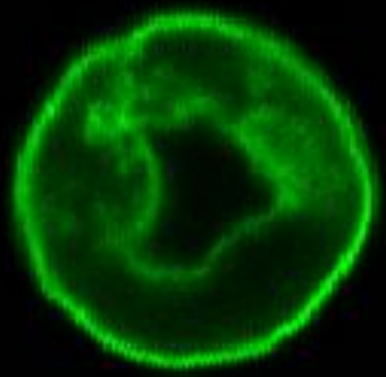
University of BRISTOL

wellcome trust

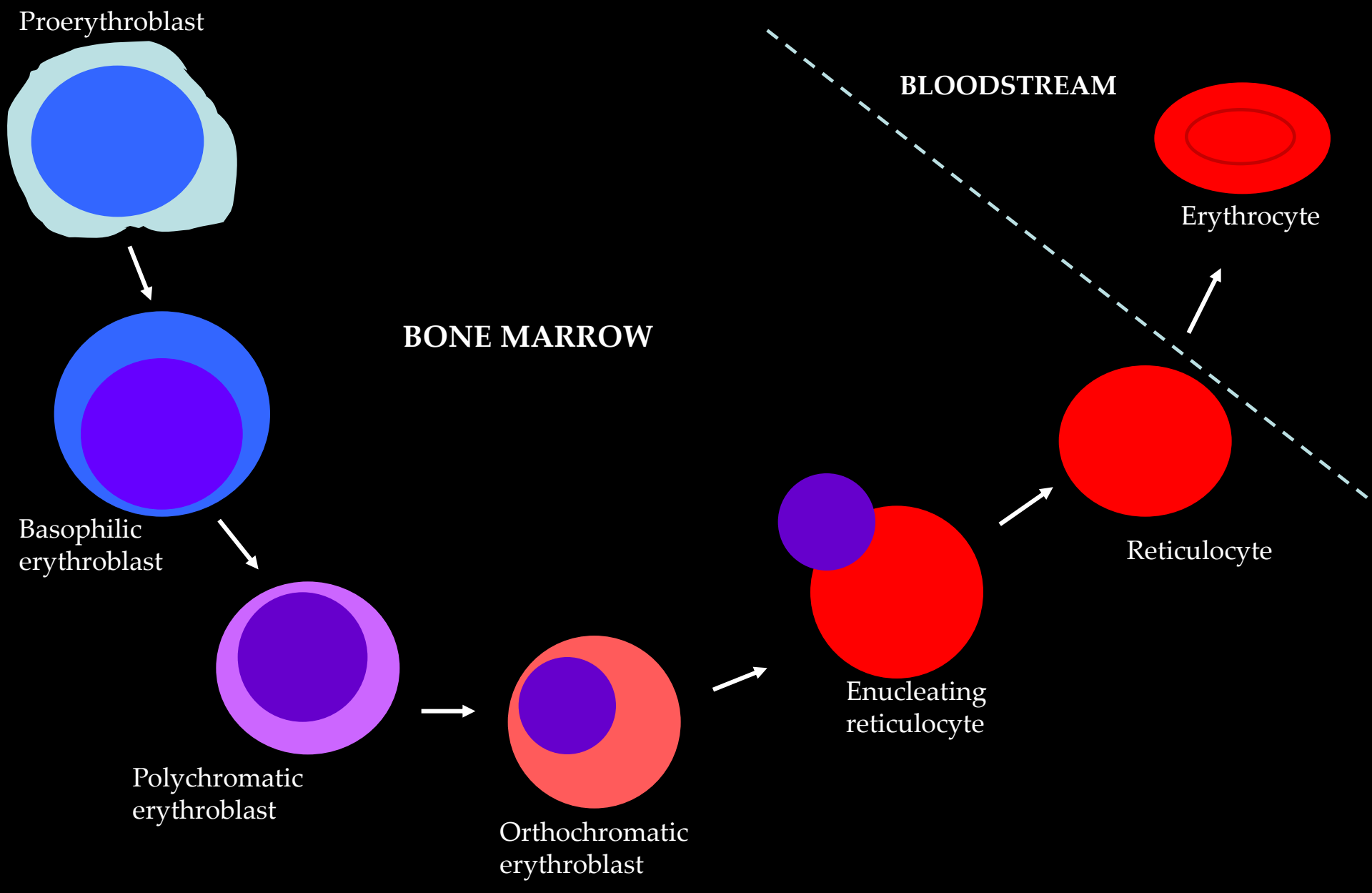
NHS Blood and Transplant

# From enucleation to mature erythrocyte:
## Imaging reticulocyte maturation in vitro



Rebecca Griffiths

# Erythropoiesis



# Erythroid cultures

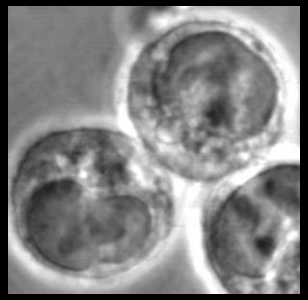
Adult peripheral blood CD34+ cells are isolated from waste blood products and cultured in a 3 stage medium.

Overall expansion of cell numbers is ≥ $10^4$ fold.

Enucleation rates are up to 95%.

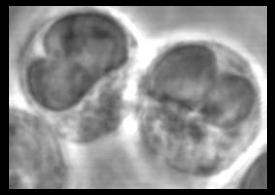
Leukocyte filtration at the end stage of the culture removes free nuclei, early R1 reticulocytes and normoblasts.

