and the assay principle is illustrated in Figure 7.

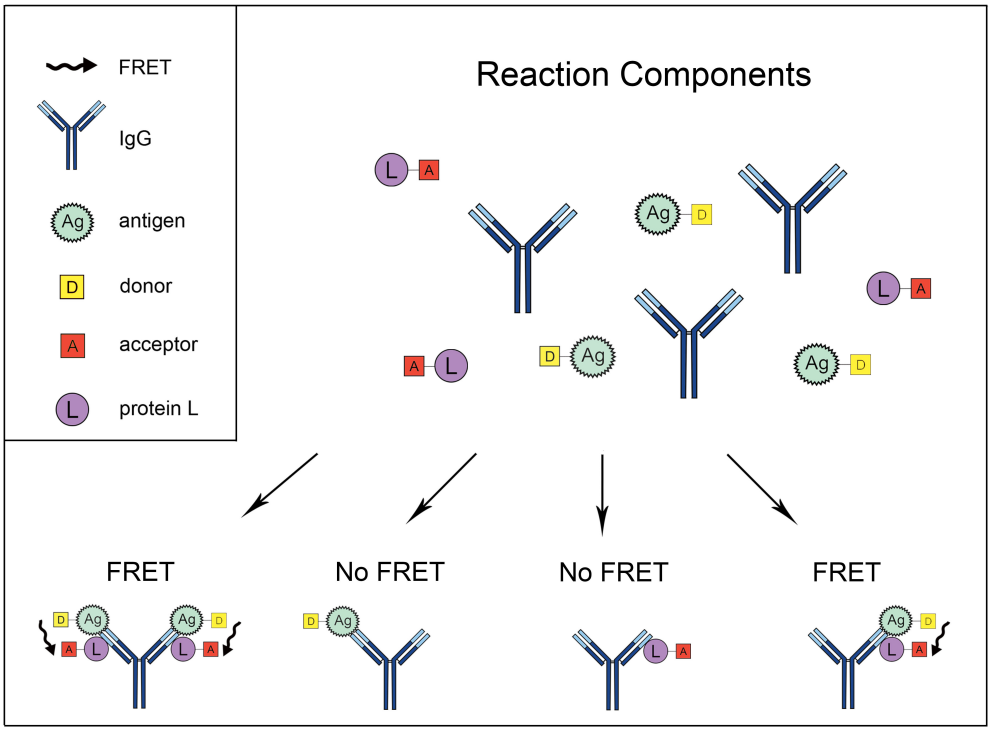

Figure 7. The LFRET assay principle. After combining the reaction components (labeled antigen, labeled protein L, and antibodies), a variety of molecule combinations form. FRET signal forms, when acceptor-labeled protein L and donor-labeled antigen are bound by the same Fab-arm of an Ig. This principle is exemplified by an IgG, although similar FRET-active complexes also form with other antibodies, such as IgM.

CFRET

The protocol was in brief: the antigen and serum sample were combined and dispensed onto a microwell plate followed by 10-min incubation at RT. Labeled anti-PUUV-N monoclonal antibody (3H9) was added to the wells followed by a second incubation period of 15 min at RT. The final reaction volume was 20 µl. Finally, the results were detected with a Wallac Victor2 fluorometer. For each sample, two preparations were examined: one with and one without IgG depletion. IgG depletion was performed as described above for LFRET, and the samples were further diluted to obtain 1/50 dilution on the plate. Measurement parameters were the same as for LFRET and FRET bridge. More details of the protocol are available (58). The assay principle is in Figure 8.

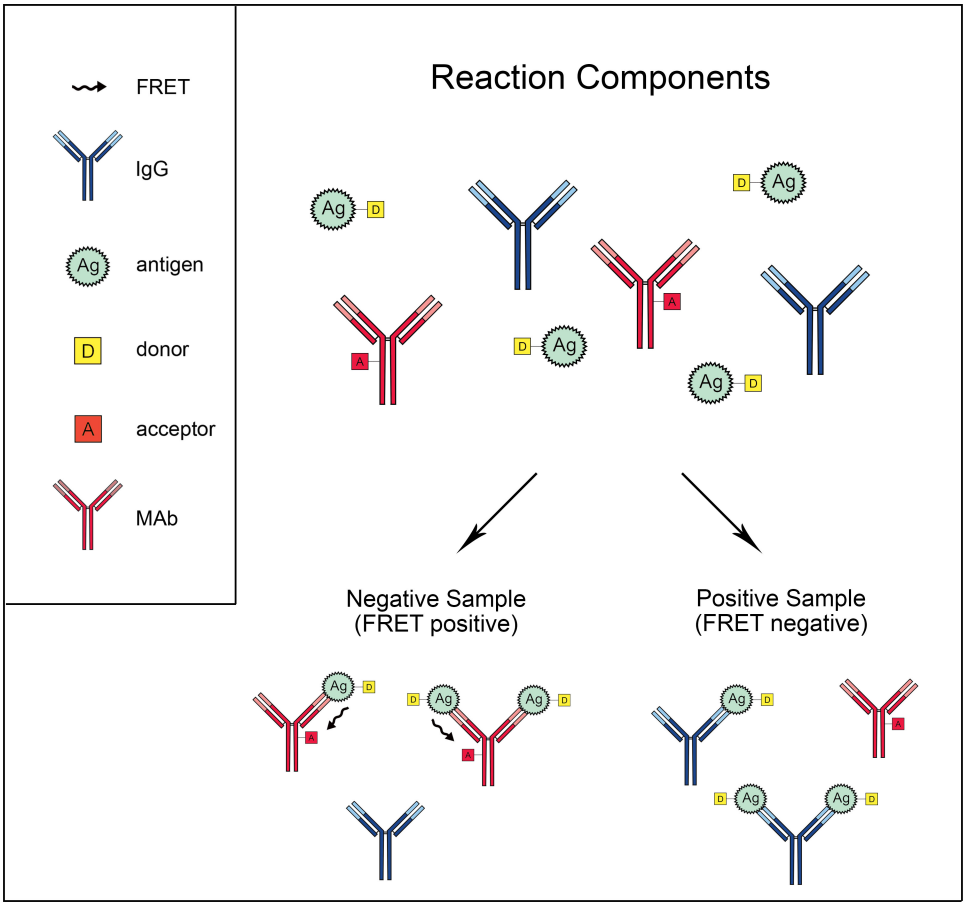

Figure 8. **The assay principle of CFRET.** After the combination of the reaction components (labeled antigen, labeled MAb, and serum antibodies), a variety of molecule combinations form. The lack of FRET signal indicates a positive sample, since the serum antibodies compete for antigen binding, thus preventing any FRET-signal formation between competitive acceptor-labeled MAb and donor-labeled antigen. This principle is exemplified by an IgG.

Reference methods

Reference methods used to evaluate the performances of CFRET and LFRET immunoassays in PUUV diagnostics were an IgM-capture enzyme immunoassay, an IgG IFA, and an IgM IFA. The IgM-capture enzyme immunoassay and the IgG IFA (49,67) are accredited (SFS-EN ISO/IEC 17025 and SFS-EN ISO 15189; Finnish Standards Association) and used in diagnostics in HUSLAB. All of the reference tests are manufactured in-house, and the assay times range from 3 to 4 hours. The antigen for the IgM-capture enzyme immunoassay was

manufactured similarly to the one we used in LFRET- and CFRET assays (129). For more details and the protocol for PUUV IgM IFA see Hepojoki et al. (58).

Statistics

The statistical tool for analyses in the LFRET assay was IBM SPSS software version 21. The SAS 9.3 program served as the tool for calculating the Spearman correlations for the CFRET.

RESULTS

Introduction

The four studies of this thesis project, which explored rapid antibody detection by FRET-based applications, developed three distinct approaches, two of which were also successfully applied in clinical virology. Study I introduced a concept utilizing FRET-pair-forming fluorophore-labeled antigens for antibody detection in a homogeneous assay format. Simultaneous binding of the antigens to an antibody induced a FRET signal. We designated this assay the “FRET bridge.”

Study II and Study III dealt with another FRET-based homogeneous immunoassay, designated “LFRET,” which utilized fluorophore-labeled protein L and fluorophore-labeled antigen to detect antibodies. Here, a FRET signal was detectable when both labeled molecules, protein L and antigen, were bound to the same Fab arm of an Ig. Study II introduced the proof of concept using SA as an antigen, whereas in Study III, the LFRET was applied for diagnosis of acute PUUV infection.

In Study IV, a competitive FRET-based immunoassay detected antibodies by fluorophore-labeled MAb in combination with a fluorophore-labeled antigen. The labeled MAb competes with serum antibodies in binding to the antigen, which in the case of a positive sample resulted in FRET-signal inhibition. This assay was designated “CFRET”, and also here the first clinical application was developed for diagnosis of PUUV infection.

FRET bridge

Study I examined the possibility of detecting antibodies in a homogeneous assay format by FRET-pair-forming fluorophore-labeled antigens. Initially, the functionality of the FRET-bridge assay was examined by a series of cross-titration assays. With both antigens (donor-labeled and acceptor-labeled streptavidins) at 8 nM, the specific antibodies (PAb and MAb) induced TR-FRET signals, whereas the control produced only background fluorescence. The signals recorded with the PAb increased dose-dependently, whereas with the MAb, a

peak was observable at a concentration of 17 nM, after which the signals began to decrease. Also with the PAb, at lower antigen concentrations than 8 nM, a similar peak was observable as was with the MAb (Figure 9). Fab fragments of the anti-SA MAb served as a control, which showed no FRET activity above the recorded background signals.

