



**Stefanie Schmeiser**  
Institute of Virology  
University of Veterinary  
Medicine Hannover



**Jan Mast**  
CODA-CERVA  
Ukkel, Belgium



**Matthias König**  
Institute of Virology  
Faculty of Vet. Med.  
JLU Giessen

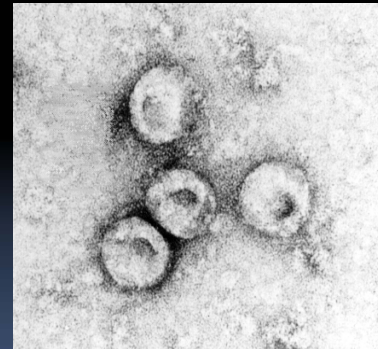
Rome 8. Nov. 2013

# MORPHOGENESIS OF PESTIVIRUSES: NEW INSIGHT BY ULTRASTRUCTURAL STUDIES

# Overview

- Introduction to Pestiviruses
- History of EM Studies on Pestiviruses
- Objectives and results of the study
  - Technical issues
    - Modell system
    - *Tokuyasu* technique
    - EM tomography
  - Morphogenesis of Pestiviruses by EM
- Summary

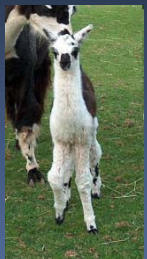
# PESTIVIRUSES



# Pestiviruses

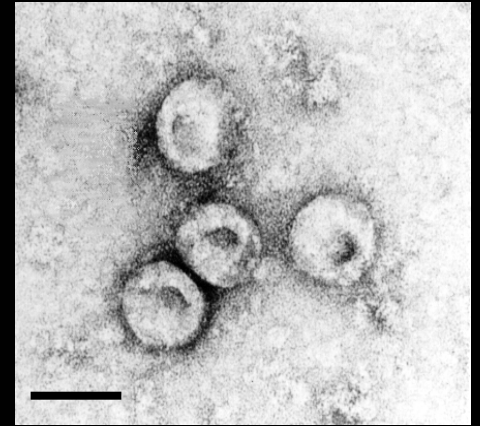


- do not cause disease in humans
- are economically important pathogens of farm animals
  - e.g. classical swine fever epidemic 1997-1998 in The Netherlands – culling of 12 mio pigs (\$2.3 billion)
- are widespread in farm animals
- have a broad host range (cloven hoofed animals)
- fascinating - but difficult to work with (EM)





# Pestivirus Taxonomy

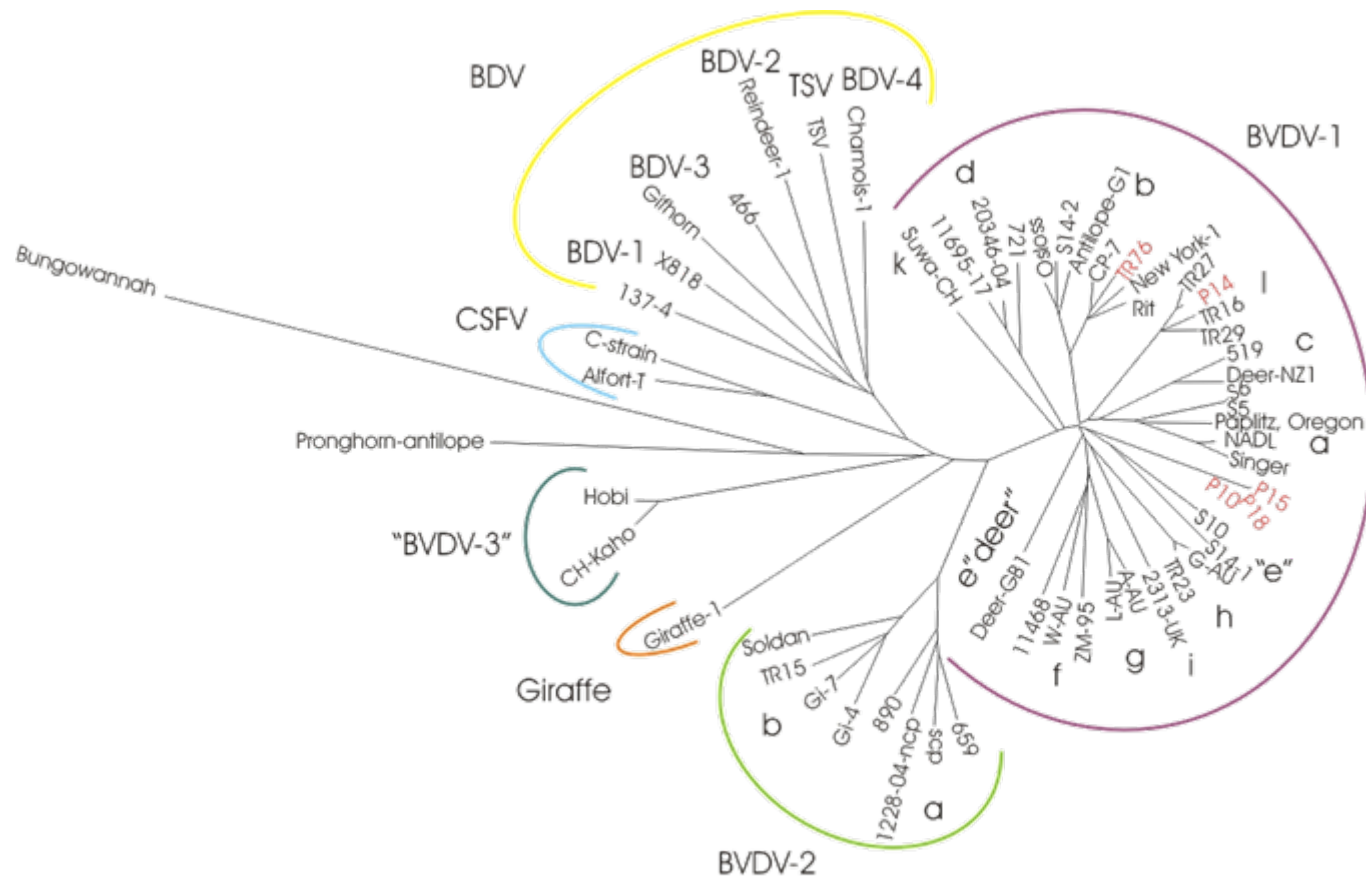


Bar 50 nm

- Flaviviridae
  - Flavivirus (e.g. Yellow fever virus, Dengue Virus)
  - Hepacivirus (Hepatitis C virus)
  - Pegivirus (eg. GB-Virus A, B, C)
  - Pestivirus
    - Classical swine fever virus (CSFV)
    - Bovine viral diarrhea virus (BVDV 1 & 2)
    - Border disease virus (BDV)



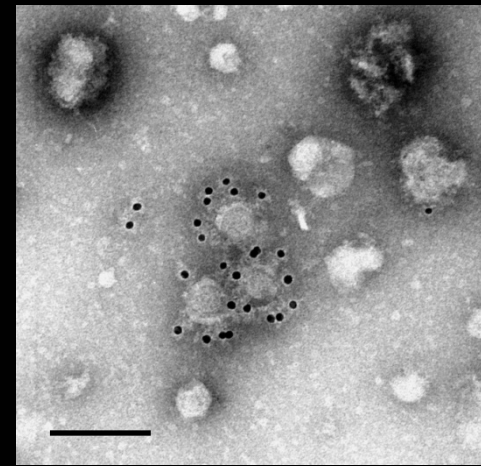
# Pestivirus Heterogeneity



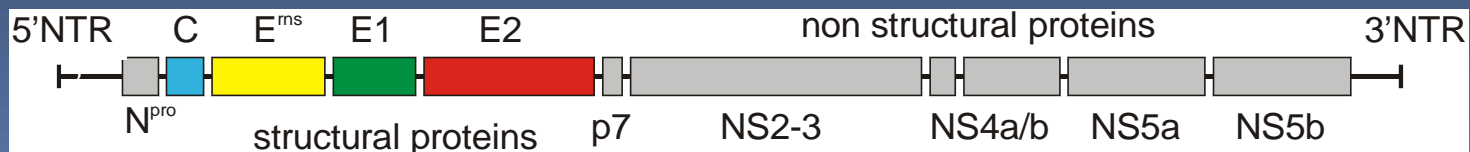
Pestivirus, Npro, 504 bp

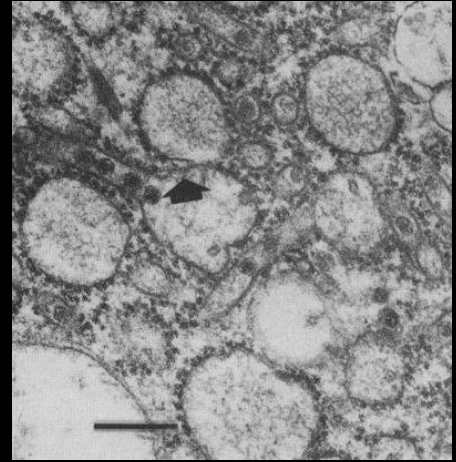
# Pestiviruses

Bar 100 nm  
immunogold  
labeling  
Anti-E<sup>rns</sup>



- Virion
  - enveloped, 40 – 50 nm, nucleocapsid ~25 nm,
- Genome
  - ss RNA, pos. polarity, 12,3 kb
- Proteome
  - single large ORF, polyprotein
    - Structural proteins C, E<sup>rns</sup>, E<sub>1</sub>, E<sub>2</sub>
    - Non structural proteins N<sup>pro</sup>, p7, NS2 - 5