**Day 6**



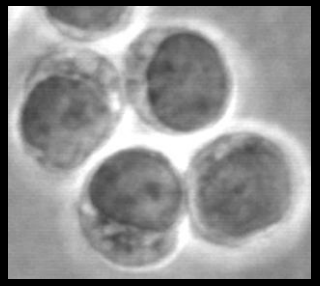
*Preproerythroblast*

**Day 8**



*Basophilic normoblast*

**Day 10**

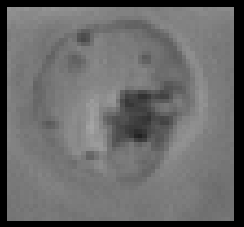


*Polychromatic normoblast*

**Day 13** →

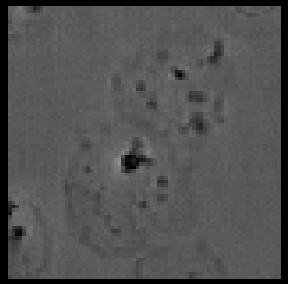
*Orthochromatic normoblast*

*Enucleating reticulocyte*



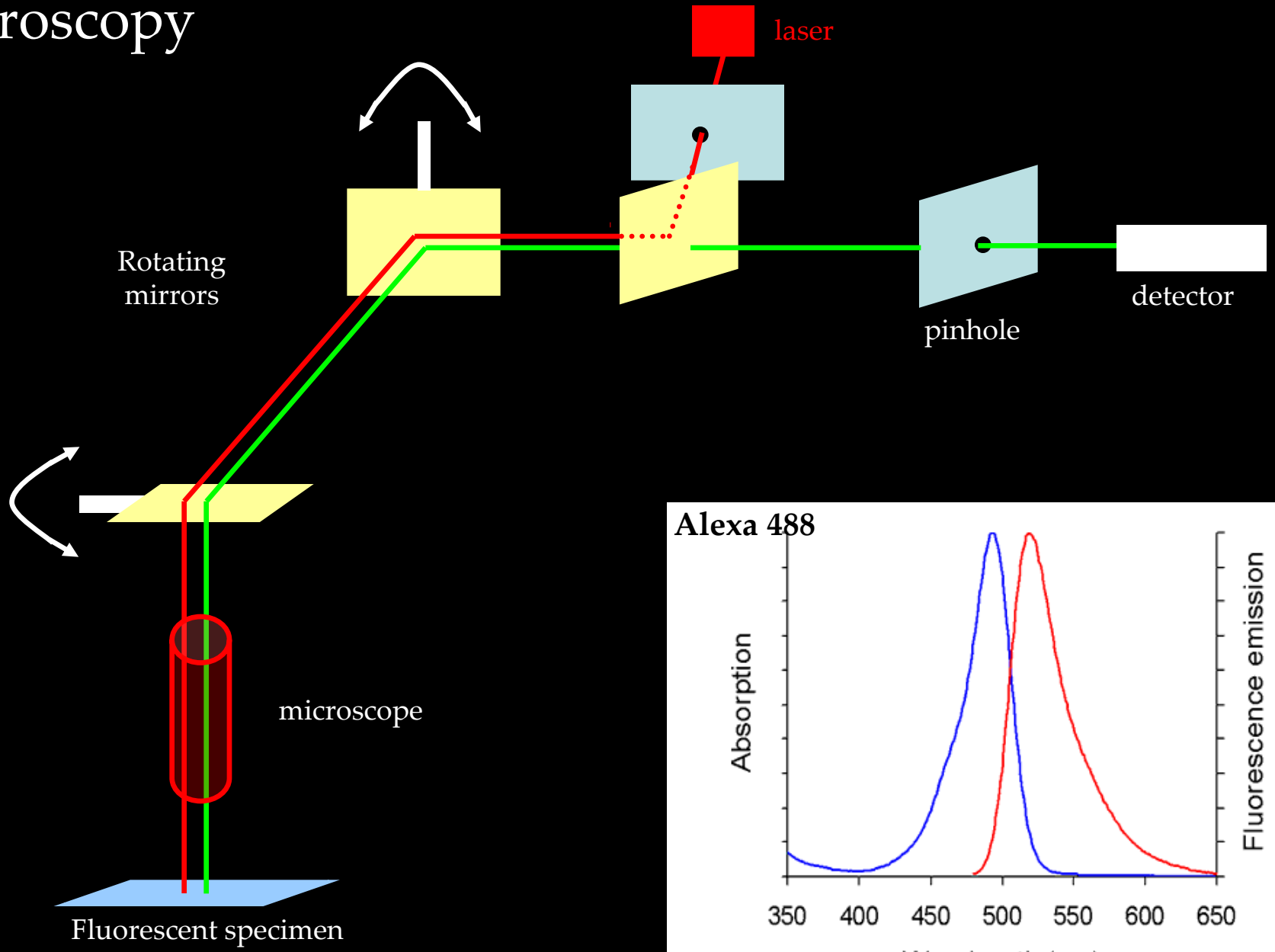
*R1 Reticulocyte*

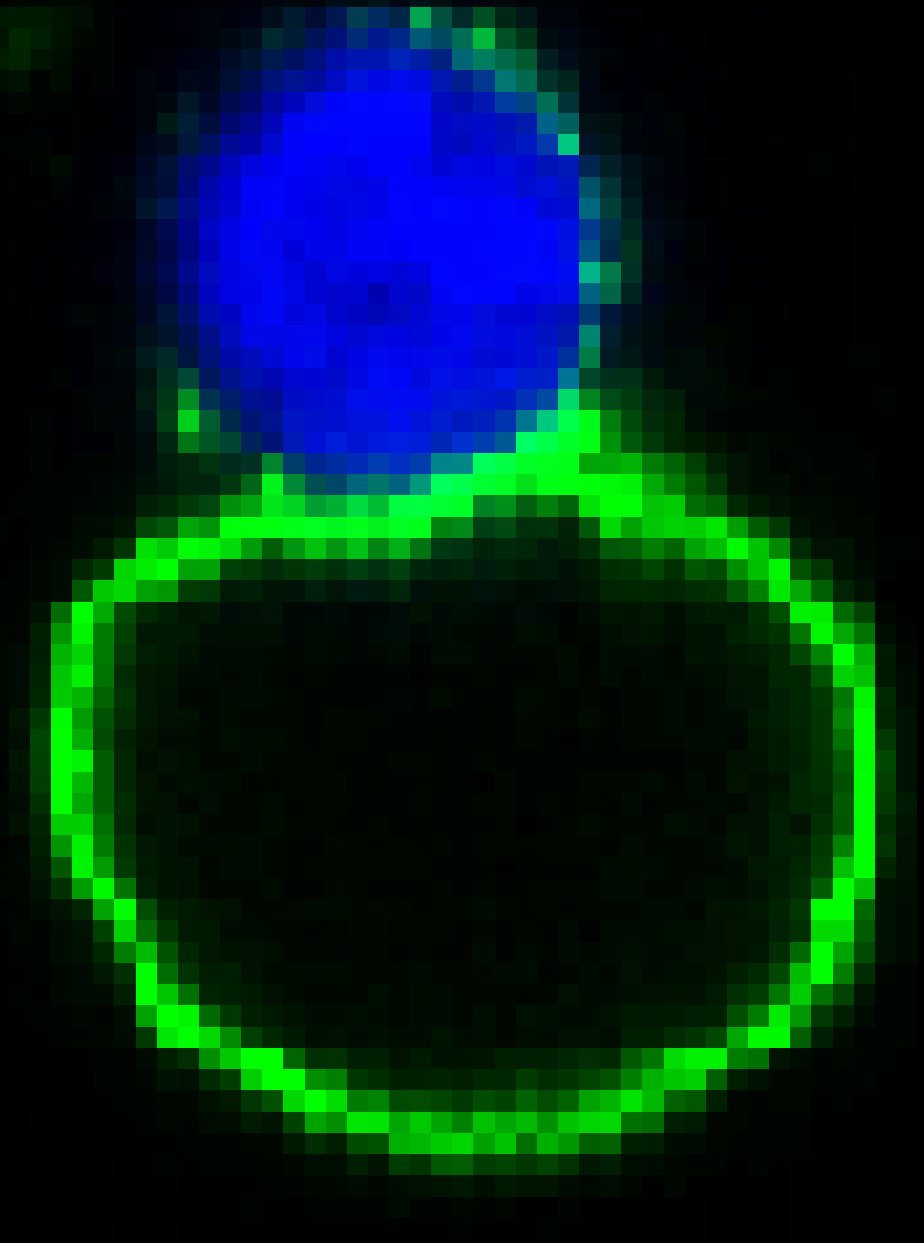