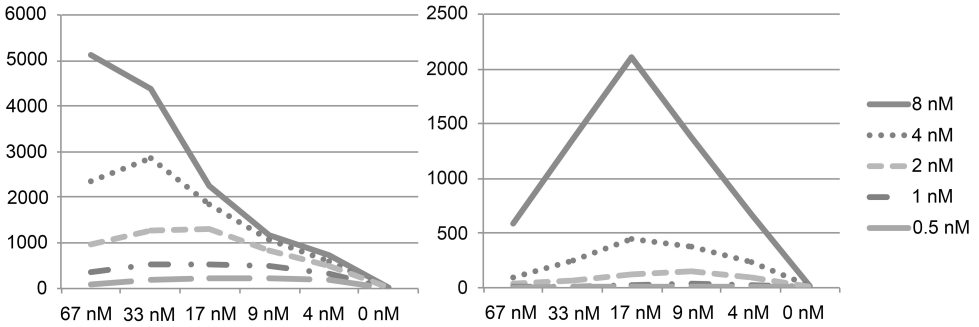

Figure 9. Antibody-antigen titration. The left graph represents results from the anti-SA PAb, while the right graph represents those from the anti-SA MAb. The antigen concentrations were from 0.5 nM to 8 nM. Y-axis: normalized response counts (FRET signal); X-axis: antibody concentration.

To examine the sizes of the TR-FRET-inducing molecular complexes, we separated them by sucrose-gradient ultracentrifugation and analyzed the fractions. By this, we sought to study whether TR-FRET signals derive from one IgG plus two antigens, the original hypothesis. Anti-SA MAb and PAb in combination with the tetravalent SA were subjected to separate examinations, both yielding similar results, indicating that TR-FRET activity derives from molecular complexes of various sizes. With MAb, most of the FRET activity derived from high-density fractions corresponding to a relative molecular mass (Mr) of 500 to >1000 kDa. Examination of distinct combinations of antibody and antigen showed the most suitable to be a 1:1 ratio, since in this case virtually no antibodies migrating without the antigen were detectable. A minor proportion of TR-FRET signal was observable in lower-density fractions corresponding to an Mr of ~300 kDa (Figure 10). As to the suitability of a monovalent antigen, we examined VP1u-GST for the FRET-bridge assay. The combination of fluorophore-labeled VP1u-GSTs and anti-GST failed to induce positive FRET signals.

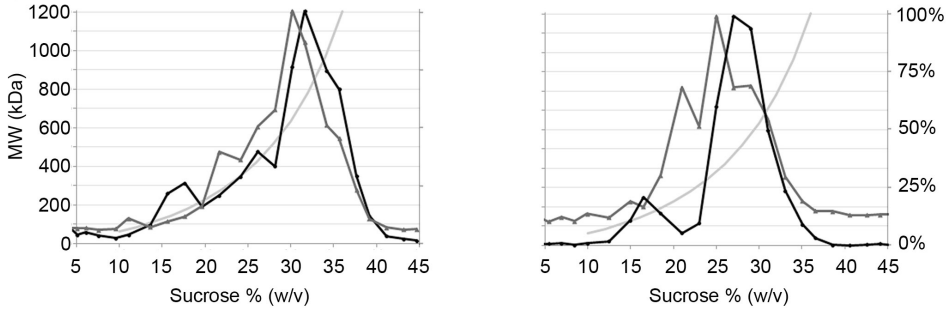

Figure 10. **Analysis of immune complexes in density-gradient ultracentrifugation**. The left graph represents results with the anti-SA MAb, whereas the right graph represents results with the anti-SA PAb (Abcam). Black line: FRET response; dark gray line: relative antibody concentration. Light gray line: estimated molecular weight (Y-axis) for each sucrose concentration (X-axis). The second Y-axis represents a relative scale for both antibody and FRET signal.

LFRET

Study II explored the proof of concept for another homogeneous immunoassay. Here, the antibodies were detected by a fluorophore-labeled antigen and a fluorophore-labeled immunoglobulin-binding molecule called protein L. Also here, the suitable assay conditions were examined by cross-titration, followed by examination of the fluorophore labels´ best positioning. The most suitable signal-to-noise (S/N) ratios resulted from the acceptor label in the protein L and the donor in the antigen. The optimization experiments were performed by using two antigens in parallel: a tetravalent SA and a monovalent VP1u-GST. To examine whether FRET signals truly originate from a single Fab arm, anti-SA Fab fragments cleaved from an anti-SA MAb were combined with protein L and SA. When compared to intact MAb, similar, although slightly weaker, dose-dependent FRET signals resulted.

PUUV nucleocapsid protein served as the first viral antigen in the LFRET assay. Study III established the proof of concept for detecting human PUUV antibodies with LFRET with a retrospective sample panel consisting of 58 serum samples characterized as 21 with acute infection, 17 as past infection, and 20 as negative. Based on results with these sera, the threshold for positivity was set at signal level 3-fold the background, which corresponds to the average + 5.79 times the

standard deviation (SD) of signals induced by the negative samples. Of the 21 acute-infection (IgG^+ IgM^+) samples, 20 induced positive TR-FRET signals, whereas of the 17 past-infection (IgG^+ IgM^-) samples, only 10 were identifiable as positive. None of the seronegative (IgG^- IgM^-) samples induced TR-FRET signals ≥3-fold the background signals.

Next came evaluation of the clinical performance of the assay with a prospective sample panel using coded (blind) samples. Altogether 211 (prospective + retrospective panels) samples were examined. Of the 61 acute-infection 59 (97%), and of the 27 past-infection samples 14 (52%), induced LFRET-positive signals, but false-positive results in the 103 seronegative samples numbered only 7. After IgG depletion, 58 of the acute-infection samples, 95.1%, induced positive signals, while all of the past-infection and seronegative samples were identifiable as negative (Table 4).

Table 4. LFRET in a total of 211 samples.

	Acute (IgG+/IgM+)		Past (IgG+/IgM-)		Negative (IgG-/IgM-)	
Test result (n)	**LFRET**	**Reference**	**LFRET**	**Reference**	**LFRET**	**Reference**
Acute (61)	58	61	0	0	0	0
Past (27)	1	0	14	27	0	0
Neg (123)	2	0	13	0	123	123
Total (211)	61	61	27	27	123	123

Pearson correlation of the reference ELISA IgM absorbance values (at 1:200 dilution) with the S/N ratios of the 40 IgG-depleted coded acute-phase samples showed a correlation coefficient of 0.19 (P = 0.24, i.e., nonsignificant). However, after ignoring one outlier with an exceptional signal-to-noise ratio (24), the Pearson correlation (r) was 0.35, which is statistically significant (P = 0.0276).

CFRET

In selection of the competing PUUV-N antibodies, performances were compared of two MAbs, 3H9 and 1C12, in the CFRET assay. Whereas both MAbs were inhibited by sera of PUUV-seropositive individuals (both acute- and past-

infection), the MAb 3H9 was inhibited proportionately more than was MAb 1C12. Hence, the following experiments were performed only with MAb 3H9.

While setting up the assay parameters, we observed that all of the 21 acute-phase samples, examined with and without IgG depletion, induced relative TR-FRET inhibition >20%. Of the 19 past-infection samples, 15 induced relative TR-FRET inhibition >20% without IgG depletion, whereas with IgG depletion, all of the samples exhibited inhibition values <20%. All 21 seronegative samples induced <20% inhibition values regardless of IgG depletion.

Based on these data, we set the threshold for positivity at 20% relative TR-FRET inhibition, which corresponds to the mean + 3.6 SDs of the inhibition values of negative samples without GullSORB treatment and the mean + 3.8 SDs of the inhibition values of negative samples with GullSORB treatment. Diagnosis of acute-phase PUUV infection was achieved by comparing results from serum samples with and without IgG depletion. Samples inducing >20% relative TR-FRET inhibition with or without IgG depletion represented acute-phase infection, whereas past infection samples induced inhibition values >20% only when IgG was present. Those samples that induced inhibition values <20% regardless of IgG depletion were considered negative. In total, we analyzed 128 retrospective samples.

In the prospective study, 201 coded samples underwent analysis. By comparing the inhibition values with and without IgG depletion, all of the 40 acute-phase samples were identified, whereas of the 14 past-infection samples, only 12 induced positive signals with IgG present and none induced signals without IgG. Of the 142 seronegative samples, 2 induced positive signals; one was identified as acute and the other as past infection. Of a total of 329 samples tested, the CFRET assay identified acute PUUV infection with 100% sensitivity and 99.1% specificity, and the respective values for past-infection samples were 78.5% and 99.7%. Results of the retrospective and prospective sample panels are summarized in Table 5.

Table 5. Prospective and retrospective sample panels. CFRET results obtained from the 329 samples compared by reference methods. Modified with the permission of the American Society for Microbiology, from Hepojoki et al., "Competitive Homogeneous Immunoassay for Rapid Serodiagnosis of Hantavirus Disease," J Clin Microbiol ;53:2292-2297 2015.