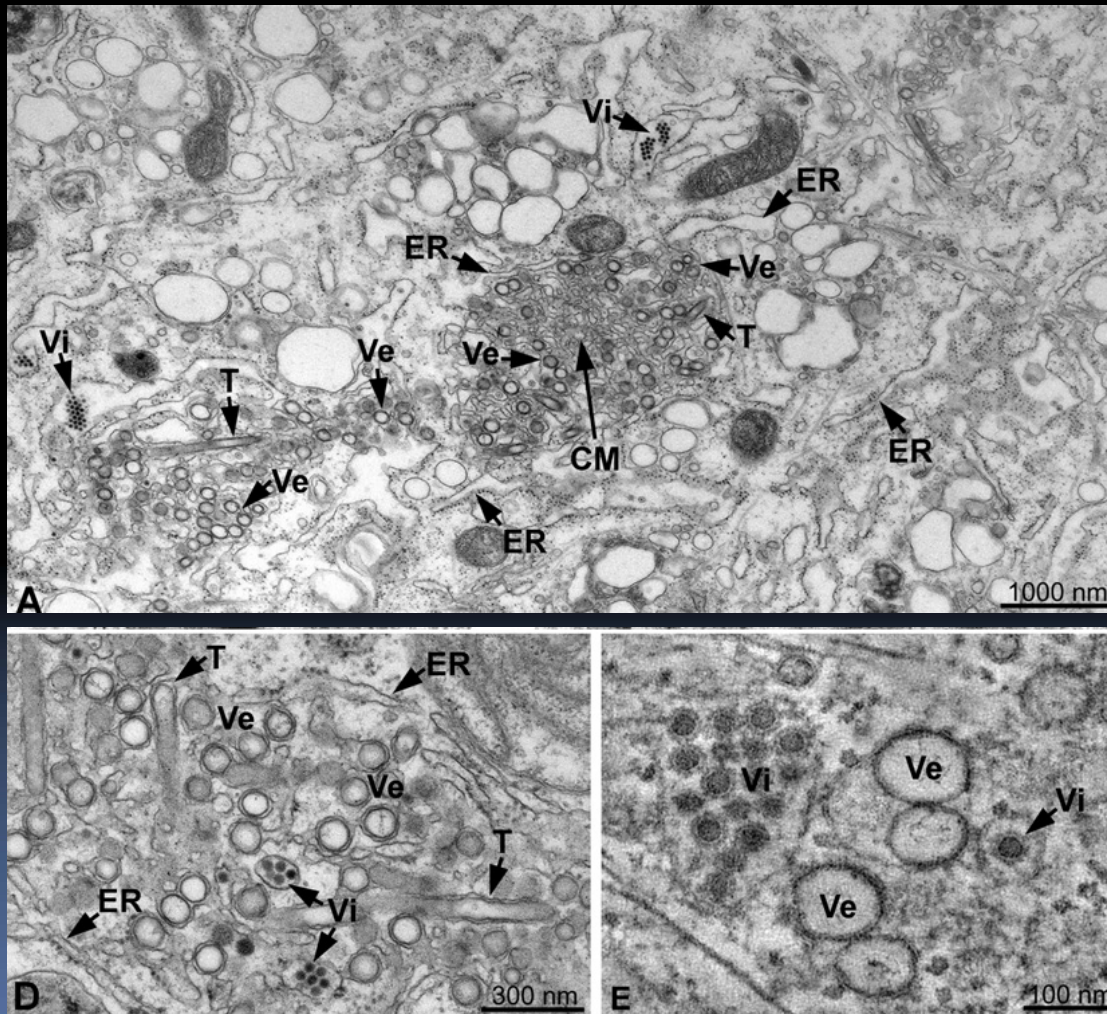


A brief history of

# EM STUDIES ON PESTIVIRUS MORPHOGENESIS



# Dengue Virus Morphogenesis (Flavivirus)

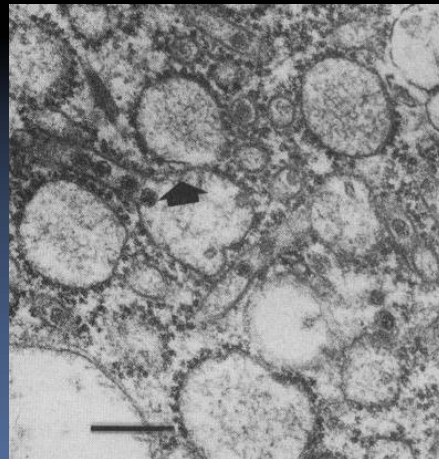


Welsh et al., 2009

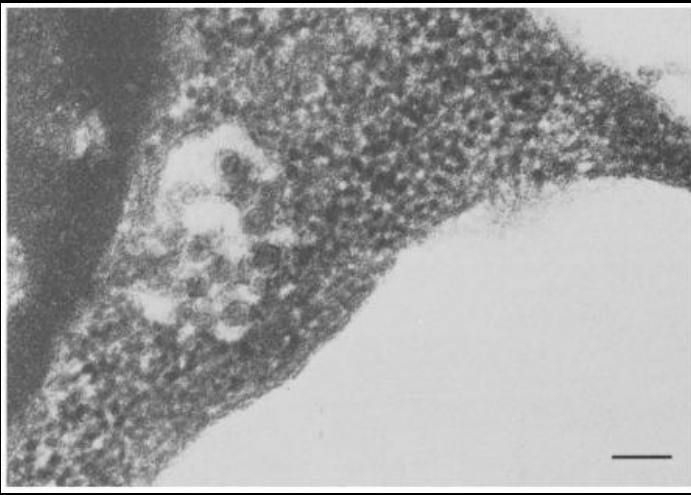
# EM studies on Pestiviruses

- biochemical evidence for intracellular pathways of Pestivirus replication
- few reports on ultrastructure of pestivirus infected cells
  - ▣ low replication rate of pestiviruses

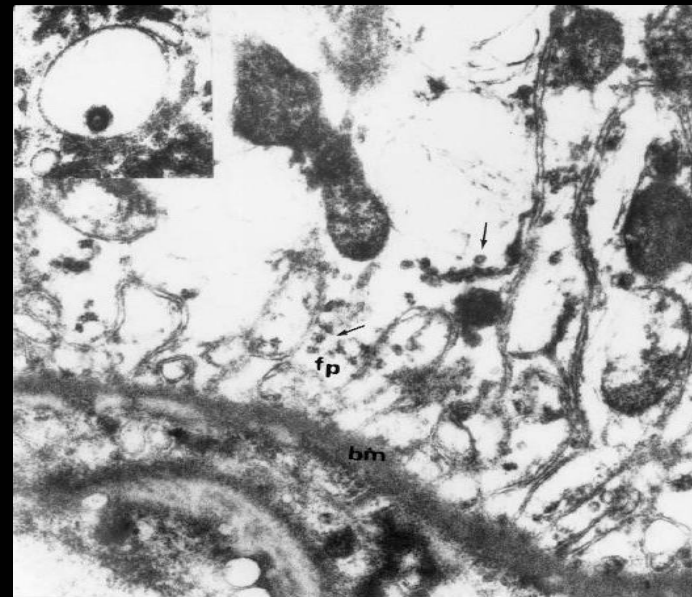
bar 200nm  
Gray and Nettleton, 1987







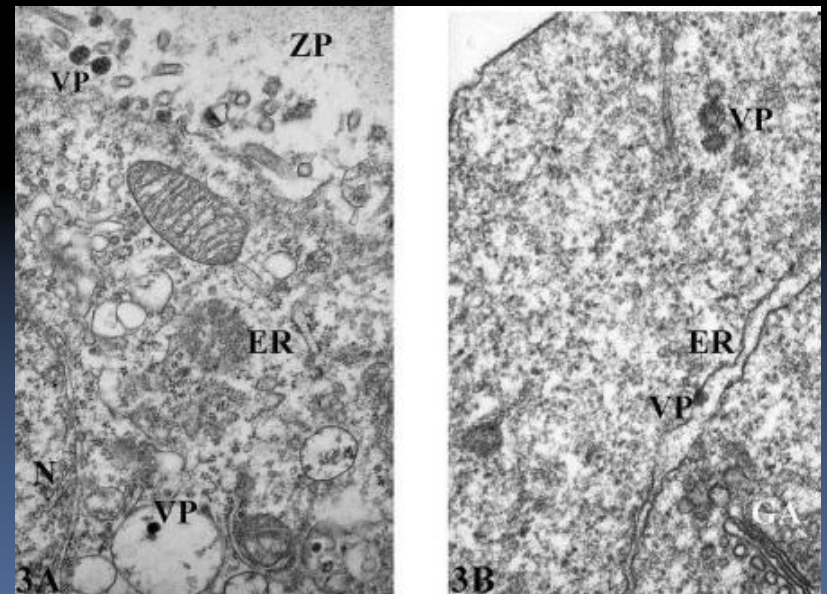
bar 100nm  
Bielefeldt Ohmann et al., 1987



Calderon et al. 2000



bar 100nm  
Weiland et al., 1999

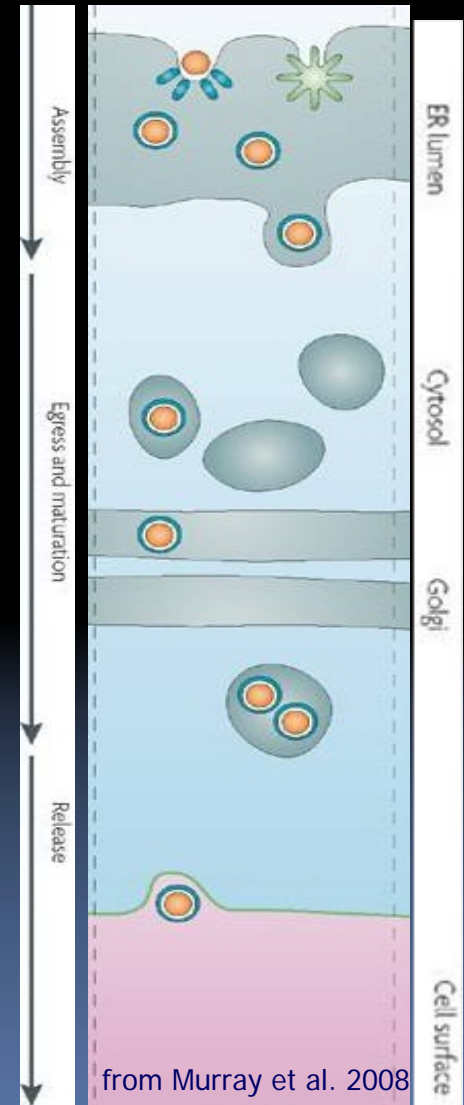


Kubovicova 2008



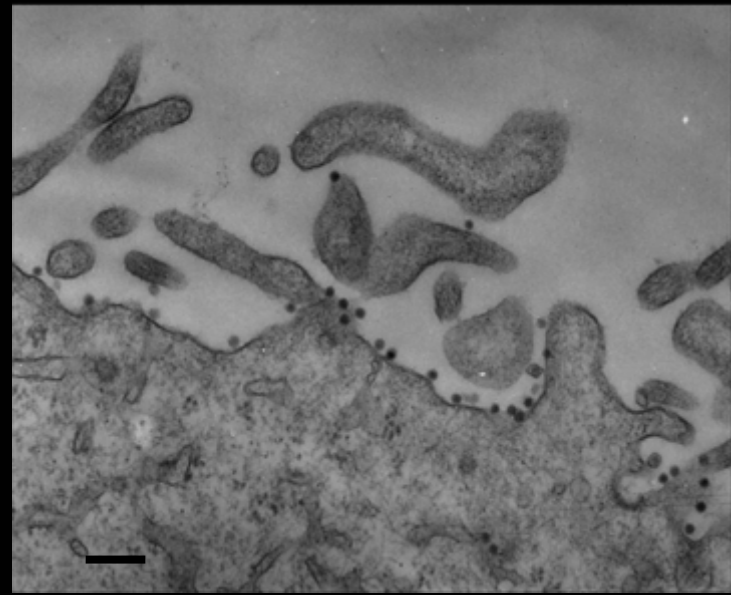
# Objectives of the study

- RNA replication in cytoplasm (replication complex)  
→ rearrangement of cellular membranes?
- assembly of pestivirions  
→ directly at ER membranes? Budding?
- intracellular transport  
→ secretory pathway?
- release  
→ single particle exocytosis?



# Project set up

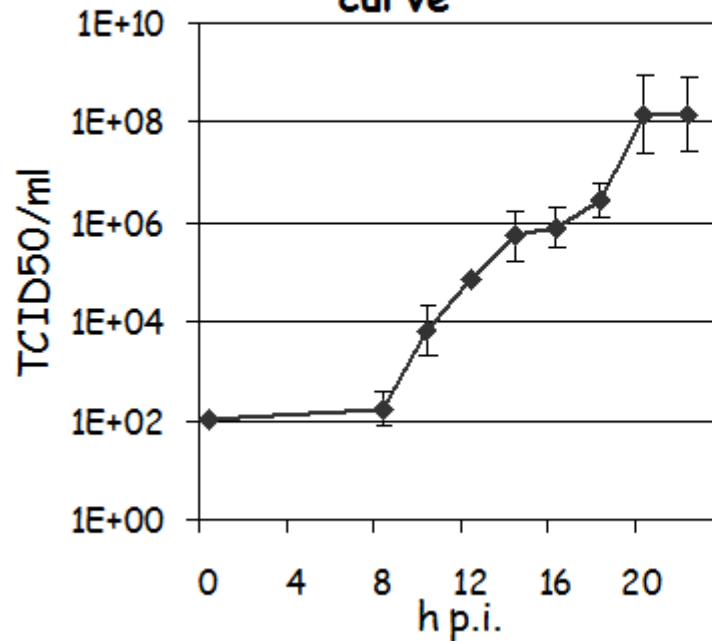
- Virus/ cell system:
  - „Giraffe-1“/ MDBK cells
  - BVDV-2 „890“/ MDBK cells
  - CSFV „Alfort-Tüb“/ PK15 cells
- Time course studies to optimize infection models:
  - synchronized infection of cells, moi. 10
  - one-step growth curves from supernatants
  - real time RT-PCR (RQ) of viral RNA (cell lysates)



Pestivirus Giraffe 1 (bar 300nm)

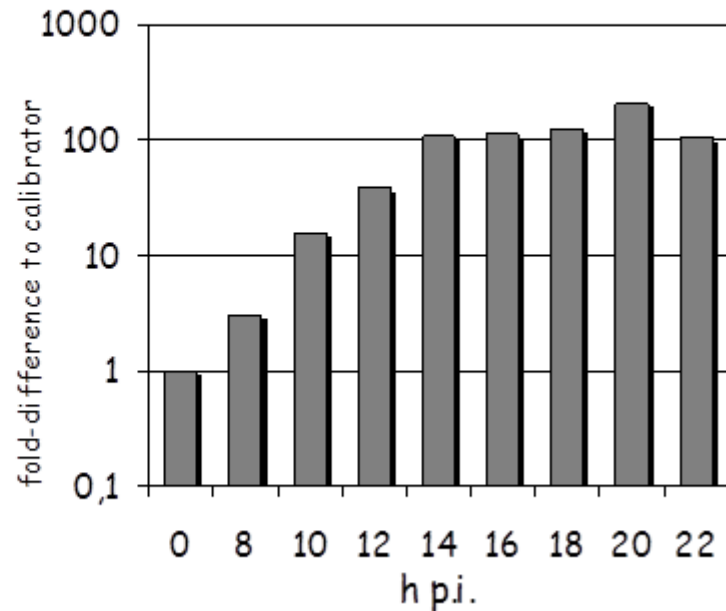
# Model Systems

Pestivirus one-step growth curve



Virus infectivity assay

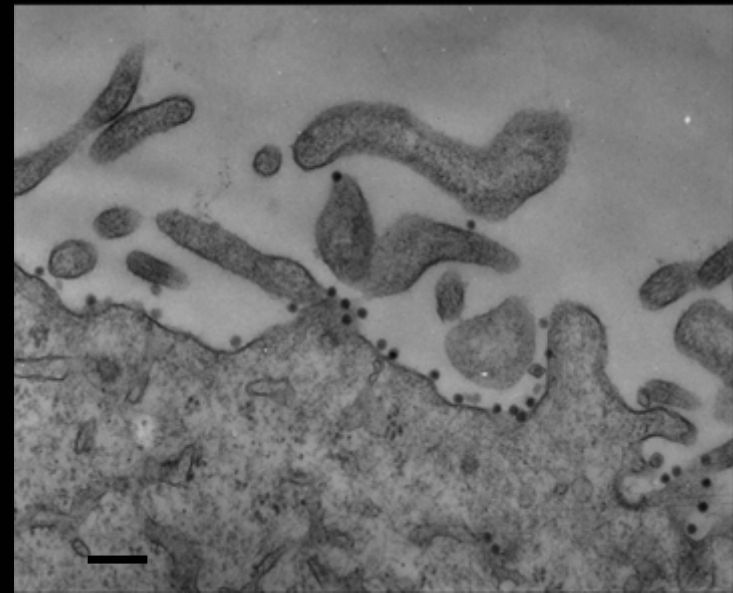
Relative quantification of pestivirus RNA