**Day 22**



*R2 Reticulocyte*

# Confocal microscopy





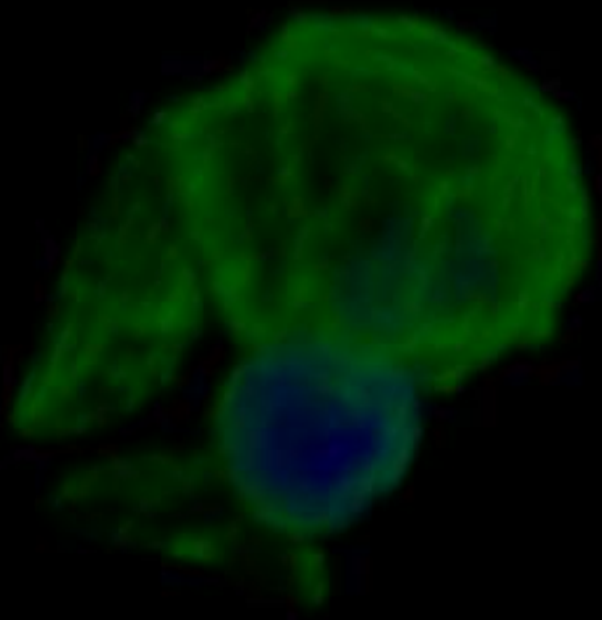
# Enucleation

Extrusion of the nucleus at terminal erythroid differentiation – a form of assymetric cytokinesis.

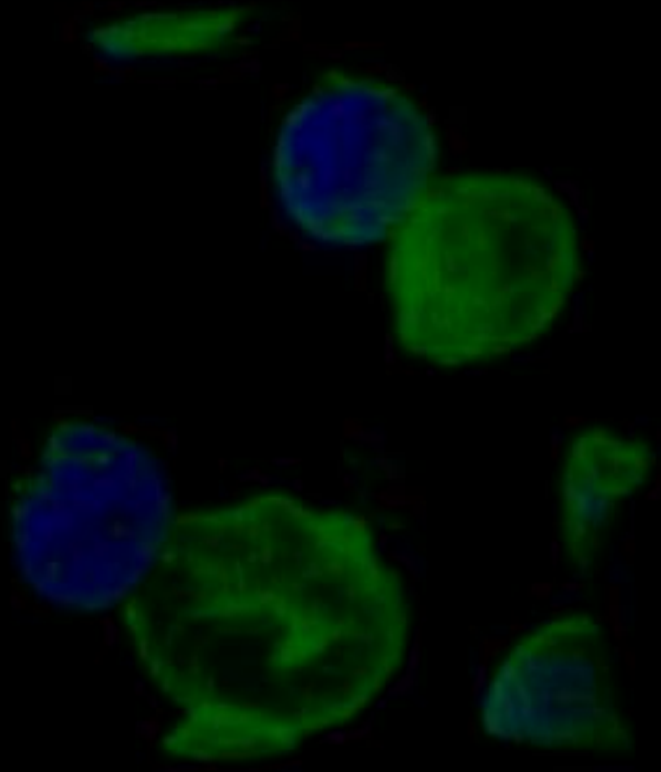
Dynamic state of reorganisation of the plasma membrane and cytoskeleton.

Molecular mechanisms of enucleation are unknown.