	Acute (IgG+/IgM+)		Past (IgG+/IgM-)		Negative (IgG-/IgM-)	
Sample type (n)	**CFRET**	**Reference**	**CFRET**	**Reference**	**CFRET**	**Reference**
Acute (101)	101	101	1	0	1	0
Past (42)	0	0	33	42	1	0
Neg (186)	0	0	8	0	184	186
Total (329)	101	101	42	42	186	186

Comparison of the sensitivities of CFRET and reference tests involved examination of randomly chosen samples representing 10 acute and 10 past infections in all assays (CFRET, IgG IFA, IgM ELISA, and IgM IFA). Results were that the highest IgM titer from the CFRET was 3,200, whereas for ELISA and IgM IFA the respective values were 25,000 and 2,560. The highest IgG titer that CFRET recognized was 200, while for the IgG IFA, it was 5,120 (Table 6). Comparison of IgG or IgM titers of the selected sera to the CFRET titers yielded poor correlations. When IgG and IgM IFA titers were summed (in 2-logarithmic scale), however, a significant correlation (Spearman's correlation coefficient [r] = 0.62; P = 0.0036) resulted with the CFRET titer. We also compared the performances of CFRET and the LFRET by analyzing 131 (60 acute-infection, 26 past-infection, and 45 seronegative) samples with both assays. The CFRET recognized 100% of acute-infection and 73% of past-infection samples, whereas the respective values with LFRET were 95% and 54%. All seronegative samples were identifiable with both assays.

Table 6. Comparison of assay sensitivities. Highest antibody titers indicated for each assay. Samples 1-10: acute infection; samples 11-20: past infection. Modified with the permission of the American Society for Microbiology, from Hepojoki et al., “Competitive Homogeneous Immunoassay for Rapid Serodiagnosis of Hantavirus Disease,” J Clin Microbiol ;53:2292-2297 2015.

Samples	IgG IFA	IgM IFA	IFAs combined	ELISA	CFRET with IgG	CFRET without IgG
1	1280	80	1360	200	800	800
2	5120	320	5440	5000	800	800
3	320	640	960	5000	200	200
4	2560	80	2640	1000	50	50
5	160	5*	165	5*	200	200
6	5120	80	5200	1000	3200	3200
7	40	2560	2600	25000	200	200
8	5120	320	5440	5000	800	800
9	640	1280	1920	25000	800	800
10	5120	1280	6400	25000	200	800
11	160	<10	160	<10	50	<10
12	80	<10	80	<10	50	<10
13	640	<10	640	<10	200	<10
14	160	<10	160	<10	50	<10
15	80	<10	80	<10	50	<10
16	320	<10	320	<10	200	<10
17	2560	<10	2560	<10	50	<10
18	1280	<10	1280	<10	200	<10
19	320	<10	320	<10	50	<10
20	320	<10	320	<10	50	<10

* This individual sample showed diagnostically low IgG avidity, despite discordantly below-cut-off levels of IgM antibodies to N by an EIA test.

DISCUSSION

This thesis presents three TR-FRET-based approaches for antibody detection. All are based on donor-labeled and acceptor-labeled components, which, when in close proximity, produce a FRET signal. In addition, all assays function in a homogeneous format.

The FRET-bridge (I) assay was optimized by means of a model antigen, streptavidin (SA). We examined the molecular determinants behind the assay principle, and observed that the multivalency of SA seems to play an important role in FRET signal induction, since most of the FRET activity derived from large-sized immunocomplexes. During application of LFRET (II and III) and CFRET (IV) for clinical diagnostics, PUUV served as the model pathogen. Both assays diagnosed acute PUUV infection with excellent specificity and very good sensitivity.

FRET bridge

The idea behind FRET bridging is based on two antigens binding to the same antibody. Antigens are separately labeled with specific fluorophores, which form a FRET pair. If these antigens are added to a solution containing their specific antibodies, they may bind to the same antibody, thus allowing FRET-signal formation.

The antigen, SA, used while setting up this assay, is a tetravalent molecule; this means that, in theory, it is able to bind four identical IgG molecules. In the case of PAb (or serum antibodies), numerous antibodies can bind to SA, limited primarily by steric conditions, since unlike MAbs, which are a mixture of identical antibodies with the same epitope-specificity, PAbs and serum antibodies are a mixture of antibodies with distinct epitope specificities. This means that a PAb mixture (or serum antibodies) may contain numerous binding sites to a given antigen. In theory, to test the original FRET-bridge idea of a single IgG binding two antigens, one would actually need a monovalent antigen and a monoclonal antibody. However, since serum always contain a variety of antibodies with

distinct specificities, it is probable that even a monovalent antigen combined with serum samples would induce formation of FRET-active immunocomplexes.

For both, monoclonal and polyclonal anti-SA antibodies, combining them with their labeled antigens induced FRET signals. With both antigens at 8 nM, the PAb induced dose-dependent FRET signals, while with the Mab, the signals increased dose-dependently only until a certain concentration. This is most likely explained by the prozone effect, which manifests in the excess of antibody in relation to the antigens, since more antibodies exist with only one bound antigen, and fewer antibodies with FRET activity. This was more evident with the MAb, since the concentration of the specific antibody in PAb is lower than it is in MAb (I, Fig. 3).

We compared the performance of a PAb and a MAb in the assay, and observed that using a PAb produced much higher TR-FRET signals than did using the MAb. We also studied the mechanism behind these results by fractionating the reagent components in a density gradient; we observed that the TR-FRET signals originated from molecular complexes of various sizes. Use of PAb seems to favor the formation of bulky immune complexes via non-covalent cross-linking of antigen molecules. Also with the MAb, most of the signals derived from immunocomplexes >700 kDa, corresponding to, for example, three IgG molecules (ca. 450 kDa) and four antigen molecules (ca. 250 kDa). Some FRET activity was also induced from fractions containing immunocomplexes corresponding to one IgG and two antigens in size. These results suggest that the FRET-bridge assay would greatly benefit from a multivalent antigen. Such an antigen could simultaneously bind several antibodies, and taking into account the bivalent nature of antibodies, they could also bind more than just one antigen. This would enable the formation of highly FRET-active immunocomplexes, in which several antigen-FRET pairs could form, when in close enough proximity.

Performance of the FRET-bridge assay we also studied by use of a monovalent (or bivalent) antigen, GST-VP1u (53 kDa), in combination with an anti-GST MAb (II). This antigen was produced as monovalent, but because GST has a tendency to form dimers, bivalent forms are also possible (38). The GST-VP1u was originally produced for other purposes, but as it is close to SA in size, we hypothesized that it would also suit our experiments. However, no FRET activity occurred in these experiments (II, Fig. 8), supporting the finding that only a

multivalent antigen may induce proper FRET signals in the FRET-bridge assay. It is also possible that the degree of labeling (DOL) or positioning of the labels in the GST-VP1u was sub-optimal.

Liu et al. (82) introduced a homogenous immunoassay for anti-BSA detection based on two-photon excitation similar to the FRET bridge. Although the detection technologies in two-photon excitation and TR-FRET differ, the assays resemble each other, since the polyclonal anti-BSA was detectable with the BSA antigens carrying distinct labels. Their results are in line with ours with SA and anti-SA, albeit the molecular properties of BSA differ from those of SA. At the concentrations that they used, BSA exists in monomeric forms (80), implying that the FRET-bridge assay could also function with monomeric antigens, even though the two techniques are not directly comparable. It is nevertheless possible that in their assay, as well, some sorts of immunocomplexes were formed that induce higher fluorescence signals than would only one IgG plus two antigens.

Two-photon excitation-based POC assays are available also for infectious diseases, ones such as the mariPOC® assays developed by ArcDia International Ltd. These assays employ microspheres, which bind immunocomplexes formed by microbial antigens, a capture antibody, and fluorescent antibodies on their surfaces. Two-photon excitation serves as the detection method to homogeneously measure the fluorescence from individual microspheres, which corresponds to the amount of the analyte. The sensitivities and specificities of the mariPOC® resp assay, one that detects eight respiratory viruses, are a respective 85.4% and 99.2%; 84.6% of the positive results are identifiable in 20 min (107). Unlike FRET bridge and the assay introduced by Liu et al., methods that detect antibodies, the mariPOC® assays detect microbial antigens, making these assays not directly comparable, for example, in terms of sensitivity.

To further develop the FRET-bridge concept for infectious disease diagnostics, one would need to produce several microbial antigens in multivalent forms to examine their suitability for this assay format. Tailored recombinant antigens that would have several binding sites for antigens, such as the tetravalent SA, could enable the formation of highly FRET-active immunocomplexes. One challenge is the fluorophore labeling, since the labels attach randomly to free (and suitable) amino acids. Suitable binding sites depend on the labeling chemistry, but most often reactions target free amines available in the side-chains of lysines

(sometimes also N-terminus). More selective labeling can be achieved through use of thiol-reactive chemistries that target free thiol groups of cysteine residues. This means that in order for one to succeed in labeling the antigen, the labels should incorporate into amino acids that do not locate in immunologically important epitopes; moreover, the DOL (the number of label molecules per target molecule) should be optimal. For example, if the antigen is over-labeled, the spill-over of the donor label into the acceptor channel becomes too high, and thus the specific signal diminishes. Finding the most optimal conditions for each antigen requires that the labeling reactions be optimized for both donor and acceptor. The inefficiency of the FRET-bridge assay is in part also due to the fact that in addition to binding FRET-pair-forming antigens, the antibodies can bind two acceptor-labeled or two donor-labeled antigens. This reduces the number of antibodies taking part in FRET signal formation.

LFRET

The second homogeneous immunoassay introduced (II, III) is based on a labeled immunoglobulin-binding molecule called protein L and a labeled antigen. Since FRET efficiency is dependent on the distance between the interacting donor and acceptor molecules, in an ideal assay the binding sites for both fluorophores are located in the Fab arm of the Ig molecule. As each IgG contains two Fab arms, two FRET pairs are possible within one IgG. In this assay, protein L and antigen were labeled separately with either a donor or an acceptor fluorophore, and functioned as a FRET pair. If these components bind to the same Fab arm of an Ig, a FRET signal forms in an assay time of 30 minutes.