qPCR

# Project set up

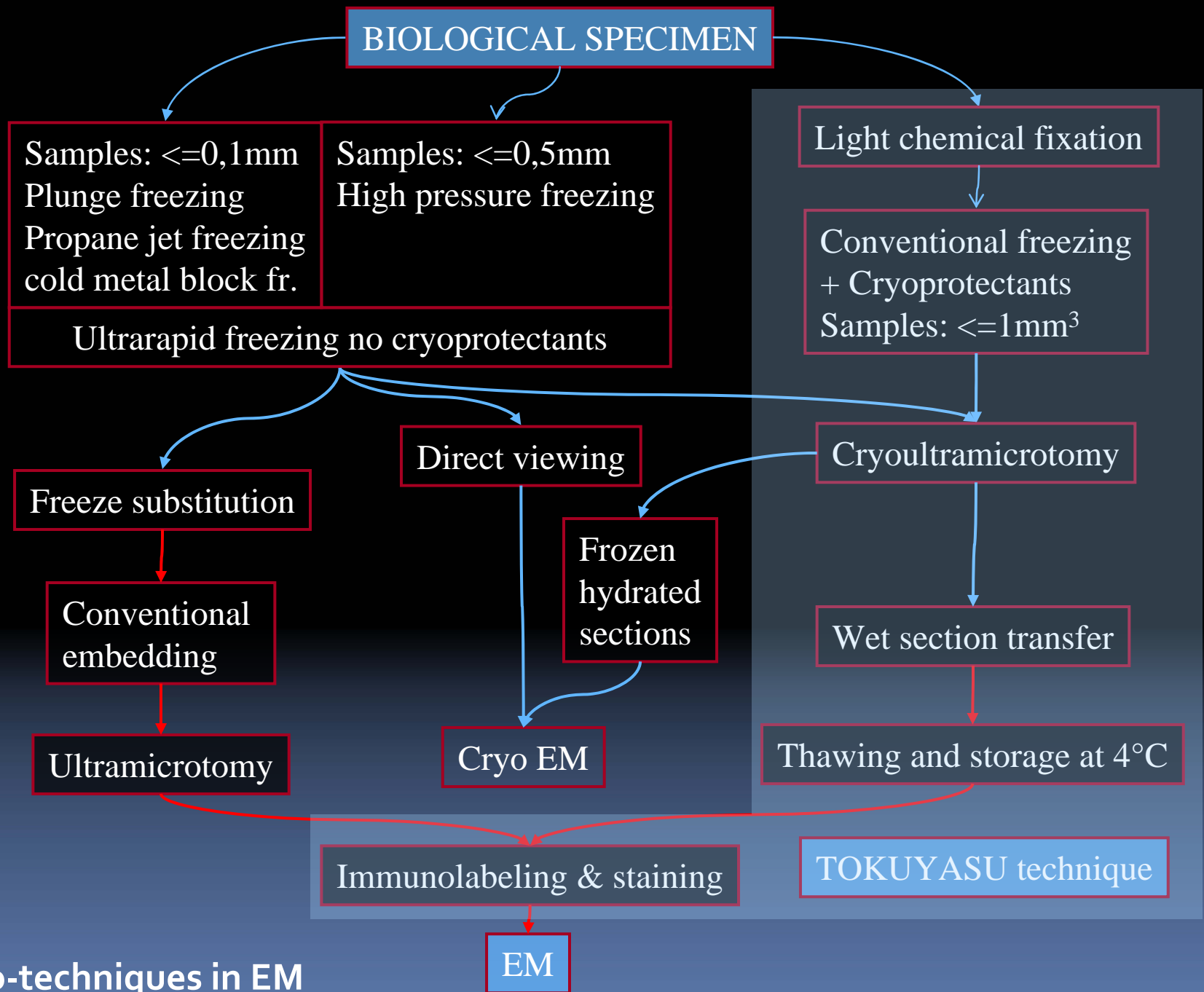
- Virus/ cell system:
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- Time course studies to optimize infection models:
  - synchronized infection of cells, moi. 10
  - one-step growth curves from supernatants
  - real time RT-PCR (RQ) of viral RNA (cell lysates)
  - Time range to study virus assembly and release: 10-16h p.i.
- EM techniques:
  - epoxid resin embedding for studies on pestivirion morphogenesis
  - cryoultramicrotomy (Tokuyasu) for immunogold labelling studies
  - electron tomography



Pestivirus Giraffe 1 (bar 300nm)

Exploring the *Tokuyasu* technique

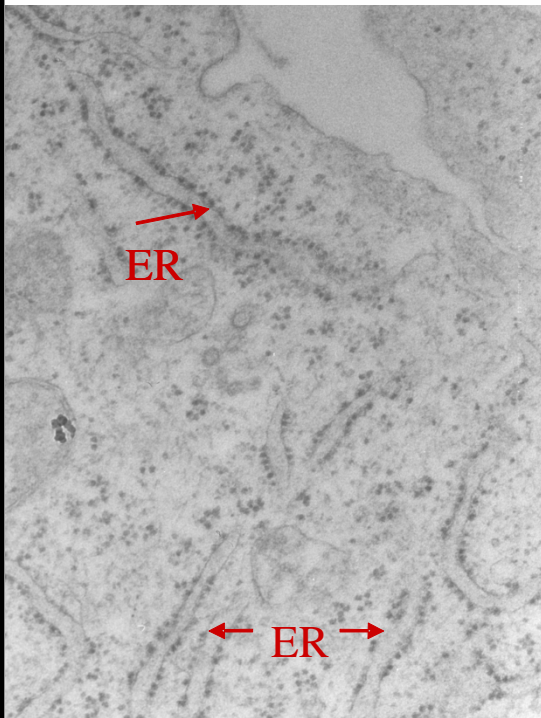
# IMMUNOLABELING OF FROZEN SECTIONS





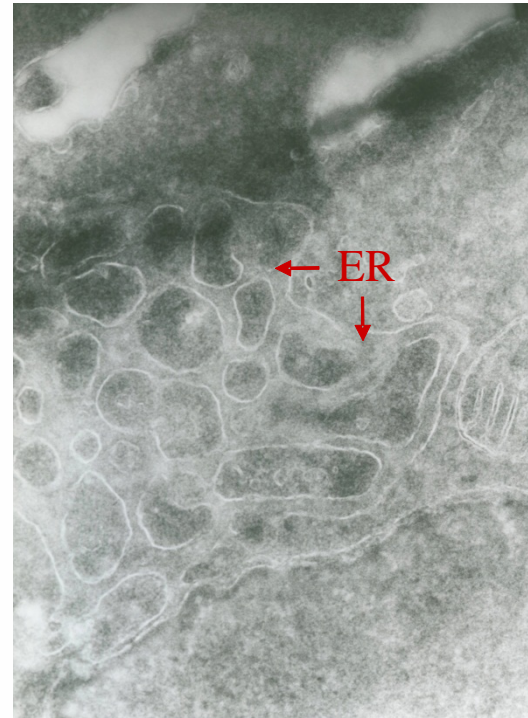
# Morphology of Tokuyasu Sections

Epoxid Resins



Positive staining of  
membranes

Tokuyasu



Negative staining of  
membranes



# Immunolabeling of Tokoyasu sections

„a long road to go“

- Visiting a workshop at the beginning is most important
  - learning the basics as well as the tricks
  - getting into contact with tutors as well as with other beginners
- regular practice (at least 1x/ week) helps to get routine in sectioning and picking up sections

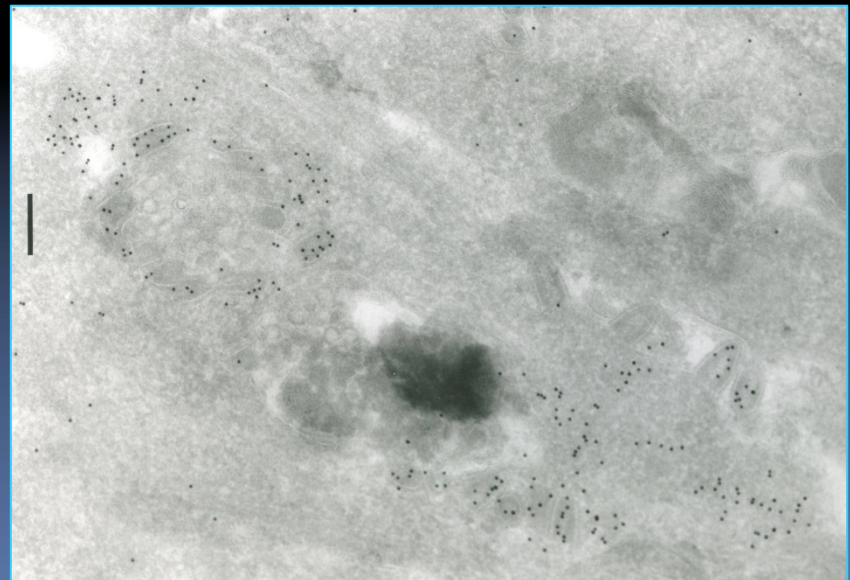
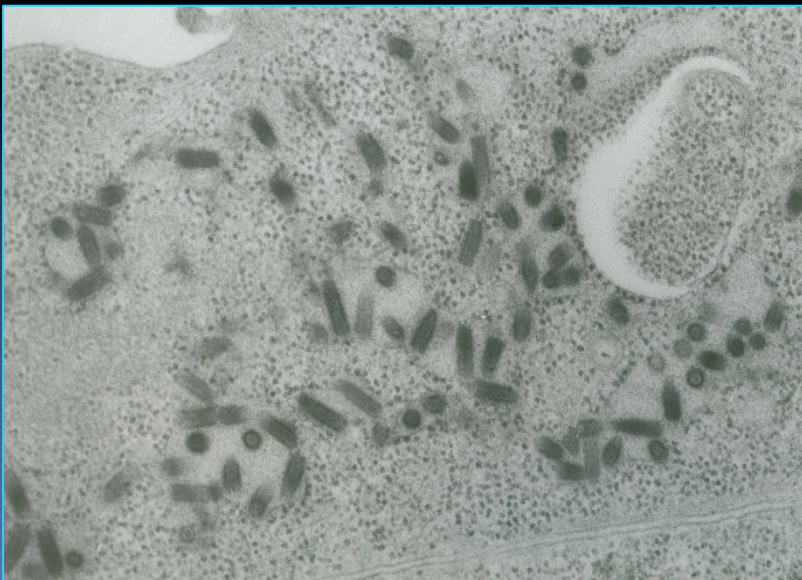
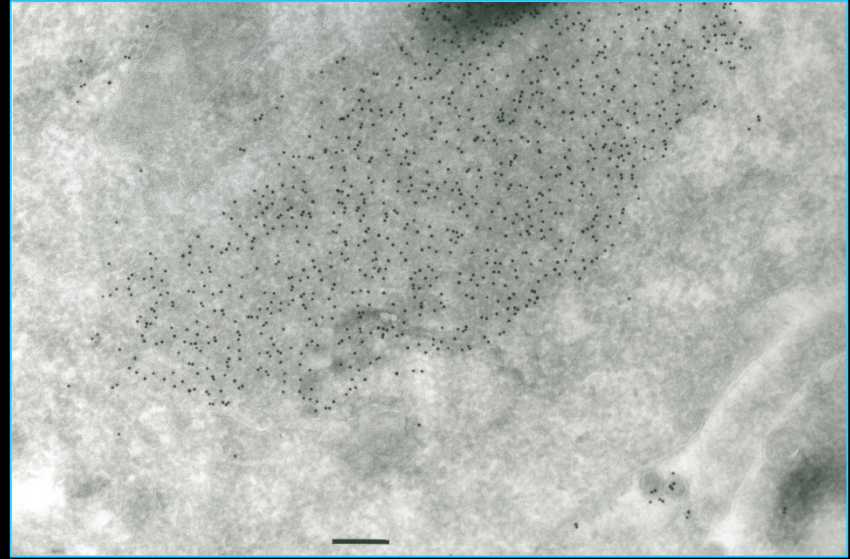
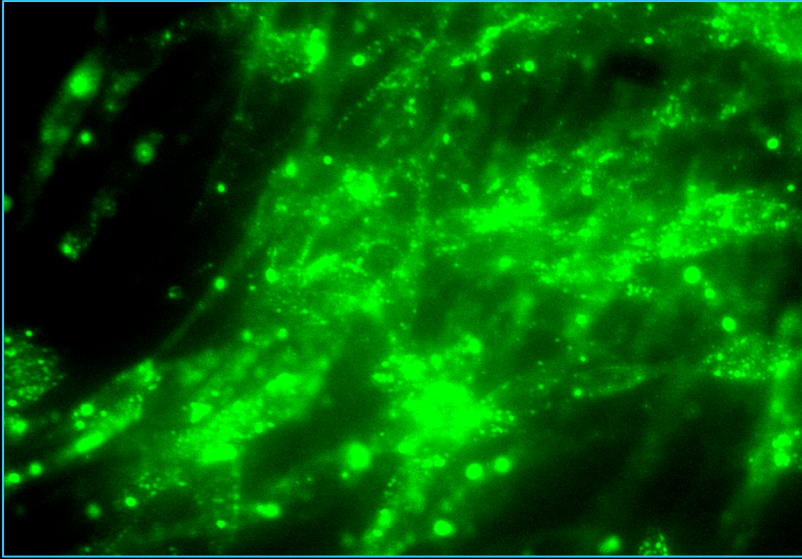
# Immunolabeling of Tokoyasu sections