GPA &
DAPI



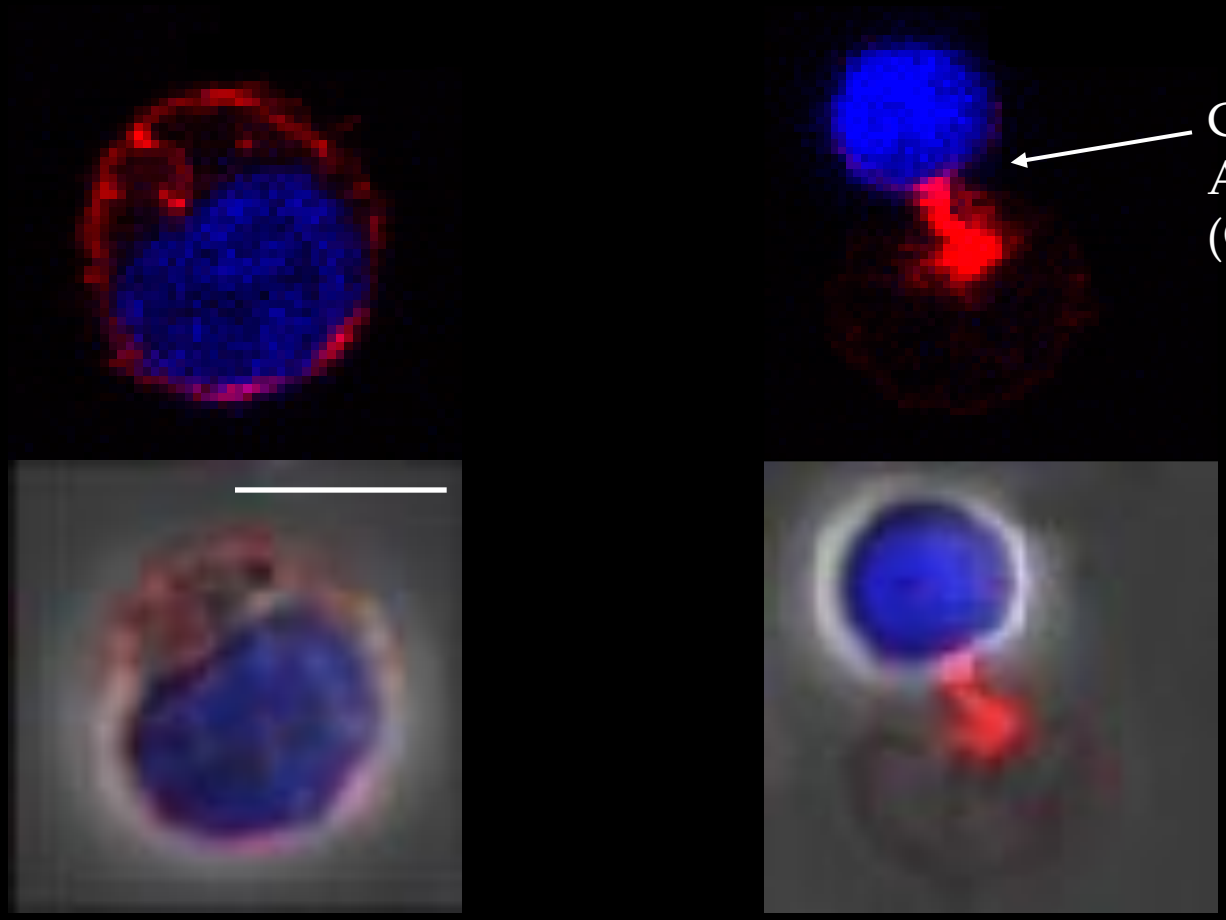
GPA &
DAPI



# Actin reorganisation at enucleation

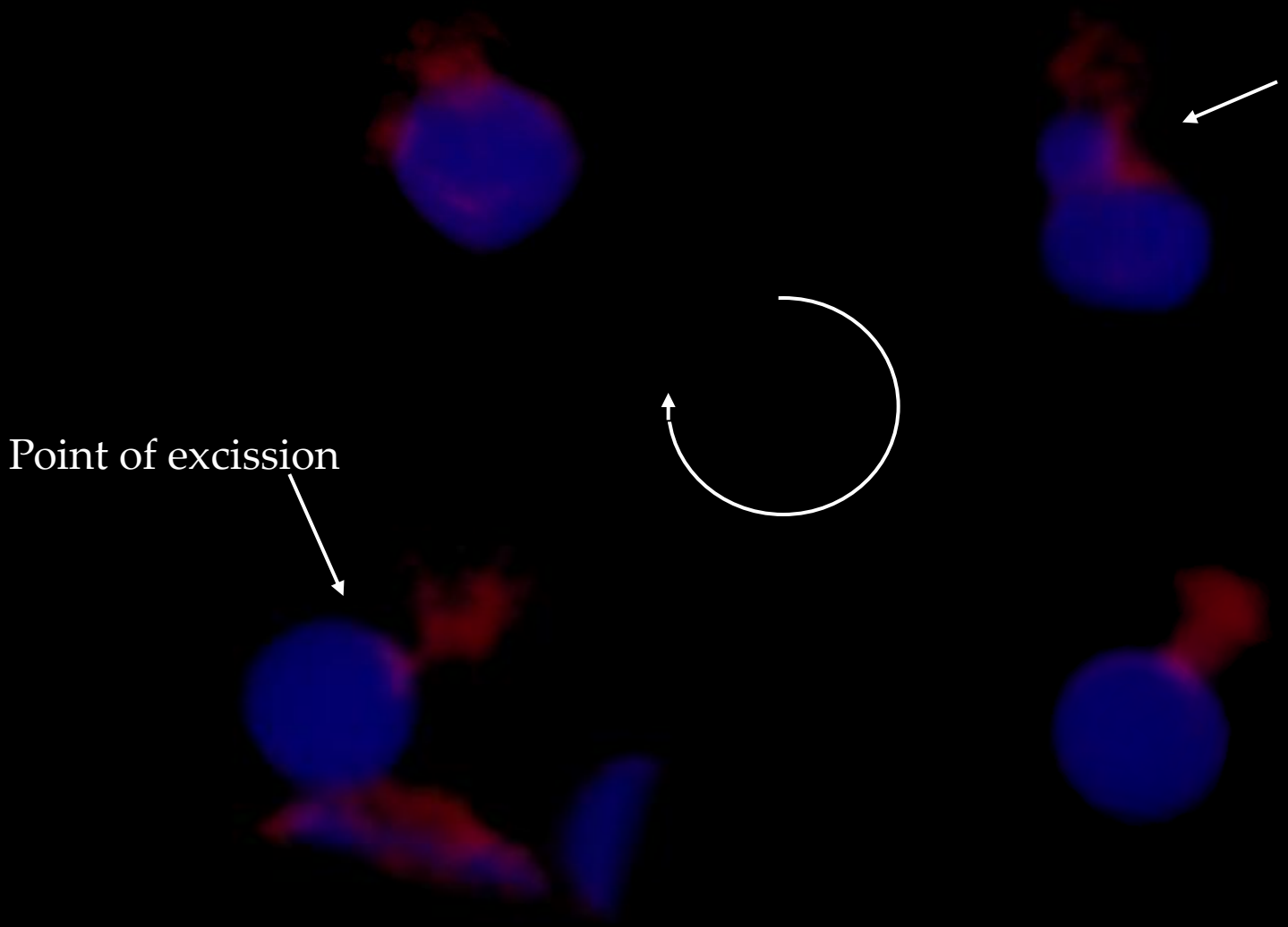
**erythroblast**

**enucleating erythroblast**



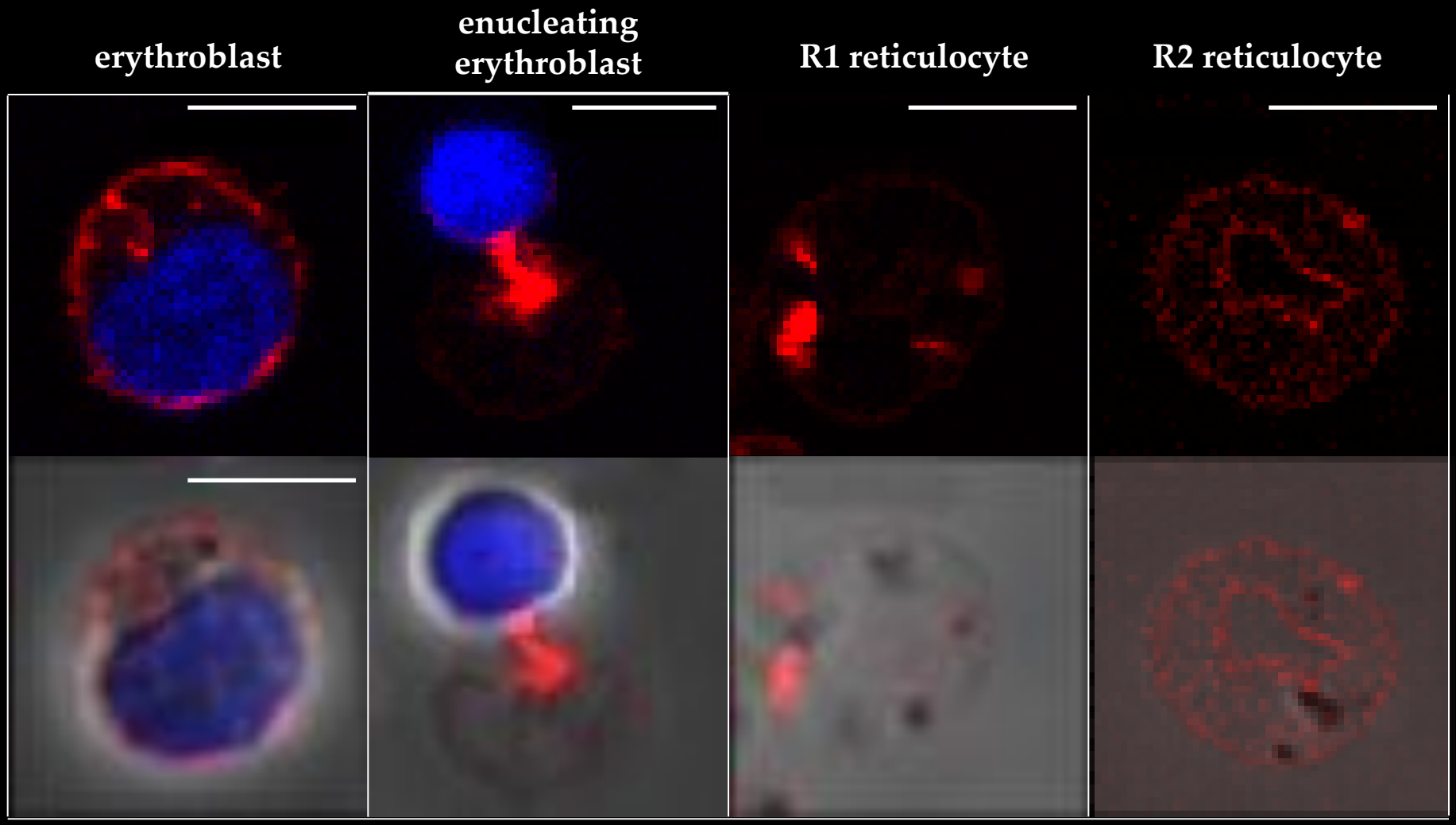

scale bars 5μm

# Actin reorganisation at enucleation – the contractile actin ring (CAR) process



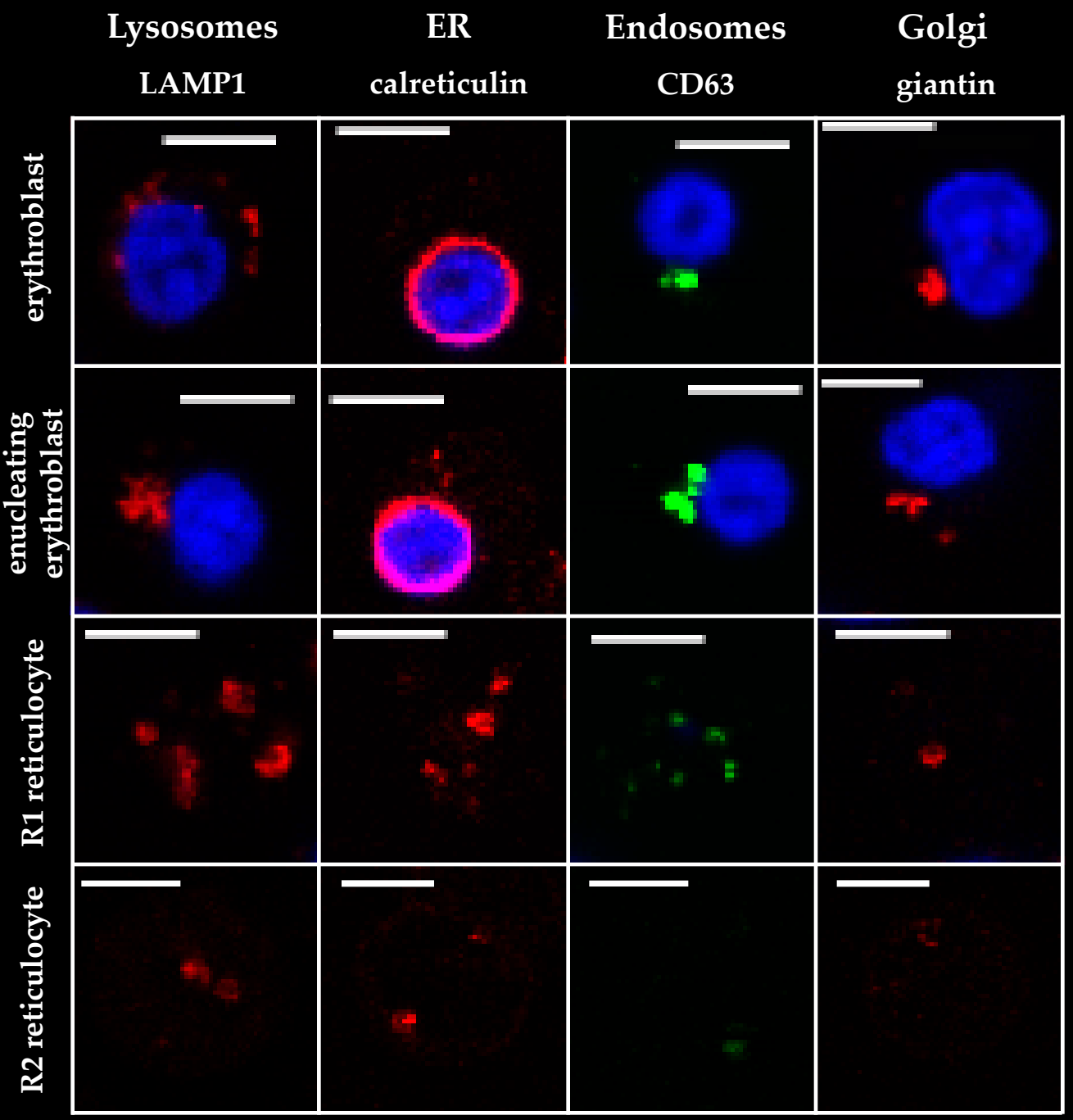