In the preliminary experiments, the highest S/N ratios appeared when protein L carried the acceptor fluorophore, and the antigen carried the donor (II, Fig. 3). This is most likely because an increase in the concentration of the acceptor label does not cause an increase in the background signal that is as large as when the increase occurs in the concentration of the donor label, due to the spill-over of the donor into the acceptor channel. Since only a fraction of the IgGs in human sera will be specific to a given antigen, it is beneficial to use the protein L in excess to saturate the binding to Ig molecules in the sample. Hence, we decided to continue the experiments with donor in the antigen and acceptor in the protein L.

Here, as well, SA served as the test antigen. The S/N ratios recorded were even higher than those with the FRET bridge. Several factors can contribute to this higher S/N ratio. The smaller distance between the interacting FRET pairs in LFRET enables a more efficient energy transfer, and each IgG molecule can also form two FRET pairs. In addition, in LFRET both antigens binding to an IgG can form a FRET pair, as opposed to FRET bridge, in which an IgG can also form "homo-pairs" composed of two donors or acceptors, neither of which is FRET active.

To show that the FRET signals indeed originate from the same Fab arm of an Ig, and originate not only between the two Fab arms, which is also possible, we used anti-SA Fab fragments prepared from an anti-SA MAb. A mixture containing the anti-SA Fab fragments combined with the labeled protein L and SA was able to induce similar dose-dependent FRET signals as an intact MAb, albeit at somewhat lower intensity. This is most likely due to the bivalent nature of an antibody, since one MAb can form two FRET pairs. In theory, FRET in an intact MAb may also occur between the two Fab arms, due to the flexibility of the Ig hinge region (13,108,120). Moreover, the procedure for creating the Fab fragments may have caused structural damage that affects the antigen binding.

The nucleocapsid protein (N) of PUUV served as the first viral antigen in the LFRET assay (III). Puumala hantavirus induces a strong humoral IgM and IgG antibody response against the structural proteins (67), specifically the N protein, in the acute phase of infection, whereas in the past-infection phase, IgM response is absent. After optimization of the assay component concentrations, serum dilutions, and incubation time, we recorded FRET signals from both acute-infection and past-infection samples. The signals recorded with acute-phase samples were in general higher than those with past infection (III, Fig. 2).

To study the role of IgM in FRET-signal formation, we depleted all samples of IgG. Since IgM is present only in the acute phase of infection, those samples positive before IgG depletion but not afterwards represented old immunity, whereas those samples positive before and after IgG depletion represented acute infection. The higher signals induced by the acute-phase samples is most likely due to the role of IgM, since in those samples both types of immunoglobulins participate in FRET-signal formation. In addition, IgM usually exhibits a

pentameric form, which means that several FRET pairs can form within one IgM as compared to IgG, which at maximum forms two FRET pairs.

The signal intensities recorded from individual samples varied within both cohorts: the acute- and the past-infection samples. This may reflect variation in antibody clonality. The relation of antibodies with κ (protein-L binding) light chain to those with λ light chain varies among individuals; human antibodies carry roughly two-thirds of κ, and one-third of λ light chain(68). We furthermore examined—using acute-phase samples after IgG depletion—whether the FRET signals would correlate with reference IgM ELISA absorbance values. To our surprise, only a weak ($r = 0.35$), yet statistically significant ($P = 0.0276$), correlation appeared. One explanation for this could be the individual variability among antibodies and their light-chain types.

We analyzed 211 serum samples originally sent to HUSLAB for suspicion of PUUV infection. The panel consisted of 61 acute-, 27 past-infection, and 123 seronegative samples, and IFA (IgG) and IgM capture EIA served as reference methods. The panel included retrospective samples as well as prospective samples that we examined blindly. This assay was optimized by use of the retrospective samples, and was further evaluated (blind) using the prospective samples. After IgG depletion, all of the past-infection and seronegative samples were identified as negative, while among the 61 acute-phase samples, 58 induced FRET signals.

To conclude, with the LFRET assay, acute PUUV infection can be diagnosed at 100% specificity and 95.1% sensitivity. The excellent performance of this assay in diagnosing acute hantavirus disease indicates that this approach could be applicable also to other important human pathogens. The assay format could be suitable for diagnosis of other disease types as well, such as allergies and autoimmunity. This is something well worth exploring.

CFRET

The third approach, CFRET, is based on competition between a fluorophore-labeled MAb and serum antibodies. In this assay (IV), a fluorophore-labeled antigen and a fluorophore-labeled MAb form a FRET pair. If the serum contains antibodies with the same specificity as the MAb, they compete for antigen

binding, resulting in a decrease in the FRET signal. A FRET signal indicates, on the other hand, that the sample lacks the corresponding antibodies. Originally, McGiven et al. introduced this assay format for veterinary diagnostics (91). Their assay was developed for detection of anti-Brucella antibodies in ruminant serum samples. With slight modifications, we adapted the idea to human diagnostics, using PUUV as a model. The main differences between the assay of McGiven et al. and CFRET were the donor- and the acceptor labels.

To optimize the assay for the detection of PUUV antibodies, two anti-PUUV-N MAbs targeting distinct epitopes, the 3H9 and 1C12, served in the preliminary experiments in parallel. While both of the MAbs were inhibited by PUUV-seropositive samples, the results differed markedly. Most of the PUUV-positive sera, both acute- and past-infection, inhibited 3H9 more efficiently; hence we chose this antibody for future experiments (IV, Fig. 2). These results could be explained by the distinct binding affinities of each antibody, 1C12 being reactive with a conserved linear epitope shared between all hantaviruses, and 3H9 being a weaker binder detecting a species-specific epitope in a variable loop of hantavirus N and being consequently easier to compete with. What is also possible is that in individuals, antibody responses against the epitope that 3H9 targets are more common than that of 1C12.

This assay was optimized in terms of the assay component concentrations, serum dilutions, and incubation time by use of a retrospective sample panel, and was further evaluated using prospective (blind) samples. PUUV IgG IFA, IgM ELISA, and IgM IFA served as reference tests. In total, we examined 329 samples consisting of 101 acute-, 42 past-infection-, and 186 seronegative samples. Preliminary experiments show the MAb binding to be inhibited more efficiently by acute-phase samples. Here, as well, IgG depletion enabled the diagnosis of acute PUUV infection, that is, by the presence of PUUV-specific IgM antibodies. Within the 30-minute assay time, acute PUUV infection was identifiable with 99.1% specificity and 100% sensitivity, whereas the respective values for past-infection samples were 99.7% and 78.5%. These diagnostic performances were close to those of McGiven et al. (91), whose homogeneous TR-FRET immunoassay for anti-bacterial antibodies was both 100% specific and sensitive. The study of McGiven and colleagues of the competitive TR-FRET immunoassay indicates that the CFRET assay format could also function well in diagnosis of human bacterial infections. The CFRET assay was optimized for

diagnosing acute infection (IgM detection), but also recognized past-infection sera, albeit at lower sensitivity. To increase its sensitivity for identifying the state of immunization, the assay would require further optimization. Combination of another antigen and its MAb may function more efficiently with past-infection samples.

Comparison of the sensitivities and detection limits of CFRET and those of the reference tests (IgG IFA, IgM ELISA, and IgM IFA) showed lower sensitivity for CFRET in detecting both IgG and IgM than shown by the IgG IFA and IgM ELISA reference tests. CFRET performance was, however, more sensitive than that of the reference IgM IFA. The highest IgG titer that CFRET recognized was 200, while for the IgG-IFA it was 5,120. The corresponding titer of IgM by CFRET was 3,200, whereas for ELISA and IgM IFA the values were respectively 25,000 and 2,560. One explanation for this may be that enzyme-based assays in general are more sensitive because the signal is amplified by the enzymatic reaction.

We also studied correlations between the recorded inhibition values and the respective IgM or IgG titers, but no correlation was apparent. However, when summing up the IgM- and IgG-IFA titers, a significant correlation (r = 0.62) emerged for the CFRET results, which possibly reflects the ability of CFRET to detect both antibody classes. The performances of CFRET and LFRET were also compared by analyzing 131 samples of serum. With this sample panel, the CFRET assay proved somewhat more sensitive (100%) than the LFRET (95%) in diagnosing acute PUUV infection, but the specificities of both assays reached 100%.

In terms of assay workflow, the LFRET is more straightforward, since all assay components are supplied at once. Compared to CFRET, the LFRET is also more robust in assay setup, since the protein L is supplied at a saturating concentration, and only the antigen concentration requires optimization, whereas in CFRET, the concentrations of antibody and antigen are nonsaturating. The IgG depletion included for both assays takes only 15 seconds of extra time, and does not much complicate sample handling. The only extra requirements for the depletion prestep are a table centrifuge and the GullSORB reagent.

Based on its excellent sensitivity and specificity in hantavirus diagnostics, the CFRET may become widely applicable in diagnosis of other human pathogens as well. The ability to target specific epitopes would seem beneficial in distinguishing

highly cross-reactive species, such as the flaviviruses. Moreover, considering experimental research and veterinary diagnostics, this assay principle offers a new tool for screening of viral antibodies among animals lacking suitable secondary antibodies.

TR-FRET as a detection method in infectious-disease diagnosis

The FRET-based immunoassays introduced in this thesis offer a simple workflow and a rapid assay time of 15 to 30 minutes. The two applications, LFRET and CFRET, which we successfully applied in diagnosis of hantavirus disease, performed with high specificities and sensitivities, both closely approaching those obtained with the reference tests. The main benefits of the LFRET and CFRET immunoassays compared to those of conventional multistep assays are the lack of separation steps and reduced reagent consumption. These not only reduce assay time, but also reagent costs. Of note, the reaction volume of both assays, LFRET and CFRET, is only 20 μl. In addition, the hands-on time of the staff is reduced, and the results appear more rapidly. With the LFRET, what is in theory possible is that individual samples may exhibit in their antibodies amounts of λ light chain that are too low. This could affect assay sensitivity.