„a long road to go“

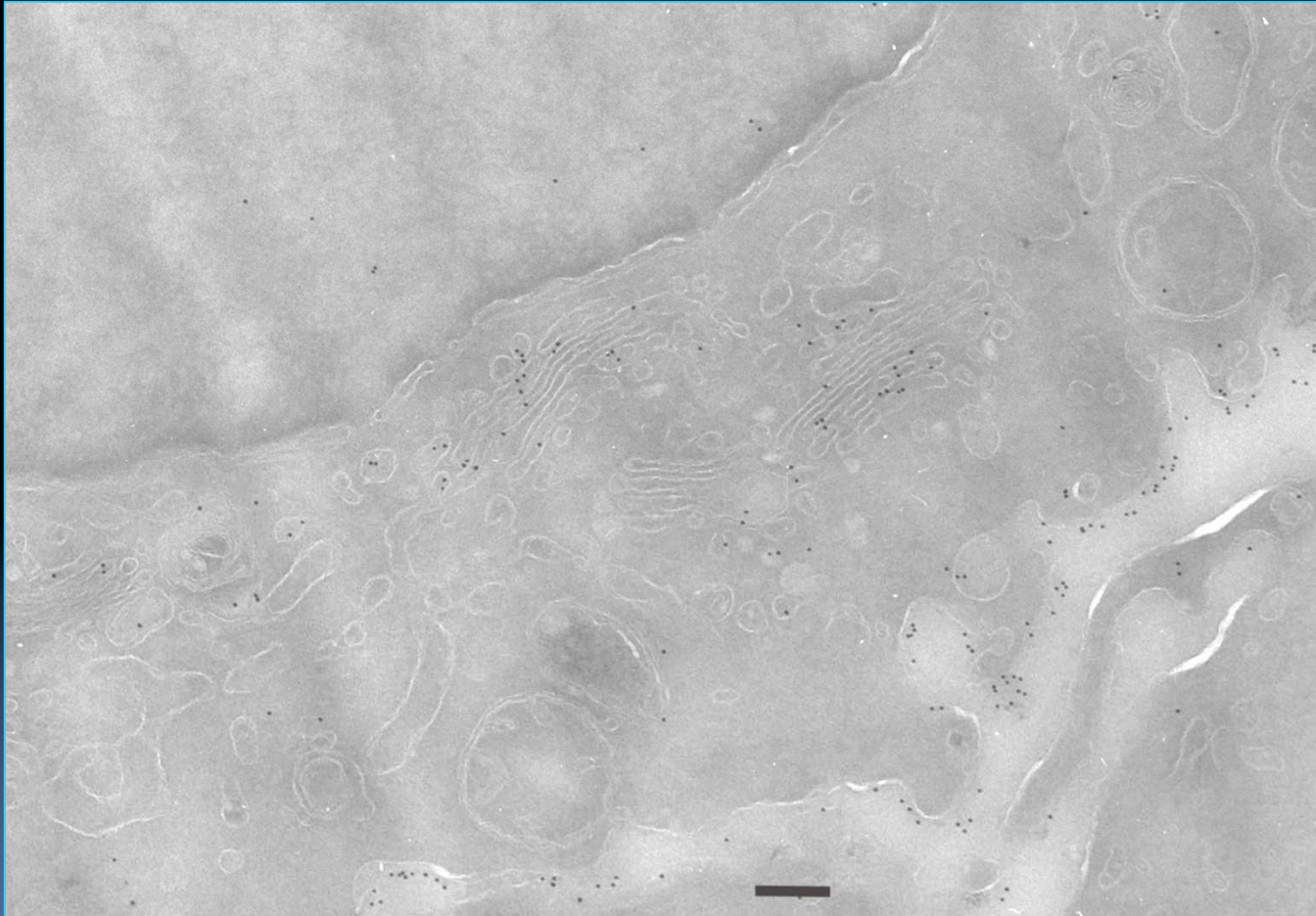
- Labeling of **rabies virus infected** culture cells  
(high antigen presence)
- Labeling of culture cells **overexpressing pestiviral proteins**  
after induction  
(high antigen presence)
- Labeling of **pestivirus infected** culture cells  
(low antigen presence)  
and  
double labeling with **cellular markers** for co-localization  
studies

# Rabies Virus

anti RV N-protein



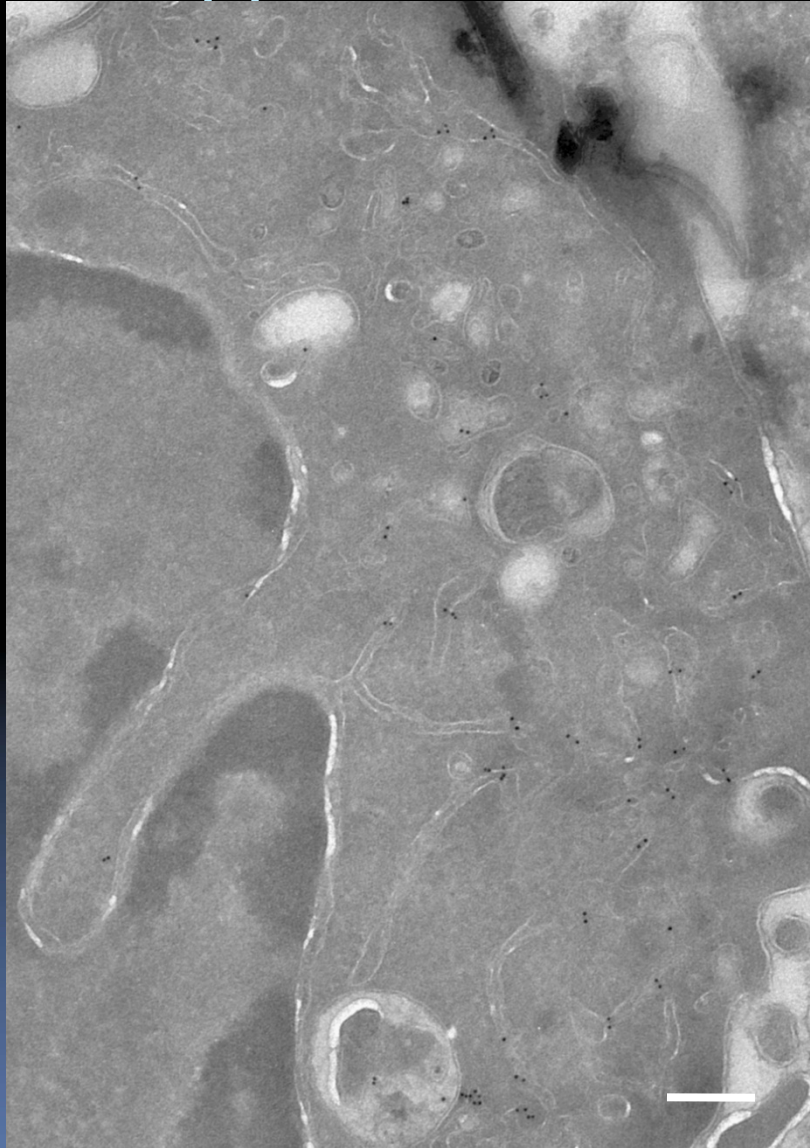
# BHK cells expressing E2 Protein of a Pestivirus



Anti-E2



# Immunolabeling of PV infected



1st ab:  
mab a E2



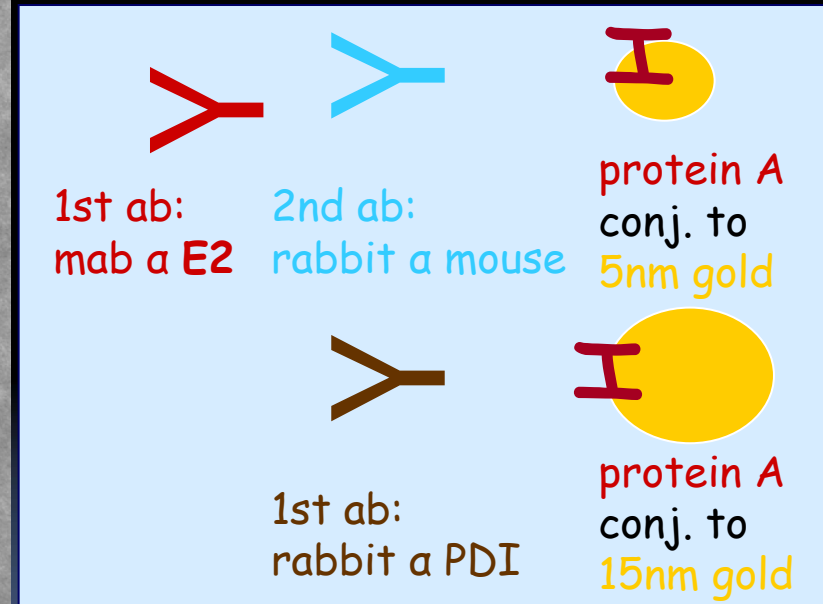
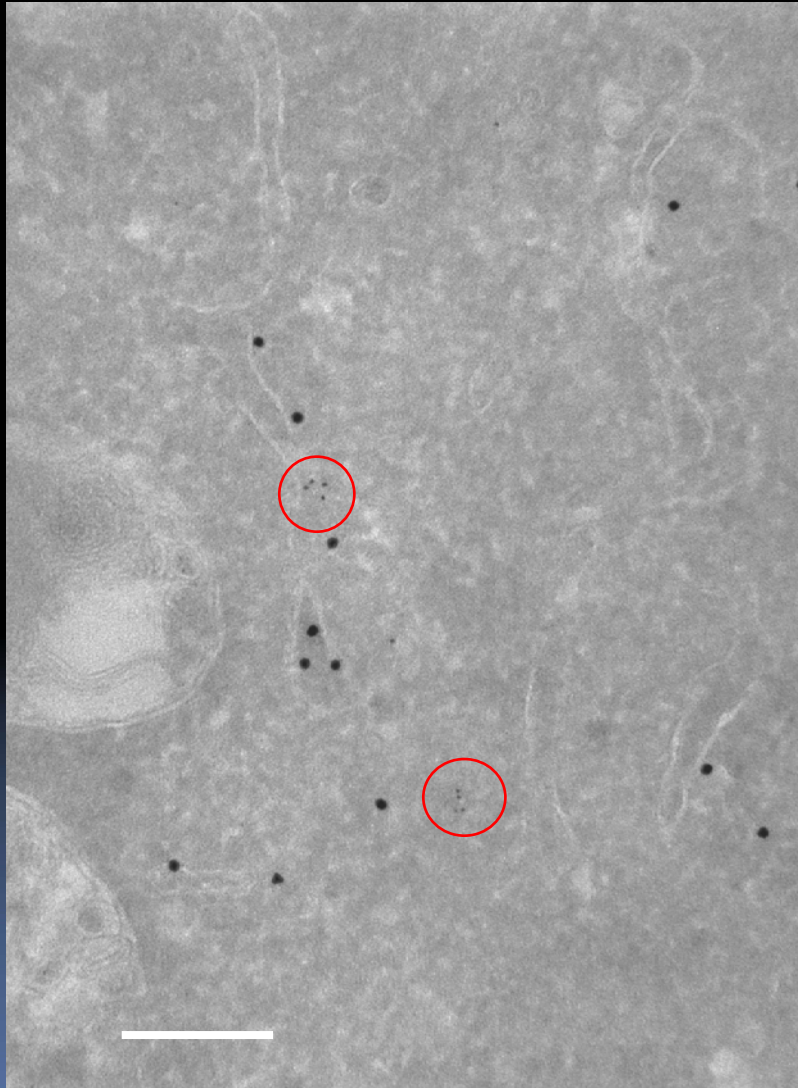
2nd ab:  
rabbit a mouse



protein A  
conj. to  
5nm gold

bar 200nm

# Double-Immunolabeling



bar 200 nm



John O'Brien, The New Yorker Magazine (1991)