# The Contractile Actin Ring

# Actin staining in erythropoiesis



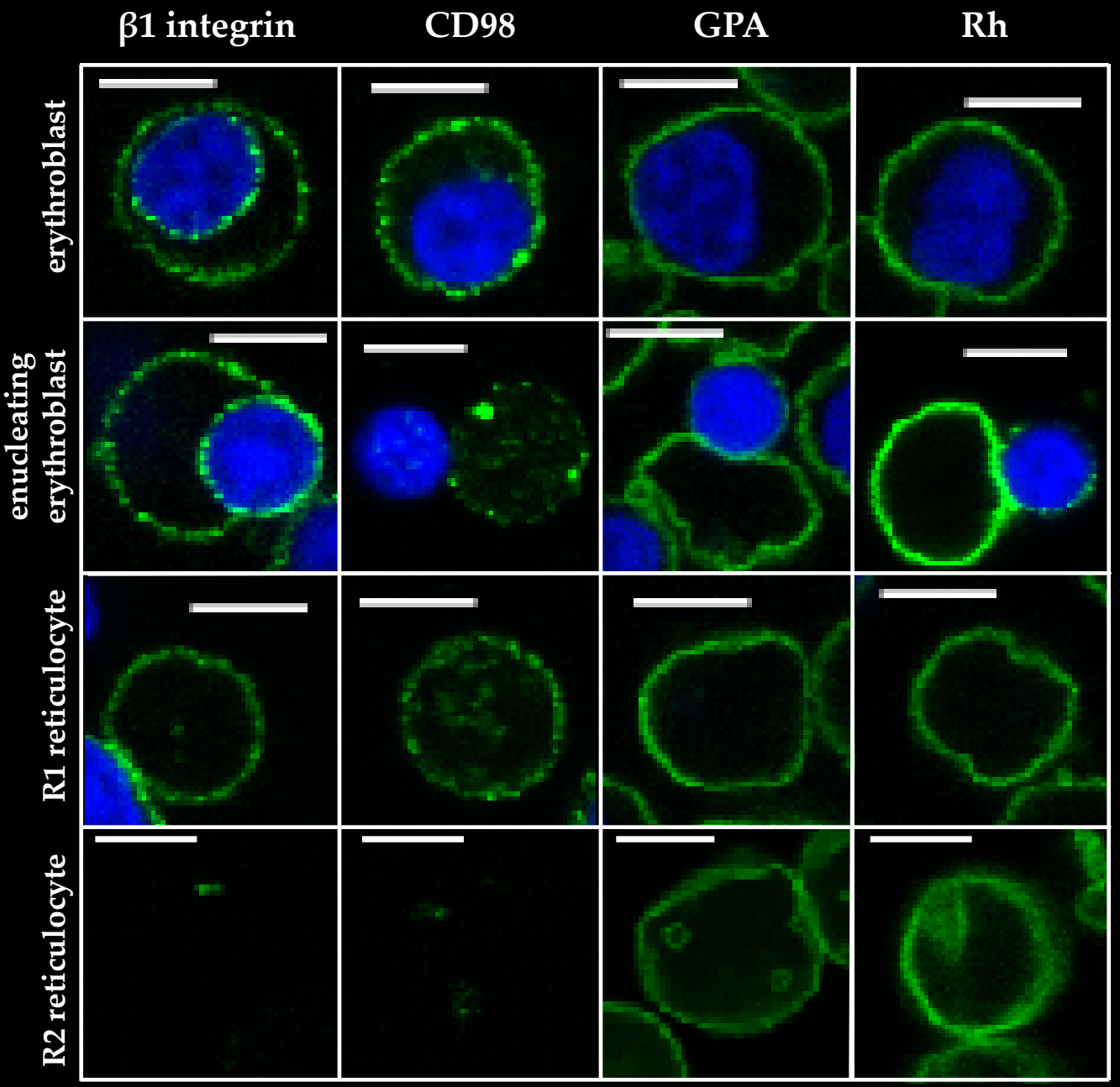

scale bars 5μm

# Organelle markers at enucleation




scale bars 5μm

# Membrane proteins at enucleation



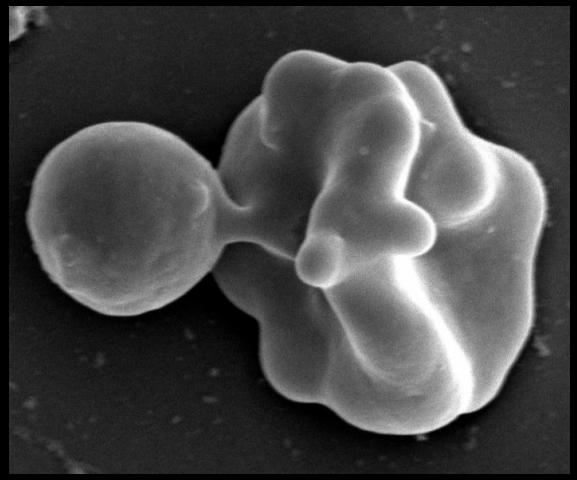

scale bars 5μm

reduction in surface area (20%) and volume

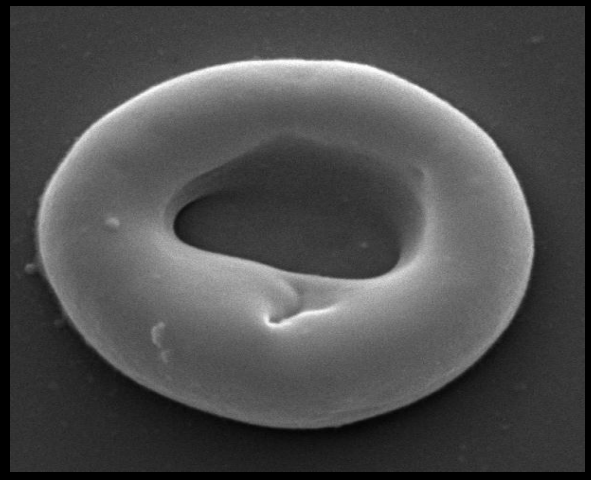
Reticulocyte → RBC

degradation / elimination of residual organelles