The possible challenge for CFRET when applied to other antigens could involve finding a suitable epitope. For CFRET to function, the requirement is that the epitope against which the MAb is targeted should be such that all individuals having had the disease would have antibodies against it. However, it is probable that even if less than 100% of the individuals have antibodies against that specific epitope, they will have antibodies that bind so closely to the epitope that competition between antibodies and MAb is possible.

One main benefit of using TR-FRET as a detection method, rather than traditional enzyme-based assays, is that fairly high signal-to-noise ratios can result without any additional washing steps. In addition, the platform can easily enable automated high-throughput screening. In terms of sensitivity, enzyme-based applications are often superior to those based on FRET. This is not only due to the signal amplification of enzyme-based assays, but other concerns also affect the sensitivity of FRET, and specifically homogeneous FRET assays. The

heterogenic assay format, which is often involved in enzyme-based assays, enables the washing of disturbing agents that possibly hinder the formation of specific signal. The homogeneous FRET assays are wash-free, which makes them rapid and simple, but also prone to disturbance by other biomolecules present in the sample other than the analytes. The spillover of the donor-label to the acceptor channel also affects sensitivity, but normalization of the FRET signals diminishes this effect.

Regarding assay time and hands-on time, the FRET-based assays were superior to the multistep reference assays we used in this study. The assay times of IFA and ELISA range from 3 to 4 hours, including several separation steps. As to the aspect of sensitivity, it is probable that the LFRET and CFRET assays perform well with pathogens that raise high antibody responses, such as PUUV (67). Despite the limitations of FRET regarding sensitivity, both LFRET and CFRET performed well in diagnosis of acute PUUV infection, with sensitivities ranging from 95 to 100%, and specificities from 99 to 100%. Based on reports regarding the accuracy of available RDTs or POC assays for infectious diseases, most of which rely on antigen detection, these numbers are excellent (15,79,103,107). Since the accuracy of the LFRET and CFRET assays in terms of sensitivity and specificity is almost as good, both should be optimized and tested in parallel for new potential antigens to see which one performs better.

Other issues also need consideration when applying FRET as the detection method. One critical step when setting up a FRET-based immunoassay is the fluorophore-labeling of the antigen (or other assay components). Not only is it important that while labeling the antigen, the immunologically important epitopes must remain intact; moreover, the DOL requires optimization. Here, the amino acid sequence of the antigen plays an important role, since the labeling reagents most commonly react with free amine groups. The labeling reactions for the Eu chelate used isothiocyanate, and the Alexa Fluor® used N-hydroxysuccinimide (NHS) ester, both of which target amines. Depending on the number of reactive groups and their location in respect to the critical epitopes, the outcome of the labeling reaction varies. This is something needing to be tested and optimized, since every molecule exhibits a unique amino acid profile. One possibility worth examining would be the expression of fluorescent tags, or linkers that would enable specific targeting of chromophores without any need for chemical labeling (133,134). The suitability of other donor-acceptor pairs should also be examined

to learn whether FRET efficiency between the donor and acceptor can be further increased.

In addition, the size of the antigen plays a role in FRET efficiency. The ideal diameter of the antigen would allow the distance between the two chromophores to be minimal. Thus, either reducing or enlarging antigen diameter would negatively affect the distance between a given FRET pair and subsequently the signals obtained. PUUV-N is a 50 kDa protein which exhibits a diameter of approximately 5 nm (34). In the natural trimeric form of PUUV-N (93), the diameter grows to approximately 15 nm. Even if the antigen seems to function very well in the LFRET and CFRET assays, one could imagine achieving better FRET efficiency with a slightly smaller protein. Depending on the location of the labels, and the orientation of the bound antigens in relation to the FRET pair, the optimal antigen size is difficult to foretell, and requires physical examination.

CONCLUDING REMARKS AND PROSPECTS

This study was conducted to provide new insights into rapid serodiagnostics. This involved introduction of three approaches for rapid antibody detection, two of which, the LFRET and CFRET, were applied in clinical diagnosis. Both assays performed with high specificity and sensitivity in diagnosing acute hantavirus disease, and the next stage would be the application of these assays for diagnosis of other human diseases. Interesting viral pathogens include dengue viruses and tick-borne encephalitis virus. In addition to viral infections, the assays could also function in diagnosis of bacterial infections. Specifically *Mycoplasma pneumoniae* and the *Borrelia burgdorferi* sensu lato would be interesting targets to examine. Furthermore, the suitability of the rapid FRET assays for screening vaccine coverage against certain infections such as tetanus and rabies needs study.

Both assays, LFRET and CFRET, are versatile in that they are not limited only to detection of IgG or IgM. The suitability of these assays should therefore be examined for other disease conditions as well, such as allergies and autoimmune diseases, by optimizing the assay for detection of IgE or IgA. Their applicability to other sample material in addition to serum, such as plasma, urea, saliva, and blood, also deserves examination.

FRET as a detection method enables multiplexing. This would certainly add more value to the LFRET and CFRET assays, and needs study. Moreover, optimizing the performance of a portable FRET reader for these TR-FRET assays would surely prove valuable. At the moment, the suitability of such portable devices is under evaluation for the LFRET and CFRET.

ACKNOWLEDGEMENTS

This study was carried out in the Department of Virology, at the University of Helsinki. My first thanks go to the head of the department, Professor Kalle Saksela, for providing excellent research facilities. The atmosphere in the department of virology has always been warm. The members of my thesis committee, Dr. Urpo Lamminmäki and Professor Seppo Meri, earn my gratitude for their valuable comments and insightful discussions. My appreciation and thanks go the reviewers of this thesis, Dr. Urpo Lamminmäki and Dr. Petri Susi, for their critical comments and suggestions. Professor emeritus Antti Vaheri also deserves thanks for his inspiring enthusiasm towards science.

I warmly thank my supervisors Professors Klaus Hedman and Olli Vapalahti; it has been an honor to work with the two of you. Besides being such excellent scientists, you also have strong personalities. I have learned a lot from both of you. Klaus, you have taught me much about critical thinking and new perspectives. Your passion for scientific writing, and those numerous hours spent revising my texts, have played a central role on my journey towards being a scientist. I thank you for putting up with my anxiety caused by this “growth project.” Olli, you are one of the most versatile human beings I have ever known. No matter what the question was, no matter what virus was in question, you always had an answer. You also always had the time for discussions. Thank you for that.

Financial support came from the Integrative Life Science Doctoral Program, the Finnish Funding Agency for Technology and Innovation, the Foundation for Clinical Chemistry Research, the Otto A. Malm Foundation, the Oscar Öflund Foundation, and the Laboratoriolääketieteen edistämissäätiö. Maria Vartiainen contributed much through her lecture dealing with FRET-related research, which originally led my Professor Klaus into the world of FRET. Anne Lahtinen and Ilkka Hemmilä deserve thanks for their aid in the early steps of this project, and Carol Norris for author-editing the language of this book.

Zoonoses, the weirdest and coolest research group in the whole Meilahti campus: thank you for being who you are, and those who are, know who they are. Special thanks go to Liina, Satu K., and Suvi, with whom we have had so

many great adventures outside work. I also thank my ex- and present room-mates for your friendship and support. For all the lovely ex-members of the Zoonosis group: I really miss you! The members of Monday and Wednesday sähly, thank you for taking me into your group. The weekly sähly hours were always such a pleasure! Thanks also go to our inspiring FRET team.

My girls: Alina, Dora, Linda, Lumi, and Saara. Most of you I have known for half my life, and I am privileged to have grown up with you. Thank you for your friendship; you mean so much to me.

Last but not least, my deepest appreciation goes to my family. Ever since I was a child, my parents have always encouraged me to follow my heart and passion. My mother, Marita, and my father, Matti, thank for your endless love and support. You have always been there for me. Heartfelt thanks also go to my little sister, Mari, and my cousin, Eeva, as well as the whole Hautala and Saraheimo families for their friendship and support.

Jussi, my beloved husband, you are an example of what every scientist should be. I admire you in every way. Thank you for your love and support. You are my inspiration! Anton, our son, I thank you with all my heart for showing that not all Reality is Virtual.

Helsinki, 2016

Satu Hepojoki

REFERENCES

(1) A timeline of pregnancy testing. Available at: https://history.nih.gov/exhibits/thinblueline/timeline.html. Accessed 09/04, 2016.

(2) Abbas AB, Lichtman AH, Pober JS editors. **Cellular and molecular immunology**. fourth ed. USA: W.B. Saunders company; 2000.

(3) Åkerstrom B, Björck L. Protein L: an immunoglobulin light chain-binding bacterial protein. Characterization of binding and physicochemical properties. **J Biol Chem** ;264:19740-19746 1989.

(4) Arnold JN, Wormald MR, Sim RB, Rudd PM, Dwek RA. The impact of glycosylation on the biological function and structure of human immunoglobulins. **Annu Rev Immunol** ;25:21-50 2007.

(5) Baron S editor. **Medical microbiology**. fourth ed.: University of Texas Medical Branch; 1997.

(6) Barzon L, Lavezzo E, Costanzi G, Franchin E, Toppo S, Palu G. Next-generation sequencing technologies in diagnostic virology. **J Clin Virol** ;58:346-350 2013.