# EM - TOMOGRAPHY



# Tomography 1

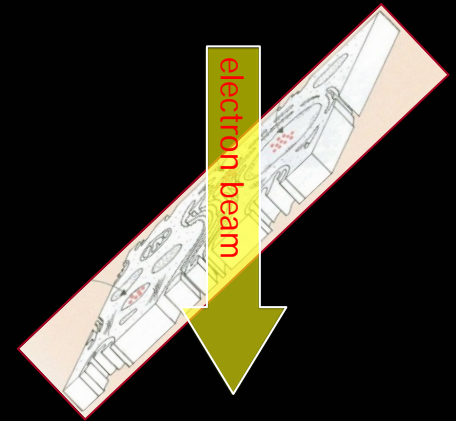
## Selection of Candidate picture

- Sections from Epon-embedded infected culture cells
  - ▣ Screening for suitable areas for tomography

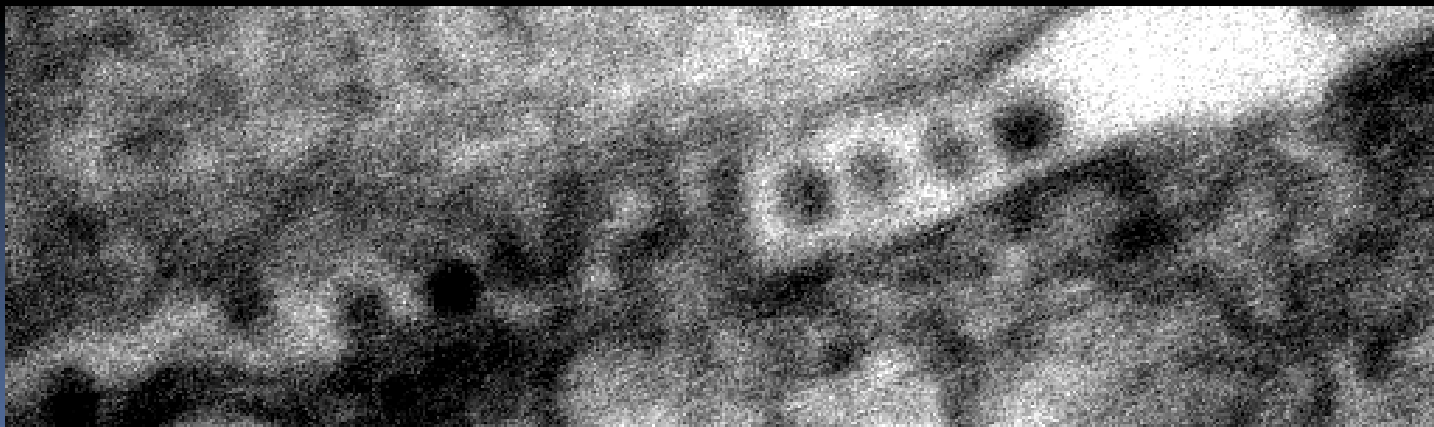


# Tomography 2

## Data Acquisition

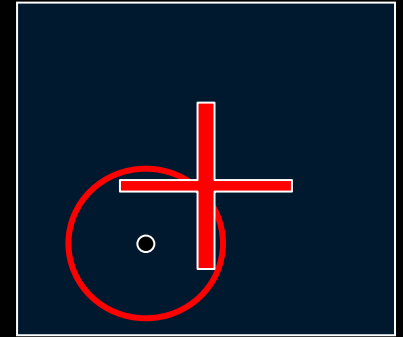


- Electron tomography: (CODA-CERVA, Belgium)
  - Tecnai® Spirit, Biotwin, LaB6-filament, 120 kV
  - tomography holder (FEI, Eindhoven, The Netherlands)
  - tilt-series were recorded semi-automatically assisted by the Xplore 3D tomography-module, tilt range of at least  $70^\circ$  at intervals of  $1^\circ$ ; Mag. 26,500 to 49,000

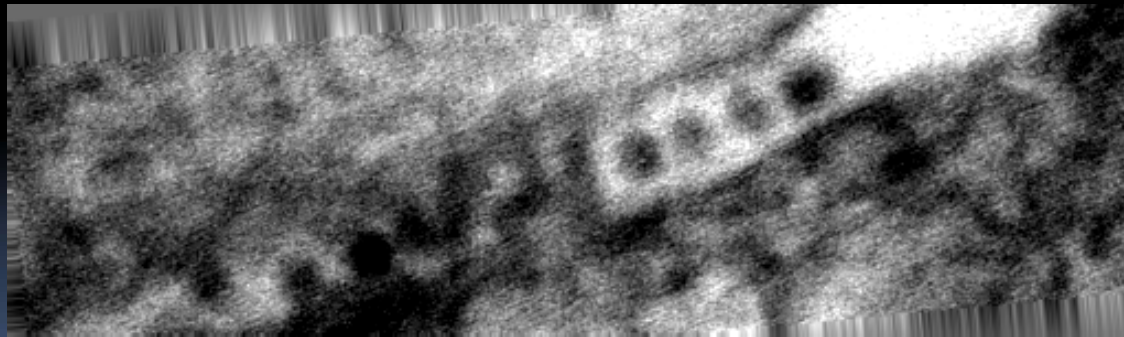


# Tomography 3

## Alignment



- Inspect 3D software (FEI), iterative cross correlation (10-20 cycles)

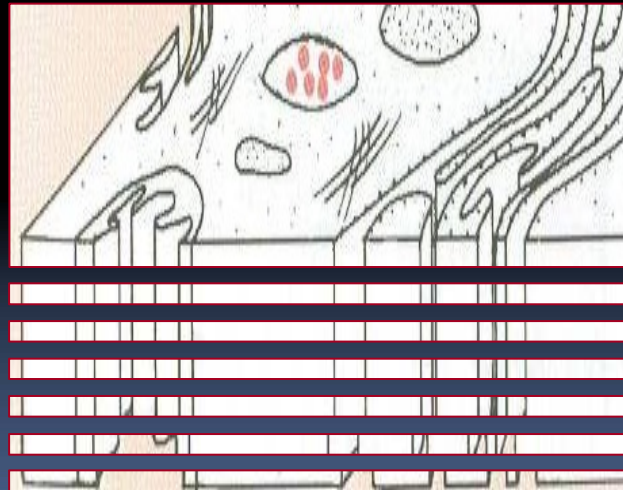


# Tomography 4

## Reconstruction

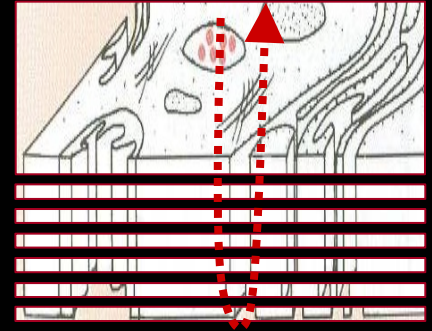
- simultaneous iterative reconstruction technique (SIRT) algorithm (15 cycles)

thin section  
of 60 nm →  
120 orthoslices  
(0.5 nm thick)

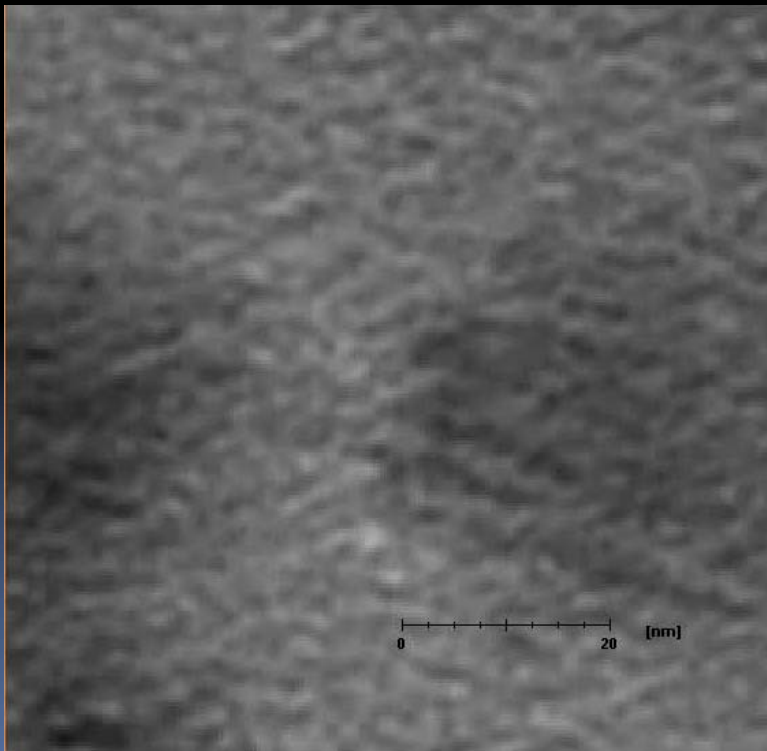


# Tomography 5

## Visualization



- Amira software version 4.1.2 (Mercury Computer Systems, France)



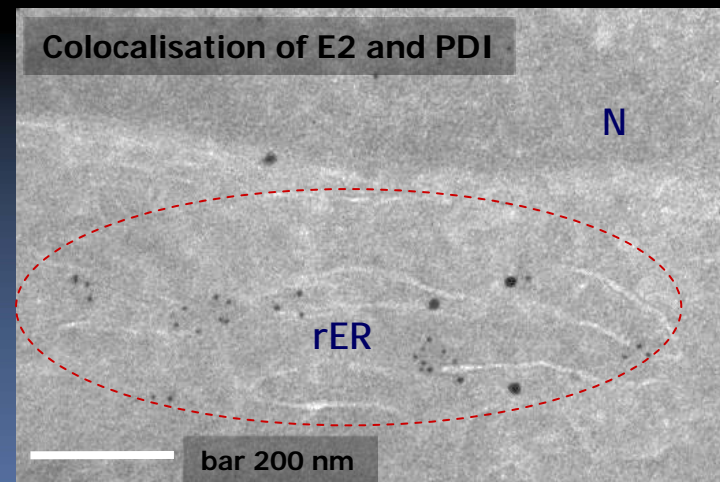
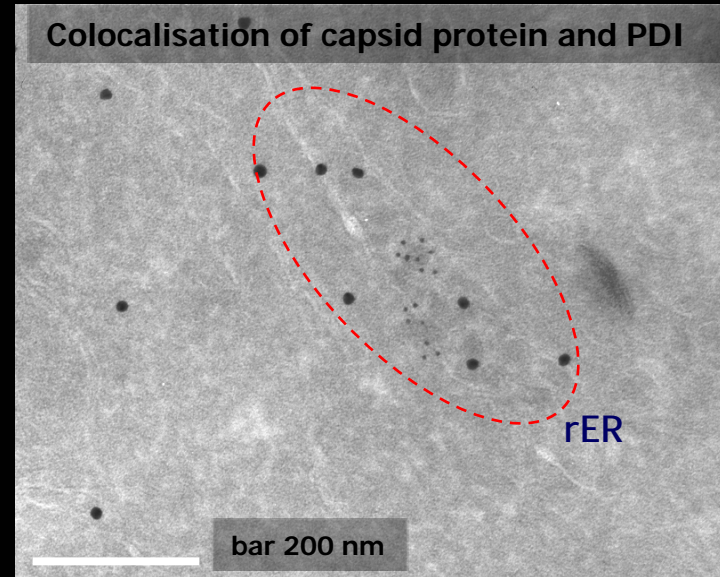
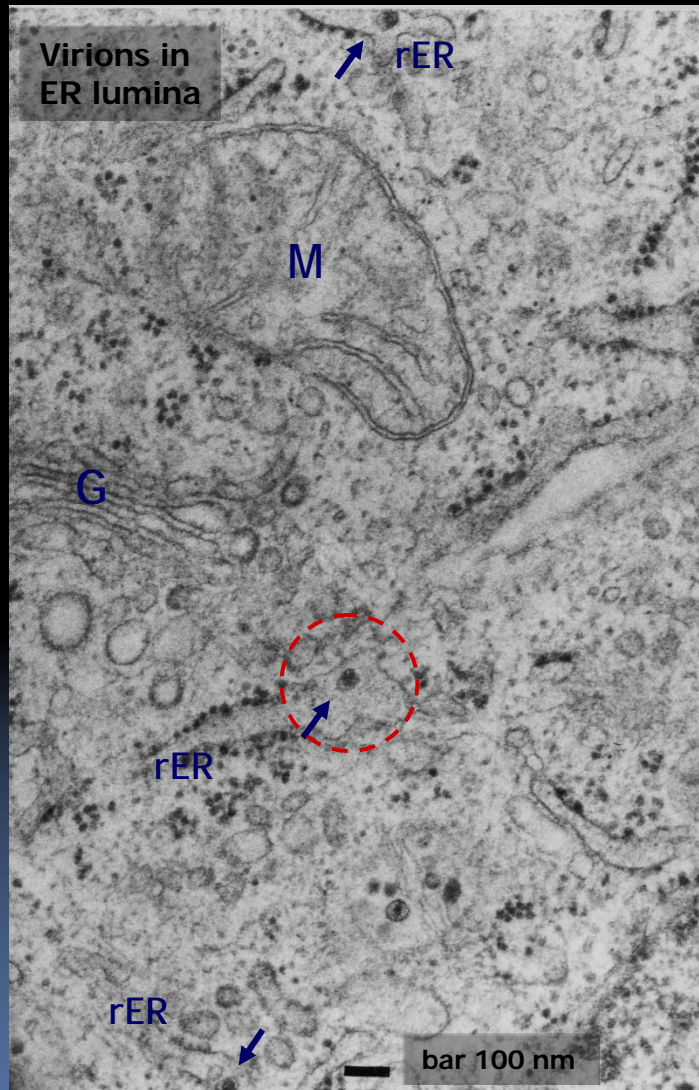
[J24A T2\\_Rek3\\_orthoslices.mpg](#)