**R1**
**Motile and multi-lobular**



**R2**
**Non-motile**



Sabine Kupzig, NHSBT

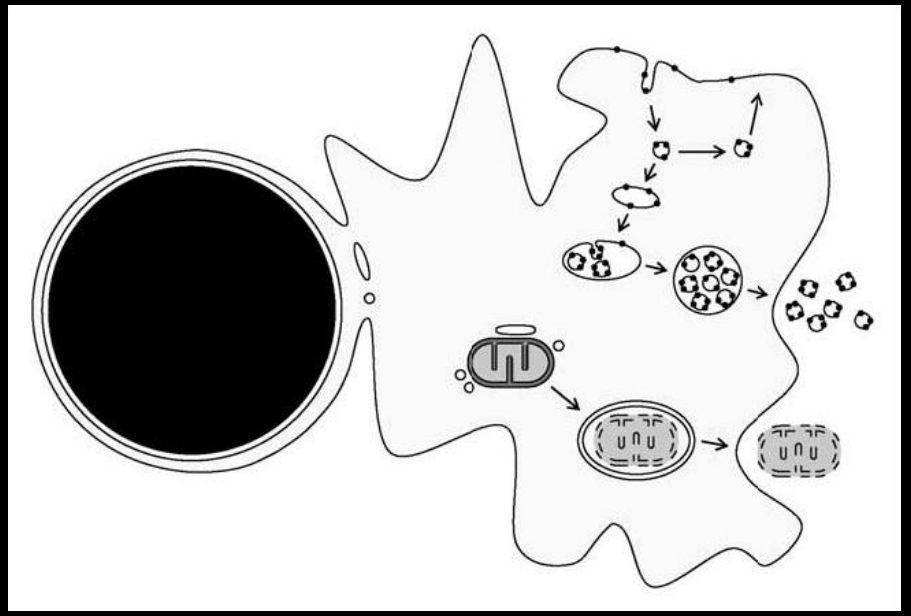
# Reticulocyte maturation

**<u>Prevailing view</u>**

**Two distinct mechanisms**

- Loss of plasma membrane surface area by the endosome-exosome pathway
- Degradation / elimination of residual organelles by autophagy

**Ney P 2011**
***Curr Opin Hematol***



We have identified a distinct, final stage of reticulocyte maturation .