(7) Bazin H, Preaudat M, Trinquet E, Mathis G. Homogeneous time resolved fluorescence resonance energy transfer using rare earth cryptates as a tool for probing molecular interactions in biology. **Spectrochim Acta A Mol Biomol Spectrosc** ;57:2197-2211 2001.

(8) Berson SA, Yalow RS, Bauman A, Rothschild MA, Newerly K. Insulin-I131 metabolism in human subjects: demonstration of insulin binding globulin in the circulation of insulin treated subjects. **J Clin Invest** ;35:170-190 1956.

(9) Beveridge TJ. Use of the gram stain in microbiology. **Biotech Histochem** ;76:111-118 2001.

(10) Biswas S, Rolain JM. Use of MALDI-TOF mass spectrometry for identification of bacteria that are difficult to culture. **J Microbiol Methods** ;92:14-24 2013.

(11) Björck L. Protein L. A novel bacterial cell wall protein with affinity for Ig L chains. **J Immunol** ;140:1194-1197 1988.

(12) Blomberg K, Hurskainen P, Hemmilä I. Terbium and rhodamine as labels in a homogeneous time-resolved fluorometric energy transfer assay of the beta subunit of human chorionic gonadotropin in serum. **Clin Chem** ;45:855-861 1999.

(13) Bongini L, Fanelli D, Piazza F, De Los Rios P, Sandin S, Skoglund U. Freezing immunoglobulins to see them move. **Proc Natl Acad Sci U S A** ;101:6466-6471 2004.

(14) Bouvier NM, Palese P. The biology of influenza viruses. **Vaccine** ;26 Suppl 4:D49-53 2008.

(15) Caliendo AM, Gilbert DN, Ginocchio CC, Hanson KE, May L, Quinn TC, Tenover FC, Alland D, Blaschke AJ, Bonomo RA, Carroll KC, Ferraro MJ, Hirschhorn LR, Joseph WP, Karchmer T, MacIntyre AT, Reller LB, Jackson AF, Infectious Diseases Society of America (IDSA). Better tests, better care: improved diagnostics for infectious diseases. **Clin Infect Dis** ;57 Suppl 3:S139-70 2013.

(16) Cannon GA, Carr MJ, Yandle Z, Schaffer K, Kidney R, Hosny G, Doyle A, Ryan J, Gunson R, Collins T, Carman WF, Connell J, Hall WW. A low density oligonucleotide microarray for the detection of viral and atypical bacterial respiratory pathogens. **J Virol Methods** ;163:17-24 2010.

(17) Carbonnelle E, Mesquita C, Bille E, Day N, Dauphin B, Beretti JL, Ferroni A, Gutmann L, Nassif X. MALDI-TOF mass spectrometry tools for bacterial identification in clinical microbiology laboratory. **Clin Biochem** ;44:104-109 2011.

(18) Charbonniere LJ, Hildebrandt N, Ziessel RF, Lohmannsroben HG. Lanthanides to quantum dots resonance energy transfer in time-resolved fluoro-immunoassays and luminescence microscopy. **J Am Chem Soc** ;128:12800-12809 2006.

(19) Chawla A, Murphy G, Donnelly C, Booth CL, Johnson M, Parry JV, Phillips A, Geretti AM. Human immunodeficiency virus (HIV) antibody avidity testing to identify recent infection in newly diagnosed HIV type 1 (HIV-1)-seropositive persons infected with diverse HIV-1 subtypes. **J Clin Microbiol** ;45:415-420 2007.

(20) Chen K, Xu W, Wilson M, He B, Miller NW, Bengten E, Edholm ES, Santini PA, Rath P, Chiu A, Cattalini M, Litzman J, B Bussel J, Huang B, Meini A, Riesbeck K, Cunningham-Rundles C, Plebani A, Cerutti A. Immunoglobulin D enhances immune surveillance by activating antimicrobial, proinflammatory and B cell-stimulating programs in basophils. **Nat Immunol** ;10:889-898 2009.

(21) Chomel B. Lyme disease. **Rev Sci Tech** ;34:569-576 2015.

(22) Clark LC,Jr, Lyons C. Electrode systems for continuous monitoring in cardiovascular surgery. **Ann N Y Acad Sci** ;102:29-45 1962.

(23) Darwish IA. Immunoassay Methods and their Applications in Pharmaceutical Analysis: Basic Methodology and Recent Advances. **Int J Biomed Sci** ;2:217-235 2006.

(24) De Chateau M, Nilson BH, Erntell M, Myhre E, Magnusson CG, Åkerstrom B, Björck L. On the interaction between protein L and immunoglobulins of various mammalian species. **Scand J Immunol** ;37:399-405 1993.

(25) De Paschale M, Clerici P. Serological diagnosis of Epstein-Barr virus infection: Problems and solutions. **World J Virol** ;1:31-43 2012.

(26) Delacroix DL, Dive C, Rambaud JC, Vaerman JP. IgA subclasses in various secretions and in serum. **Immunology** ;47:383-385 1982.

(27) Diamandis EP. Immunoassays with time-resolved fluorescence spectroscopy: principles and applications. **Clin Biochem** ;21:139-150 1988.

(28) Dickson EF, Pollak A, Diamandis EP. Time-resolved detection of lanthanide luminescence for ultrasensitive bioanalytical assays. **J Photochem Photobiol B** ;27:3-19 1995.

(29) Drain PK, Hyle EP, Noubary F, Freedberg KA, Wilson D, Bishai WR, Rodriguez W, Bassett IV. Diagnostic point-of-care tests in resource-limited settings. **Lancet Infect Dis** ;14:239-249 2014.

(30) Eliasson M, Olsson A, Palmcrantz E, Wiberg K, Inganas M, Guss B, Lindberg M, Uhlen M. Chimeric IgG-binding receptors engineered from staphylococcal protein A and streptococcal protein G. **J Biol Chem** ;263:4323-4327 1988.

(31) Enders M, Schalasta G, Baisch C, Weidner A, Pukkila L, Kaikkonen L, Lankinen H, Hedman L, Söderlund-Venermo M, Hedman K. Human parvovirus B19 infection during pregnancy--value of modern molecular and serological diagnostics. **J Clin Virol** ;35:400-406 2006.

(32) Engvall E, Perlmann P. Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. **Immunochemistry** ;8:871-874 1971.

(33) Engvall E. Perspective on the historical note on EIA/ELISA by Dr. R.M. Lequin. **Clin Chem** ;51:2225 2005.

(34) Erickson HP. Size and shape of protein molecules at the nanometer level determined by sedimentation, gel filtration, and electron microscopy. **Biol Proced Online** ;11:32-51 2009.

(35) Ermonval M, Baychelier F, Tordo N. What Do We Know about How Hantaviruses Interact with Their Different Hosts? **Viruses** ;8:10.3390/v8080223 2016.

(36) Erntell M, Myhre EB, Sjöbring U, Björck L. Streptococcal protein G has affinity for both Fab- and Fc-fragments of human IgG. **Mol Immunol** ;25:121-126 1988.

(37) Evangelista RA. A new europium chelate for protein labelling and time-resolved fluorometric applications. **Clin Biochem** ;21:173 1988.

(38) Fabrini R, De Luca A, Stella L, Mei G, Orioni B, Ciccone S, Federici G, Lo Bello M, Ricci G. Monomer-dimer equilibrium in glutathione transferases: a critical re-examination. **Biochemistry** ;48:10473-10482 2009.

(39) Fearon M. The laboratory diagnosis of HIV infections. **Can J Infect Dis Med Microbiol** ;16:26-30 2005.

(40) Förster T. Zwischenmolekulare Energiewanderung und Fluoreszenz. **Annalen der Physik** ;437:55-55-75 1948.

(41) Gan SD, Patel KR. Enzyme immunoassay and enzyme-linked immunosorbent assay. **J Invest Dermatol** ;133:e12 2013.

(42) Gaudy-Graffin C, Lesage G, Kousignian I, Laperche S, Girault A, Dubois F, Goudeau A, Barin F. Use of an anti-hepatitis C virus (HCV) IgG avidity assay to identify recent HCV infection. **J Clin Microbiol** ;48:3281-3287 2010.

(43) Graille M, Stura EA, Corper AL, Sutton BJ, Taussig MJ, Charbonnier JB, Silverman GJ. Crystal structure of a Staphylococcus aureus protein A domain complexed with the Fab fragment of a human IgM antibody: structural basis for recognition of B-cell receptors and superantigen activity. **Proc Natl Acad Sci U S A** ;97:5399-5404 2000.

(44) Habermehl K- editor. **Rapid Methods and Automation in Microbiology and Immunology** . : Springer; 1985.

(45) Hagan AK, Zuchner T. Lanthanide-based time-resolved luminescence immunoassays. **Anal Bioanal Chem** ;400:2847-2864 2011.

(46) Handl HL, Gillies RJ. Lanthanide-based luminescent assays for ligand-receptor interactions. **Life Sci** ;77:361-371 2005.

(47) Hawkins RC. Laboratory turnaround time. **Clin Biochem Rev** ;28:179-194 2007.

(48) Hedman K, Seppälä I. Recent rubella virus infection indicated by a low avidity of specific IgG. **J Clin Immunol** ;8:214-221 1988.

(49) Hedman K, Vaheri A, Brummer-Korvenkontio M. Rapid diagnosis of hantavirus disease with an IgG-avidity assay. **Lancet** ;338:1353-1356 1991.

(50) Hedman K, Heikkinen T, Huovinen P, Järvinen A, Meri S, Vaara M editors. **Infektiosairaudet**. Finland: Kustannus Oy Duodecim; 2013.

(51) Hemmilä I. **Fluoroimmunoassays and immunofluorometric assays**. **Clin Chem** ;31:359-370 1985.