Morphogenesis of Pestiviruses by EM

# RESULTS

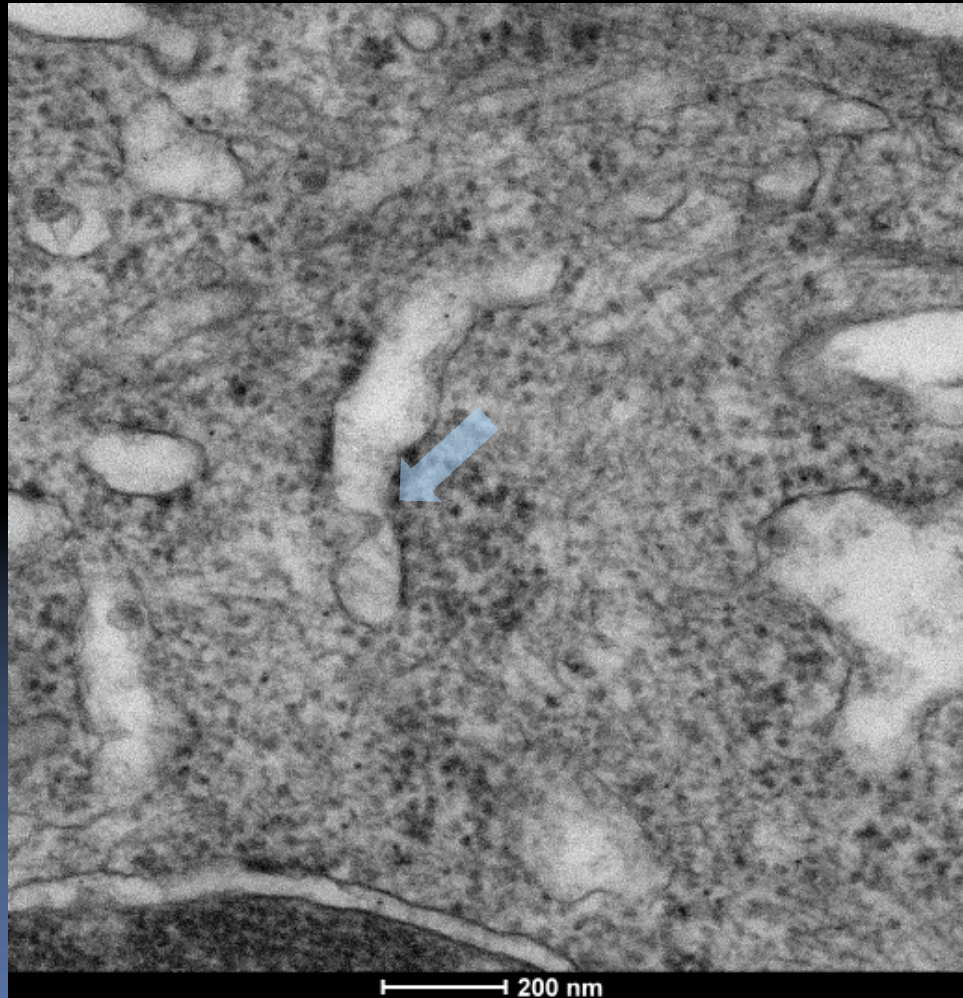


# Pestivirions arise at the ER...

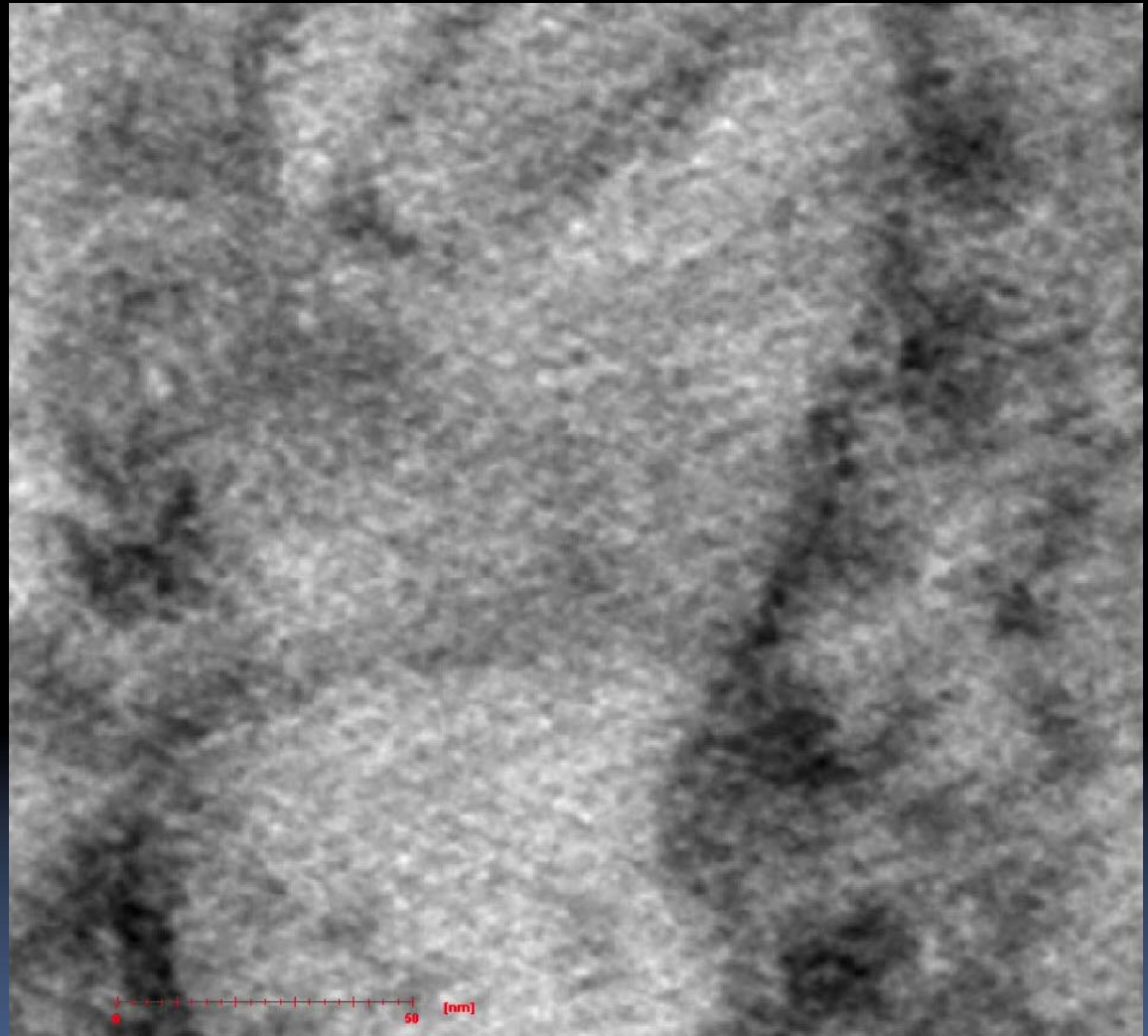




By budding into the ER lumen



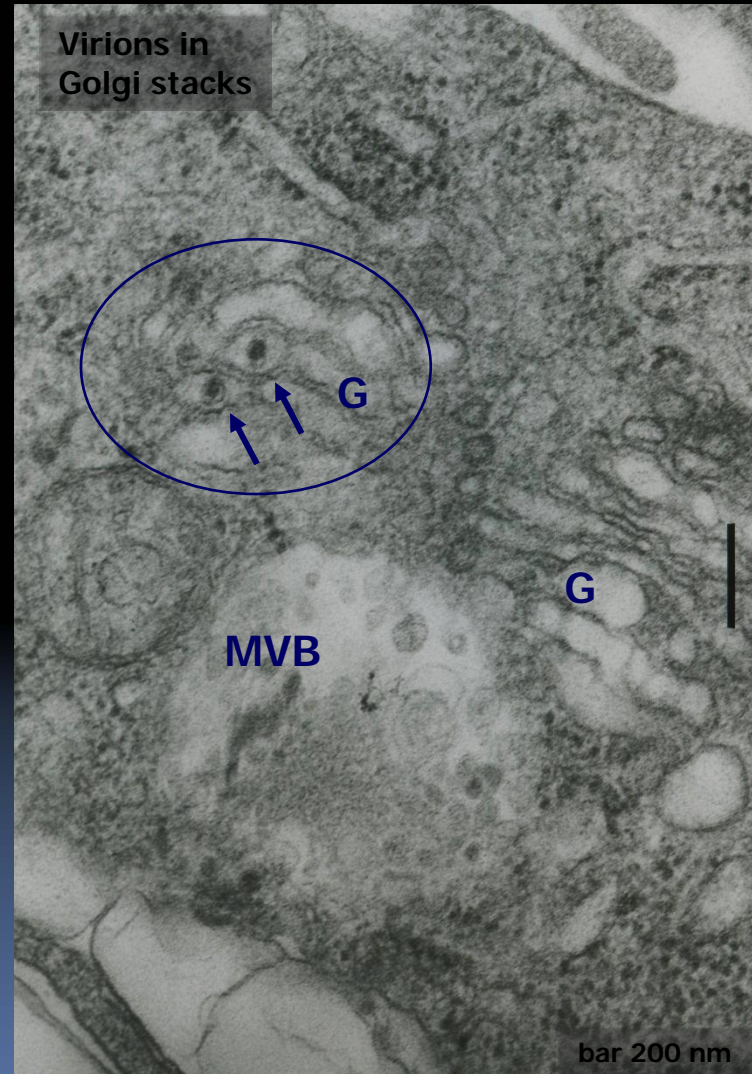
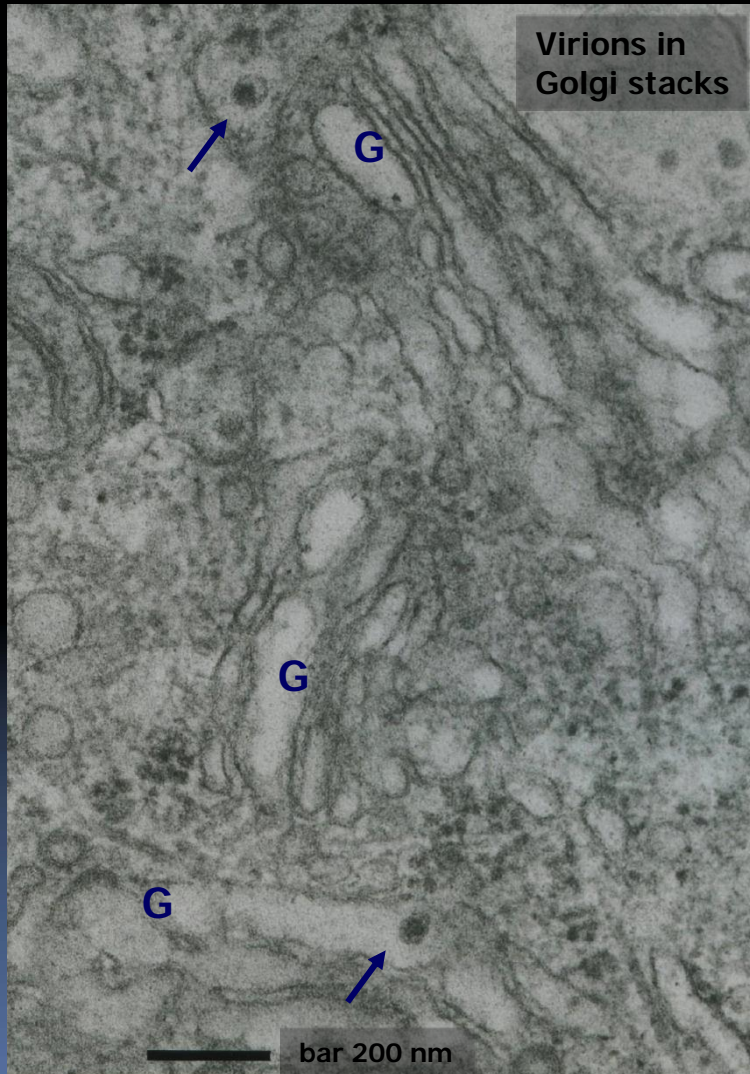
# 3D view