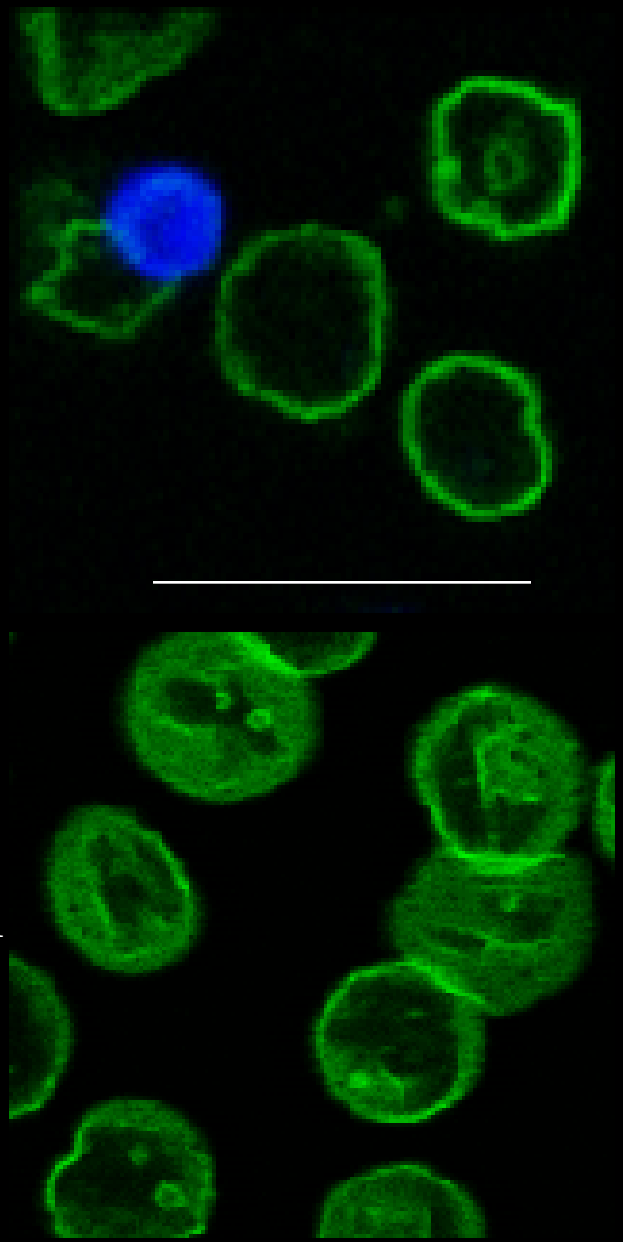
**Griffiths R *et al* 2012 *Blood***

# R2 reticulocytes exhibit large vesicles when stained with GPA

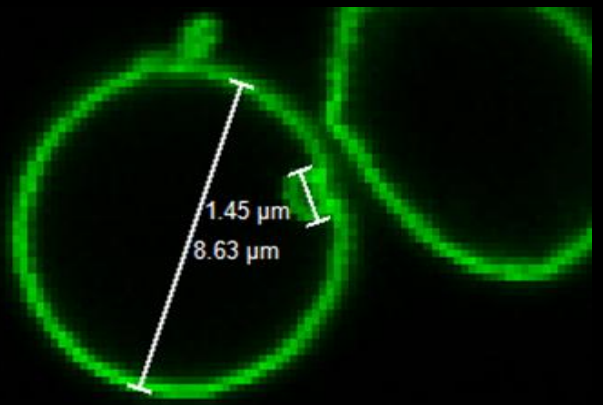
Pre-filtration

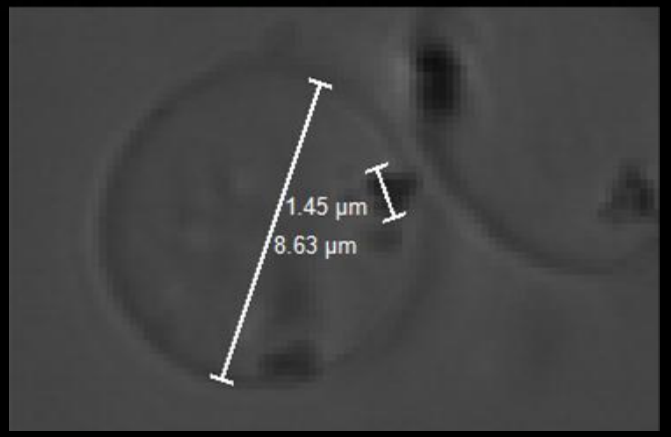
GPA and DAPI

Post-filtration

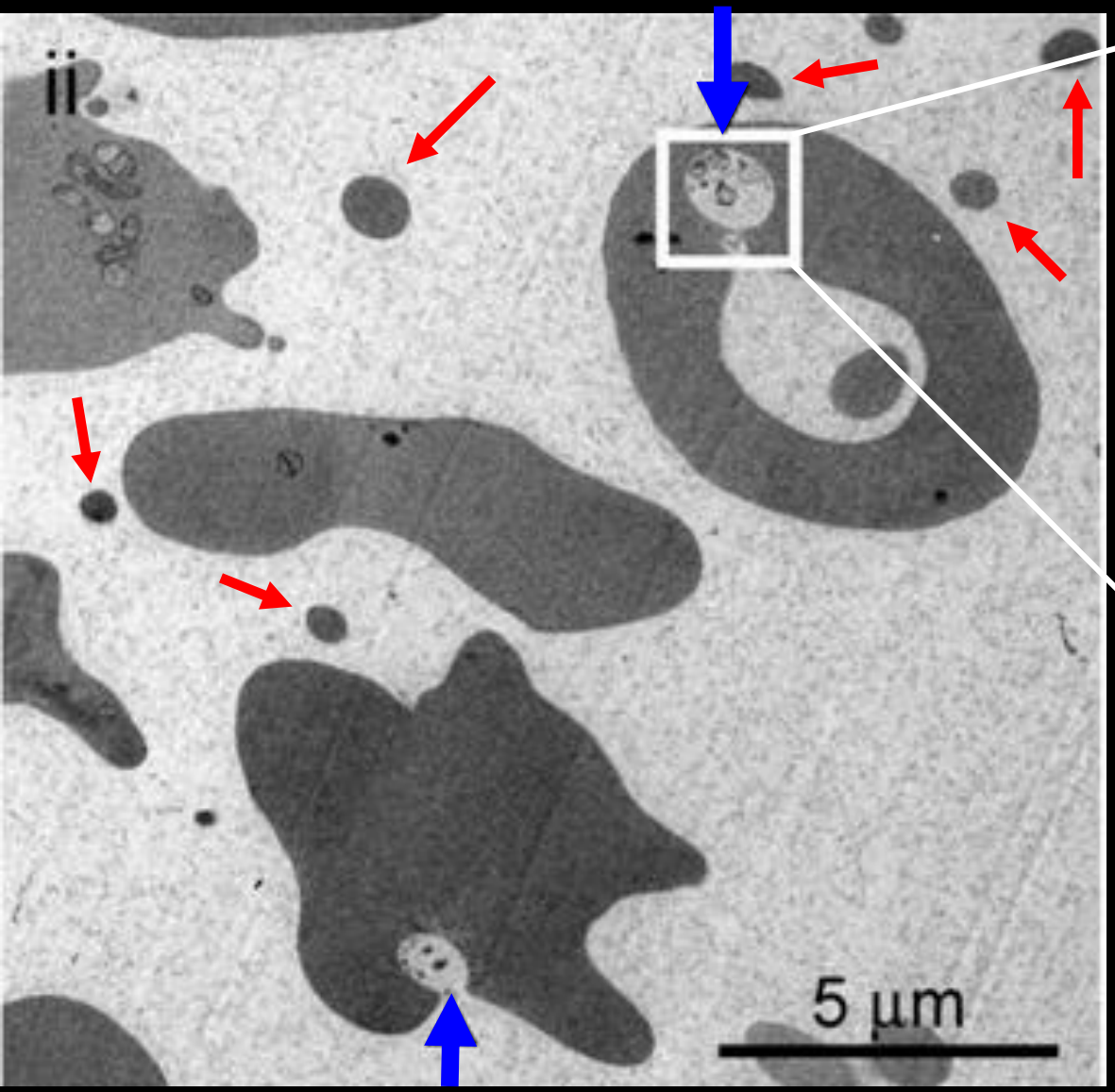


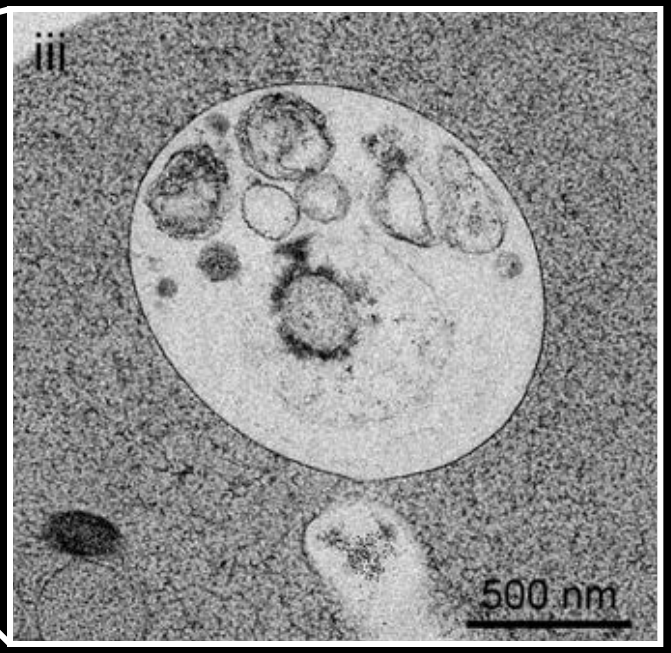