(52) Hemmilä I. Lanthanides as probes for time-resolved fluorometric immunoassays. **Scand J Clin Lab Invest** ;48:389-399 1988.

(53) Hemmilä I, Malminen O, Mikola H, Lövgren T. Homogeneous time-resolved fluoroimmunoassay of thyroxin in serum. **Clin Chem** ;34:2320-2322 1988.

(54) Hemmilä I, Laitala V. Progress in lanthanides as luminescent probes. **J Fluoresc** ;15:529-542 2005.

(55) Hemmilä I, Mukkala V, Takalo H. Twenty-first Rare Earth Research Conference Development of luminescent lanthanide chelate labels for diagnostic assays. **J Alloys Compounds** ;249:158-162 1997.

(56) Hepojoki S, Hepojoki J, Hedman K, Vapalahti O, Vaheri A. Rapid Homogeneous Immunoassay based on Time-Resolved FRET for Serodiagnosis of Acute Hantavirus Infection. **J Clin Microbiol** 2014.

(57) Hepojoki S, Nurmi V, Vaheri A, Hedman K, Vapalahti O, Hepojoki J. A protein L -based immunodiagnostic approach utilizing time-resolved forster resonance energy transfer. **PLoS One** ;9:e106432 2014.

(58) Hepojoki S, Rusanen J, Hepojoki J, Nurmi V, Vaheri A, Lundkvist Å, Hedman K, Vapalahti O. Competitive Homogeneous Immunoassay for Rapid Serodiagnosis of Hantavirus Disease. **J Clin Microbiol** ;53:2292-2297 2015.

(59) Hildebrandt N, Charbonniere LJ, Lohmannsroben HG. Time-resolved analysis of a highly sensitive Forster resonance energy transfer immunoassay using terbium complexes as donors and quantum dots as acceptors. **J Biomed Biotechnol** ;2007:79169 2007.

(60) Hofmann J, Liebert UG. Significance of avidity and immunoblot analysis for rubella IgM-positive serum samples in pregnant women. **J Virol Methods** ;130:66-71 2005.

(61) Howanitz JH, Howanitz PJ. Laboratory results. Timeliness as a quality attribute and strategy. **Am J Clin Pathol** ;116:311-315 2001.

(62) Hu LA, Zhou T, Hamman BD, Liu Q. A homogeneous G protein-coupled receptor ligand binding assay based on time-resolved fluorescence resonance energy transfer. **Assay Drug Dev Technol** ;6:543-550 2008.

(63) Hujakka H, Koistinen V, Eerikainen P, Kuronen I, Mononen I, Parviainen M, Lundkvist A, Vaheri A, Närvanen A, Vapalahti O. New immunochromatographic rapid test for diagnosis of acute Puumala virus infection. **J Clin Microbiol** ;39:2146-2150 2001.

(64) Jääskeläinen AJ, Piiparinen H, Lappalainen M, Koskiniemi M, Vaheri A. Multiplex-PCR and oligonucleotide microarray for detection of eight different herpesviruses from clinical specimens. **J Clin Virol** ;37:83-90 2006.

(65) Jääskeläinen AJ, Viitala SM, Kurkela S, Hepojoki S, Sillanpää H, Kallio-Kokko H, Bergström T, Suni J, Närvänen A, Vapalahti O, Vaheri A. Performance of a multiplexed serological microarray for the detection of antibodies against central nervous system pathogens. **J Microbiol Methods** ;100:27-31 2014.

(66) Jasir A. Applied immunology Aftab Jasir: European Centre for Disease Prevention and Control (ECDC) European Public Health Microbiology training program (EUPHEM). Available at: http://slideplayer.com/slide/741168/. Accessed 08/04, 2016.

(67) Kallio-Kokko H, Leveelahti R, Brummer-Korvenkontio M, Lundkvist A, Vaheri A, Vapalahti O. Human immune response to Puumala virus glycoproteins and nucleocapsid protein expressed in mammalian cells. **J Med Virol** ;65:605-613 2001.

(68) Katzmann JA, Clark RJ, Abraham RS, Bryant S, Lymp JF, Bradwell AR, Kyle RA. Serum reference intervals and diagnostic ranges for free kappa and free lambda immunoglobulin light chains: relative sensitivity for detection of monoclonal light chains. **Clin Chem** ;48:1437-1444 2002.

(69) Kenworthy AK. Imaging protein-protein interactions using fluorescence resonance energy transfer microscopy. **Methods** ;24:289-296 2001.

(70) Knipe DM, Howley PM editors. **Fields Virology**. 5th ed. USA: Lippincott Williams and Wilkins; 2007.

(71) Kokko T, Liljenback T, Peltola MT, Kokko L, Soukka T. Homogeneous dual-parameter assay for prostate-specific antigen based on fluorescence resonance energy transfer. **Anal Chem** ;80:9763-9768 2008.

(72) Kokko T, Kokko L, Soukka T. Terbium(III) chelate as an efficient donor for multiple-wavelength fluorescent acceptors. **J Fluoresc** ;19:159-164 2009.

(73) Kramski M, Achazi K, Klempa B, Kruger DH. Nephropathia epidemica with a 6-week incubation period after occupational exposure to Puumala hantavirus. **J Clin Virol** ;44:99-101 2009.

(74) Laitala V, Hemmilä I. Homogeneous assay based on anti-Stokes' shift time-resolved fluorescence resonance energy-transfer measurement. **Anal Chem** ;77:1483-1487 2005.

(75) Lakowicz JR editor. **Principles of Fluorescence Spectroscopy**. 3rd ed. USA: Springer; 2006.

(76) Lecuit M, Eloit M. The diagnosis of infectious diseases by whole genome next generation sequencing: a new era is opening. **Front Cell Infect Microbiol** ;4:25 2014.

(77) Lehtonen OP, Meurman OH. An ELISA for the estimation of high-avidity and total specific IgG and IgM antibodies to rubella virus. **J Virol Methods** ;5:1-10 1982.

(78) Lequin RM. Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA). **Clin Chem** ;51:2415-2418 2005.

(79) Leung AK, Newman R, Kumar A, Davies HD. Rapid antigen detection testing in diagnosing group A beta-hemolytic streptococcal pharyngitis. **Expert Rev Mol Diagn** ;6:761-766 2006.

(80) Levi V, Gonzalez Flecha FL. Reversible fast-dimerization of bovine serum albumin detected by fluorescence resonance energy transfer. **Biochim Biophys Acta** ;1599:141-148 2002.

(81) Lin B, Blaney KM, Malanoski AP, Ligler AG, Schnur JM, Metzgar D, Russell KL, Stenger DA. Using a resequencing microarray as a multiple respiratory pathogen detection assay. **J Clin Microbiol** ;45:443-452 2007.

(82) Liu L, Shao M, Dong X, Yu X, Liu Z, He Z, Wang Q. Homogeneous immunoassay based on two-photon excitation fluorescence resonance energy transfer. **Anal Chem** ;80:7735-7741 2008.

(83) Llewelyn M, Cohen J. Superantigens: microbial agents that corrupt immunity. **Lancet Infect Dis** ;2:156-162 2002.

(84) Lundkvist Å, Fatouros A, Niklasson B. Antigenic variation of European haemorrhagic fever with renal syndrome virus strains characterized using bank vole monoclonal antibodies. **J Gen Virol** ;72 (Pt 9):2097-2103 1991.

(85) Mabey D, Peeling RW, Ustianowski A, Perkins MD. Diagnostics for the developing world. **Nat Rev Microbiol** ;2:231-240 2004.

(86) Maclachlan NJ, Dubovi EJ editors. **Ferret´s Veterinary Virology**. fourth ed.: Academic Press; 2011.

(87) Majchrzykiewicz-Koehorst JA, Heikens E, Trip H, Hulst AG, de Jong AL, Viveen MC, Sedee NJ, van der Plas J, Coenjaerts FE, Paauw A. Rapid and generic identification of influenza A and other respiratory viruses with mass spectrometry. **J Virol Methods** ;213:75-83 2015.

(88) Male D editor. **Immune recognition**. United Kingdom: IRL Press Limited; 1988.

(89) Maple PA, Hedman L, Dhanilall P, Kantola K, Nurmi V, Söderlund-Venermo M, Brown KE, Hedman K. Identification of past and recent parvovirus B19 infection in immunocompetent individuals by quantitative PCR and enzyme immunoassays: a dual-laboratory study. **J Clin Microbiol** ;52:947-956 2014.

(90) Mathis G. Probing molecular interactions with homogeneous techniques based on rare earth cryptates and fluorescence energy transfer. **Clin Chem** ;41:1391-1397 1995.

(91) McGiven JA, Thompson IJ, Commander NJ, Stack JA. Time-resolved fluorescent resonance energy transfer assay for simple and rapid detection of anti-Brucella antibodies in ruminant serum samples. **J Clin Microbiol** ;47:3098-3107 2009.

(92) Mestecky J. Structure of antibodies. **J Oral Pathol** ;1:288-300 1972.

(93) Mir MA, Panganiban AT. Trimeric hantavirus nucleocapsid protein binds specifically to the viral RNA panhandle. **J Virol** ;78:8281-8288 2004.

(94) Mukkala VM, Mikola H, Hemmilä I. The synthesis and use of activated N-benzyl derivatives of diethylenetriaminetetraacetic acids: alternative reagents for labeling of antibodies with metal ions. **Anal Biochem** ;176:319-325 1989.

(95) Murphy K editor. **Janeway´s Immunobiology**. 8th ed.: Garland Science; 2012.