[Budding orthoslice 3.mpg](#)

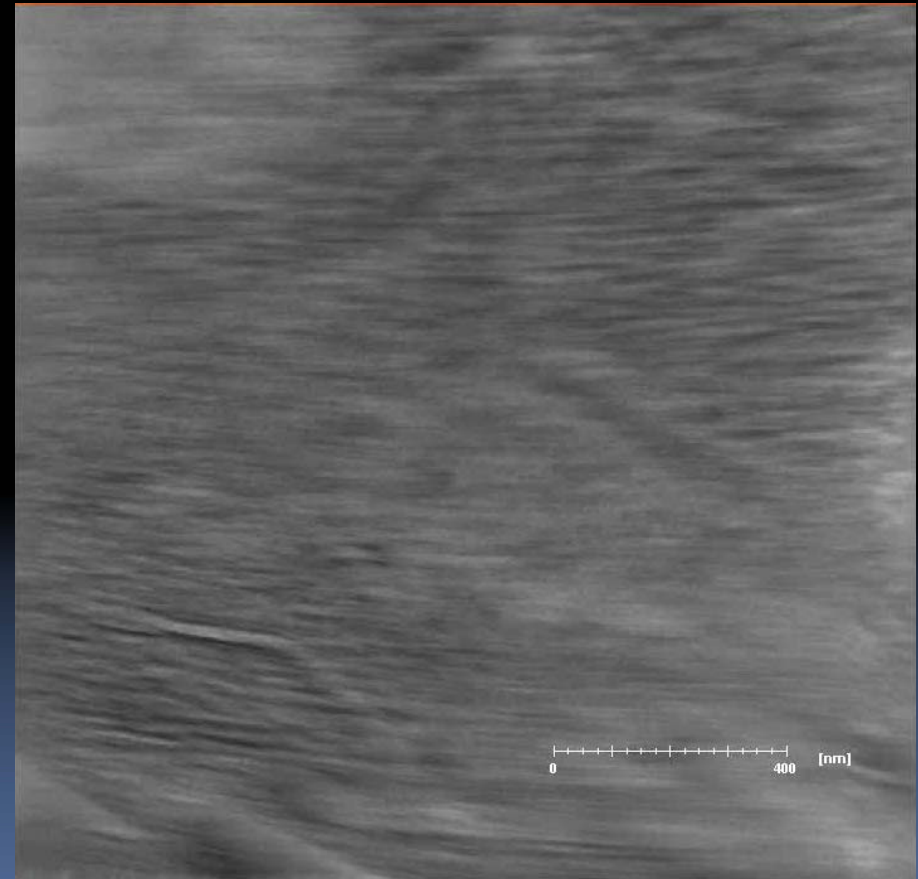
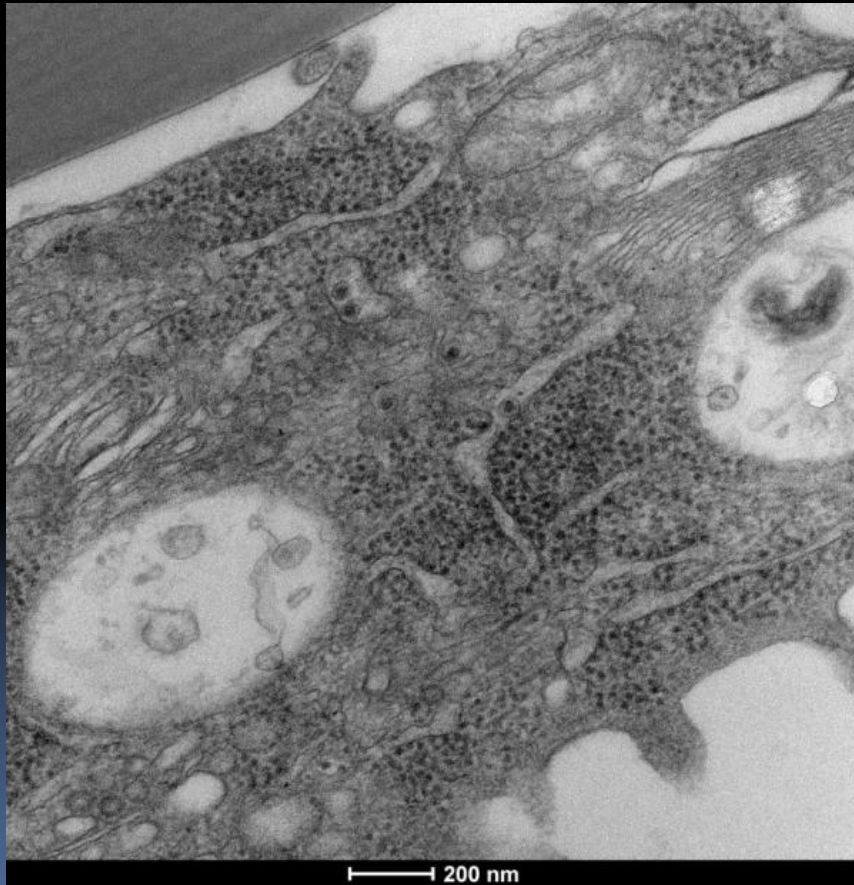
[Budding orthoslice 4.mpg](#)

# Pestiviruses use the cellular secretory pathway



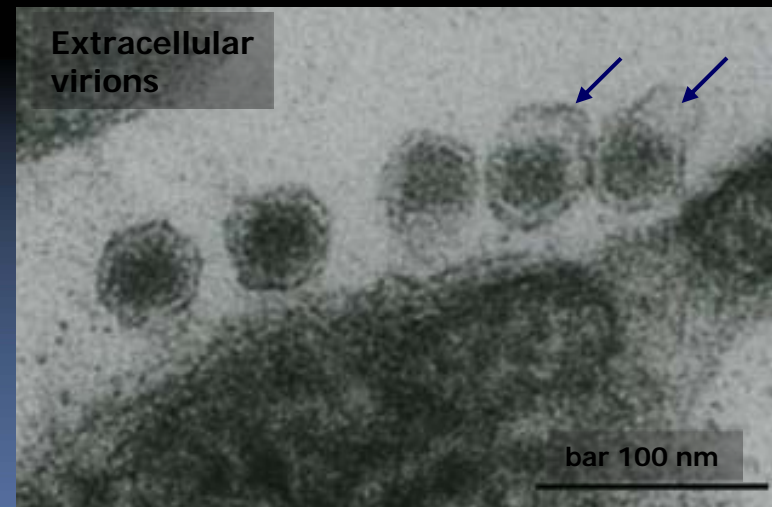
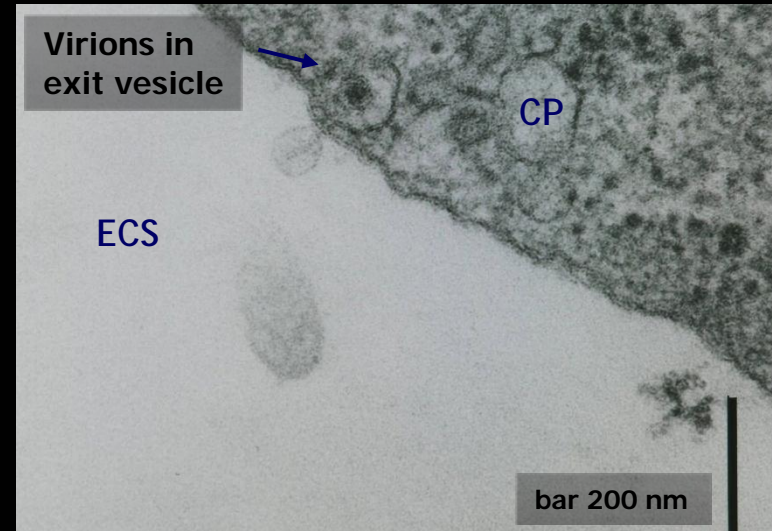
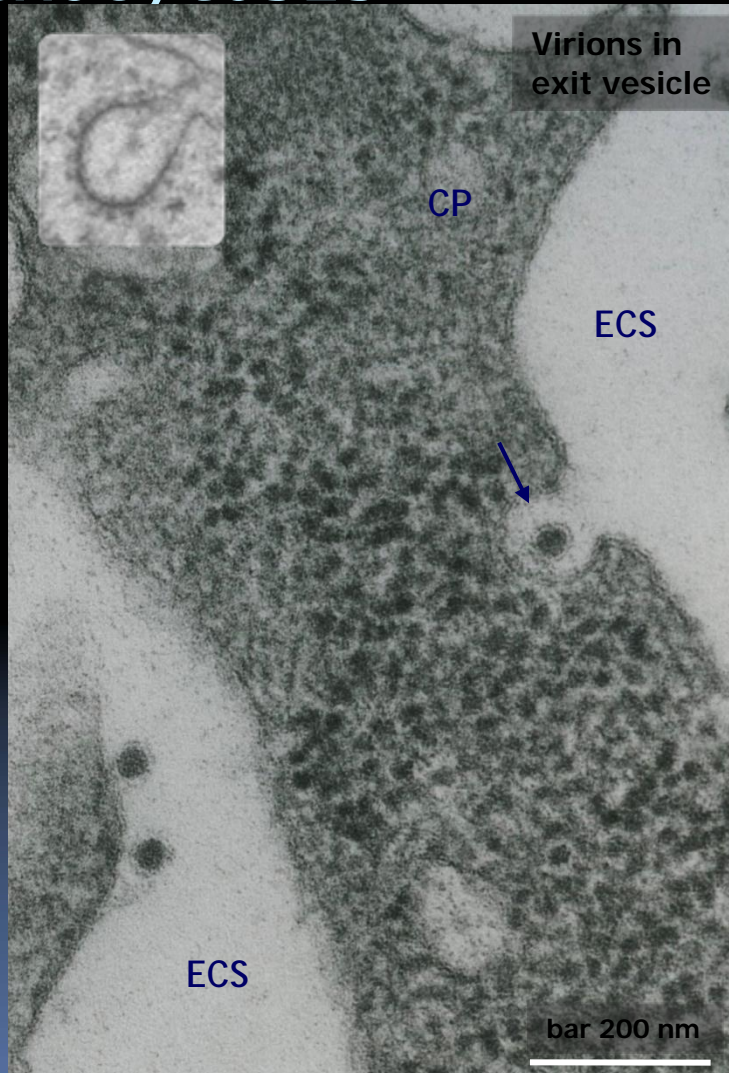


# Pestiviruses use the cellular secretory pathway



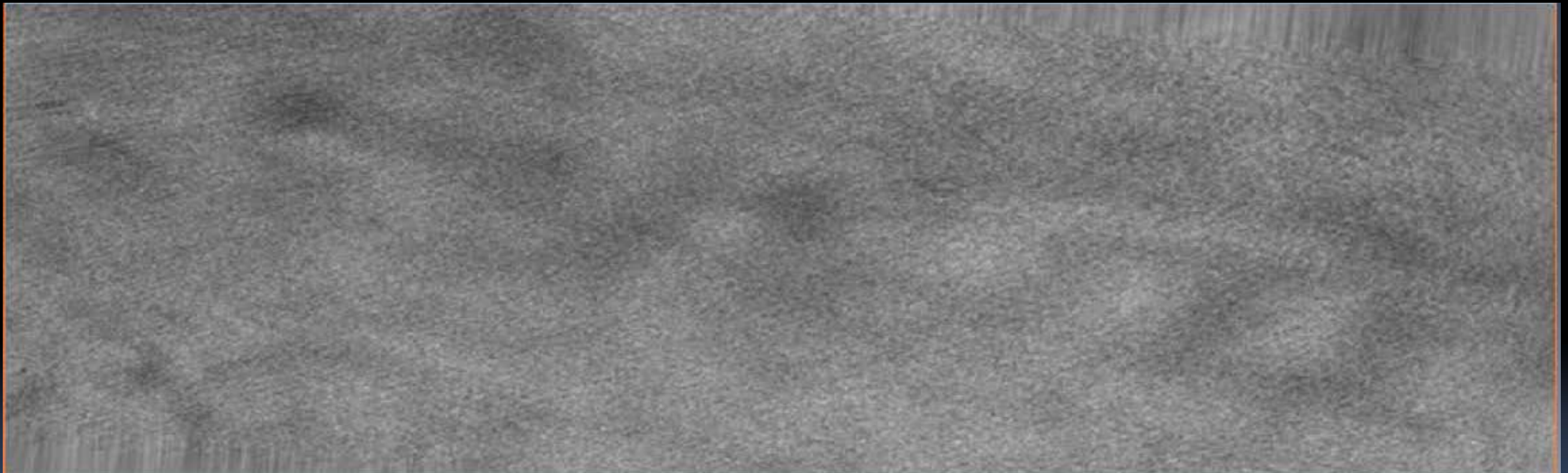
- [J29A T1\\_orthosclices.mpg](#)