(96) Nikiforov TT, Beechem JM. Development of homogeneous binding assays based on fluorescence resonance energy transfer between quantum dots and Alexa Fluor fluorophores. **Anal Biochem** ;357:68-76 2006.

(97) Nilson BH, Solomon A, Björck L, Åkerstrom B. Protein L from Peptostreptococcus magnus binds to the kappa light chain variable domain. **J Biol Chem** ;267:2234-2239 1992.

(98) Nilson BH, Logdberg L, Kastern W, Björck L, Åkerstrom B. Purification of antibodies using protein L-binding framework structures in the light chain variable domain. **J Immunol Methods** ;164:33-40 1993.

(99) Nouvellet P, Garske T, Mills HL, Nedjati-Gilani G, Hinsley W, Blake IM, Van Kerkhove MD, Cori A, Dorigatti I, Jombart T, Riley S, Fraser C, Donnelly CA, Ferguson NM. The role of rapid diagnostics in managing Ebola epidemics. **Nature** ;528:S109-16 2015.

(100) O´Gorman MRG, Donnenberg AD editors. **Handbook of human immunology**. second ed. USA: CRC press; 2008.

(101) Oldal M, Nemeth V, Madai M, Kemenesi G, Dallos B, Peterfi Z, Sebok J, Wittmann I, Banyai K, Jakab F. Identification of hantavirus infection by Western blot assay and

TaqMan PCR in patients hospitalized with acute kidney injury. **Diagn Microbiol Infect Dis** ;79:166-170 2014.

(102) Payment P, Trudel M editors. **Methods and Techniques in Virology**. USA: Marcel Dekker Inc; 1993.

(103) Peeling RW, Mabey D. Point-of-care tests for diagnosing infections in the developing world. **Clin Microbiol Infect** ;16:1062-1069 2010.

(104) Prince HE, Lape-Nixon M. Role of cytomegalovirus (CMV) IgG avidity testing in diagnosing primary CMV infection during pregnancy. **Clin Vaccine Immunol** ;21:1377-1384 2014.

(105) Rathore U, Kesavardhana S, Mallajosyula VV, Varadarajan R. Immunogen design for HIV-1 and influenza. **Biochim Biophys Acta** 2014.

(106) Roque-Afonso AM, Grangeot-Keros L, Roquebert B, Desbois D, Poveda JD, Mackiewicz V, Dussaix E. Diagnostic relevance of immunoglobulin G avidity for hepatitis A virus. **J Clin Microbiol** ;42:5121-5124 2004.

(107) Sanbonmatsu-Gamez S, Perez-Ruiz M, Lara-Oya A, Pedrosa-Corral I, Riazzo-Damas C, Navarro-Mari JM. Analytical performance of the automated multianalyte point-of-care mariPOC(R) for the detection of respiratory viruses. **Diagn Microbiol Infect Dis** ;83:252-256 2015.

(108) Sandin S, Ofverstedt LG, Wikstrom AC, Wrange O, Skoglund U. Structure and flexibility of individual immunoglobulin G molecules in solution. **Structure** ;12:409-415 2004.

(109) Saraheimo S, Hepojoki J, Nurmi V, Lahtinen A, Hemmilä I, Vaheri A, Vapalahti O, Hedman K. Time-resolved FRET -based approach for antibody detection - a new serodiagnostic concept. **PLoS One** ;8:e62739 2013.

(110) Schroeder HW,Jr, Cavacini L. Structure and function of immunoglobulins. **J Allergy Clin Immunol** ;125:S41-52 2010.

(111) Selvin PR, Hearst JE. Luminescence energy transfer using a terbium chelate: improvements on fluorescence energy transfer. **Proc Natl Acad Sci U S A** ;91:10024-10028 1994.

(112) Selvin PR. Principles and biophysical applications of lanthanide-based probes. **Annu Rev Biophys Biomol Struct** ;31:275-302 2002.

(113) Settergren B, Juto P, Trollfors B, Wadell G, Norrby SR. Hemorrhagic complications and other clinical findings in nephropathia epidemica in Sweden: a study of 355 serologically verified cases. **J Infect Dis** ;157:380-382 1988.

(114) Settergren B. Clinical aspects of nephropathia epidemica (Puumala virus infection) in Europe: a review. **Scand J Infect Dis** ;32:125-132 2000.

(115) Sibley CD, Peirano G, Church DL. Molecular methods for pathogen and microbial community detection and characterization: current and potential application in diagnostic microbiology. **Infect Genet Evol** ;12:505-521 2012.

(116) Siitari H. Dual-label time-resolved fluoroimmunoassay for the simultaneous detection of adenovirus and rotavirus in faeces. **J Virol Methods** ;28:179-187 1990.

(117) Sjöbring U, Björck L, Kastern W. Streptococcal protein G. Gene structure and protein binding properties. **J Biol Chem** ;266:399-405 1991.

(118) Soini E, Hemmilä I. Fluoroimmunoassay: present status and key problems. **Clin Chem** ;25:353-361 1979.

(119) Soini E, Kojola H. Time-resolved fluorometer for lanthanide chelates--a new generation of nonisotopic immunoassays. **Clin Chem** ;29:65-68 1983.

(120) Sosnick TR, Benjamin DC, Novotny J, Seeger PA, Trewhella J. Distances between the antigen-binding sites of three murine antibody subclasses measured using neutron and X-ray scattering. **Biochemistry** ;31:1779-1786 1992.

(121) Stewart EH, Davis B, Clemans-Taylor BL, Littenberg B, Estrada CA, Centor RM. Rapid antigen group A streptococcus test to diagnose pharyngitis: a systematic review and meta-analysis. **PLoS One** ;9:e111727 2014.

(122) Storch GA. Diagnostic virology. **Clin Infect Dis** ;31:739-751 2000.

(123) Tian X, Vestergaard B, Thorolfsson M, Yang Z, Rasmussen HB, Langkilde AE. In-depth analysis of subclass-specific conformational preferences of IgG antibodies. **IUCrJ** ;2:9-18 2015.

(124) Tipples GA. Rubella diagnostic issues in Canada. **J Infect Dis** ;204 Suppl 2:S659-63 2011.

(125) Tissari P, Zumla A, Tarkka E, Mero S, Savolainen L, Vaara M, Aittakorpi A, Laakso S, Lindfors M, Piiparinen H, Mäki M, Carder C, Huggett J, Gant V. Accurate and rapid identification of bacterial species from positive blood cultures with a DNA-based microarray platform: an observational study. **Lancet** ;375:224-230 2010.

(126) Valeur B, Berberan-Santos MN editors. **Molecular Fluorescence: Principles and Applications**. second ed.: Wiley-VCH Verlag GmbH & Co.; 2013.

(127) Valeur B, Berberan-Santos MN. A Brief History of Fluorescence and Phosphorescence before the Emergence of Quantum Theory. **Journal of Chemical Education** ;88:731-738 2011.

(128) Van Weemen BK, Schuurs AH. Immunoassay using antigen-enzyme conjugates. **FEBS Lett** ;15:232-236 1971.

(129) Vapalahti O, Lundkvist Å, Kallio-Kokko H, Paukku K, Julkunen I, Lankinen H, Vaheri A. Antigenic properties and diagnostic potential of puumala virus nucleocapsid protein expressed in insect cells. **J Clin Microbiol** ;34:119-125 1996.

(130) Vapalahti O, Mustonen J, Lundkvist Å, Henttonen H, Plyusnin A, Vaheri A. Hantavirus infections in Europe. **Lancet Infect Dis** ;3:653-661 2003.

(131) Vidarsson G, Dekkers G, Rispens T. IgG subclasses and allotypes: from structure to effector functions. **Front Immunol** ;5:520 2014.

(132) Viitala SM, Jääskeläinen AJ, Kelo E, Sirola H, Moilanen K, Suni J, Vaheri A, Vapalahti O, Närvänen A. Surface-activated microtiter-plate microarray for simultaneous CRP quantification and viral antibody detection. **Diagn Microbiol Infect Dis** ;75:174-179 2013.

(133) Vuojola J, Lamminmäki U, Soukka T. Resonance energy transfer from lanthanide chelates to overlapping and nonoverlapping fluorescent protein acceptors. **Anal Chem** ;81:5033-5038 2009.

(134) Vuojola J, Syrjänpää M, Lamminmäki U, Soukka T. Genetically encoded protease substrate based on lanthanide-binding peptide for time-gated fluorescence detection. **Anal Chem** ;85:1367-1373 2013.

(135) Wegner KD, Jin Z, Linden S, Jennings TL, Hildebrandt N. Quantum-dot-basedForster resonance energy transfer immunoassay for sensitive clinical diagnostics of low-volume serum samples. **ACS Nano** ;7:7411-7419 2013.

(136) Wegner KD, Linden S, Jin Z, Jennings TL, el Khoulati R, van Bergen en Henegouwen PM, Hildebrandt N. Nanobodies and nanocrystals: highly sensitive quantum dot-based homogeneous FRET immunoassay for serum-based EGFR detection. **Small** ;10:734-740 2014.

(137) Wikstrom M, Sjöbring U, Drakenberg T, Forsen S, Björck L. Mapping of the immunoglobulin light chain-binding site of protein L. **J Mol Biol** ;250:128-133 1995.

(138) Wu P, Brand L. Resonance energy transfer: methods and applications. **Anal Biochem** ;218:1-13 1994.

(139) Yalow RS, Berson SA. Immunoassay of endogenous plasma insulin in man. **J Clin Invest** ;39:1157-1175 1960.

(140) Zhang Y, Wang TH. Quantum dot enabled molecular sensing and diagnostics. **Theranostics** ;2:631-654 2012.