# Pestiviruses leave the cell by exocytosis





# Extracellular Virions show detached envelope

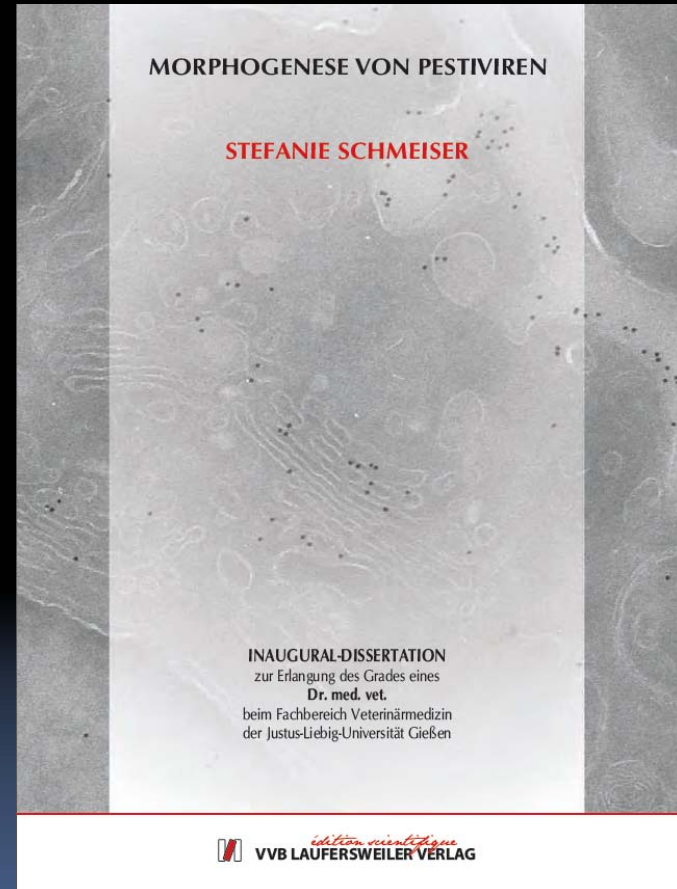


- [J24A T8\\_Orthoslices.mpg](#)

# Summary

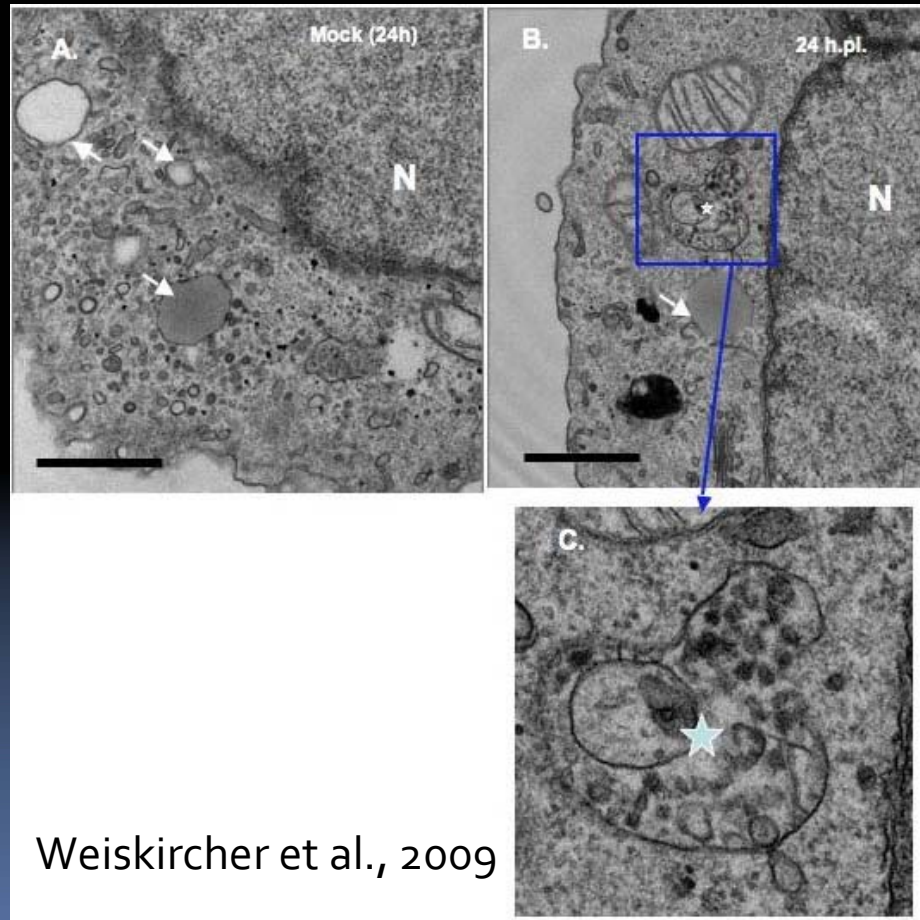
- Electron tomography (ET) in combination with cryosectioning (*Tokoyasu* technique) and conventional electron microscopy was successfully used to study pestivirus morphogenesis.
- Pestiviruses assemble at the ER and bud directly into the ER lumen.
- Pestiviruses utilize the cellular secretory pathway and are released as single particles by exocytosis.
- ET has revealed the nature of the „loosely arranged outer envelope membrane“: a detached envelope around a compact homogenously dark stained capsid

# Thank you for your attention



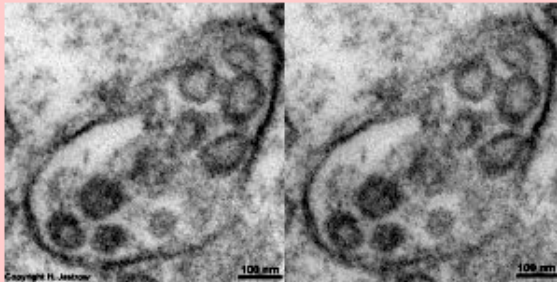
Matthias.koenig@vetmed.uni-giessen.de

# Membrane rearrangements after BVDV Infection

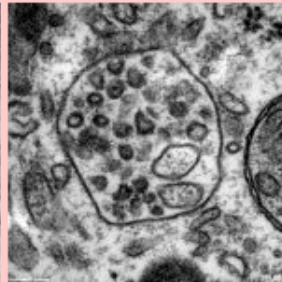


## Overview multivesicular body (MVB; Corpusculum multivesiculare):

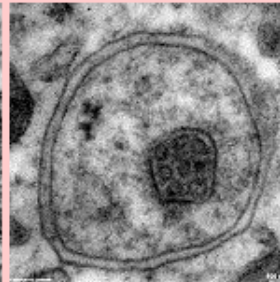
Pages with explanations are linked to the text below the images if available



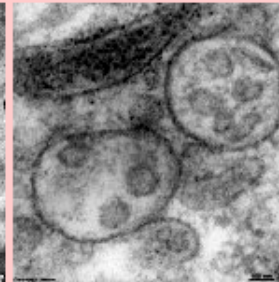
multivesicular bodies  
stereo image (hair-cell, rat)



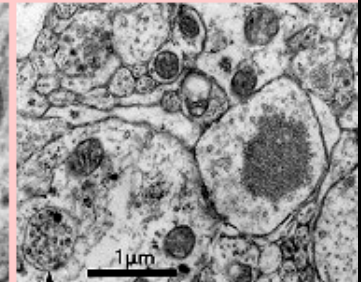
MVB of an  
inner hair cell (rat)



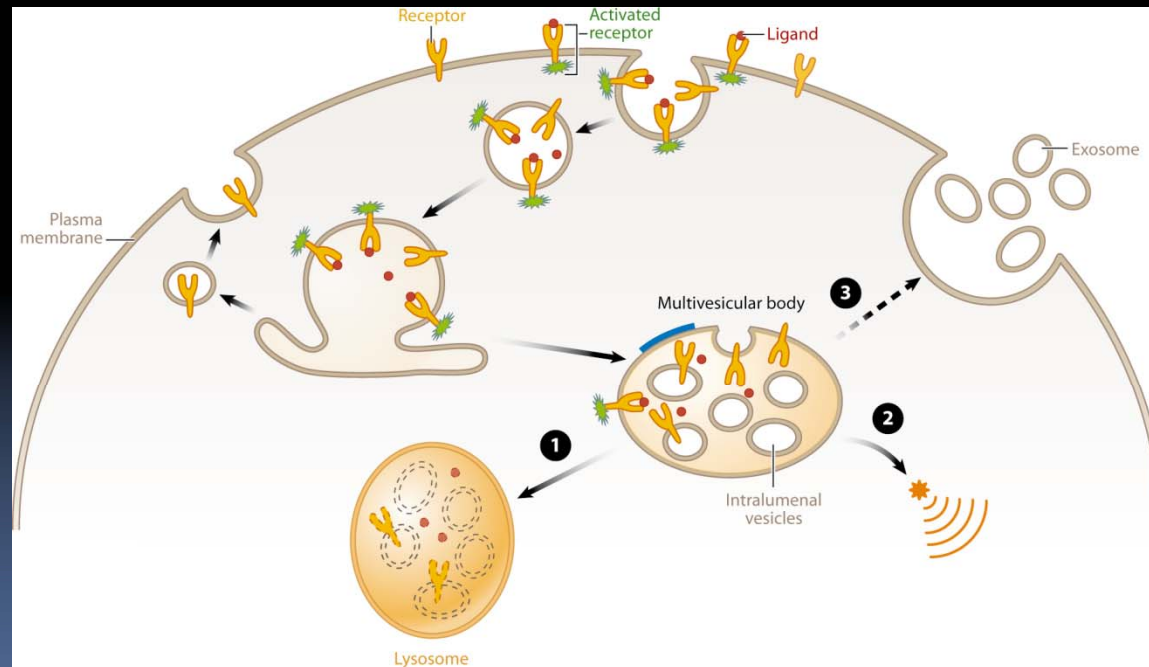
MVB with dark  
matrix (rat)




2 MVB of an  
inner hair cell (rat)



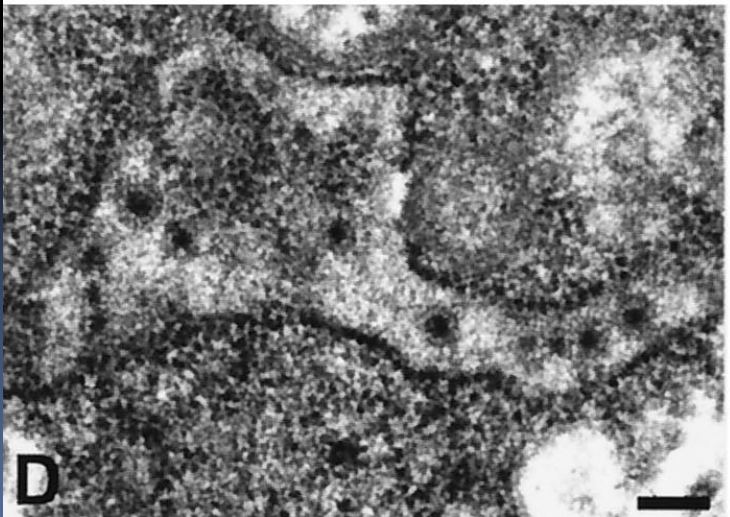
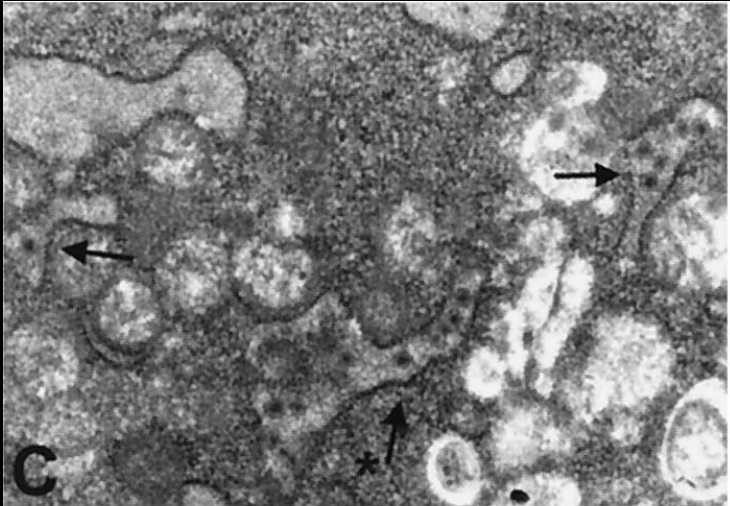
MVB in the cytoplasm  
of a glial cell (rat)



 Hanson PL, Cashikar A. 2012.  
Annu. Rev. Cell Dev. Biol. 28:337–62



BHK cells infected with recombinant  
VSV expressing HCV proteins



Ezelle et al., 2002

# HCV Morphogenesis

Non infected BHK cells



Blanchard et al., 2003