After filtration 64.55% $^{\pm 9.37\%}$ reticulocytes contain at least one GPA positive vesicle.



# Transmission Electron Microscopy of Filtered Reticulocytes



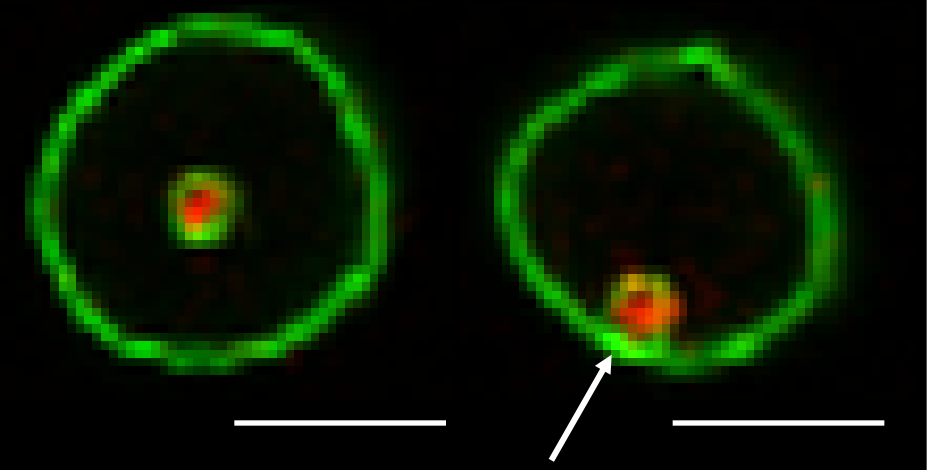




Autophagic vesicles (blue arrows)

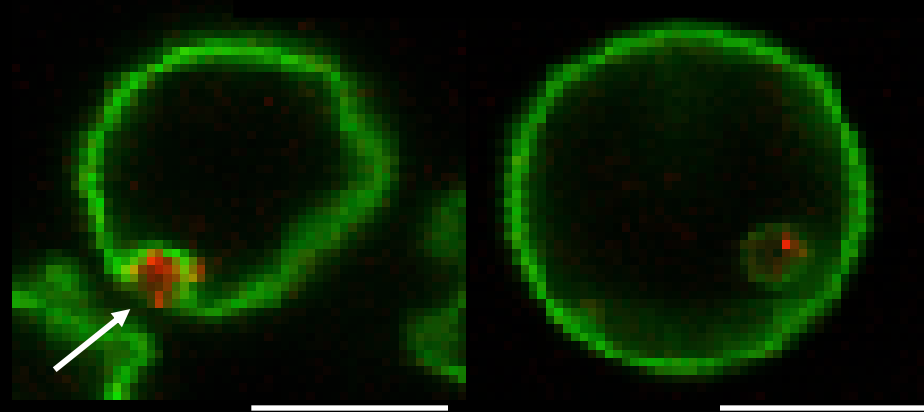
Cellular fragments (red arrows)

Virginie Betin, University of Bristol

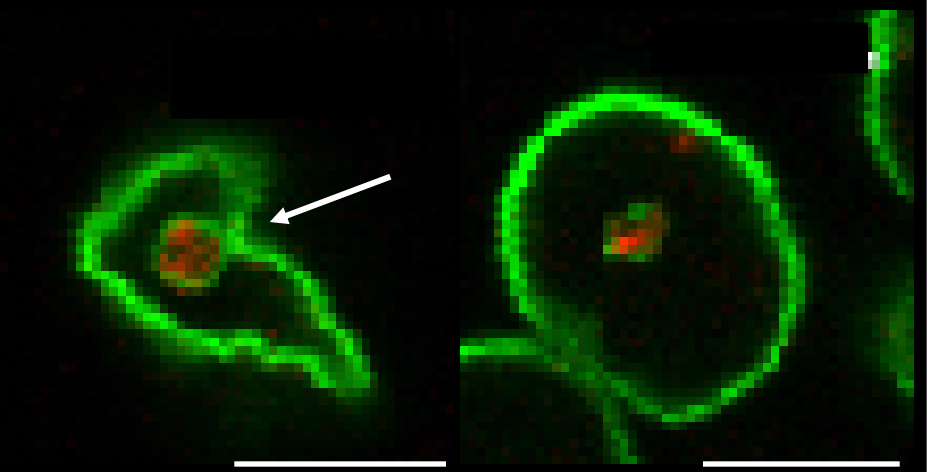
GPA & autophagosome marker LC3



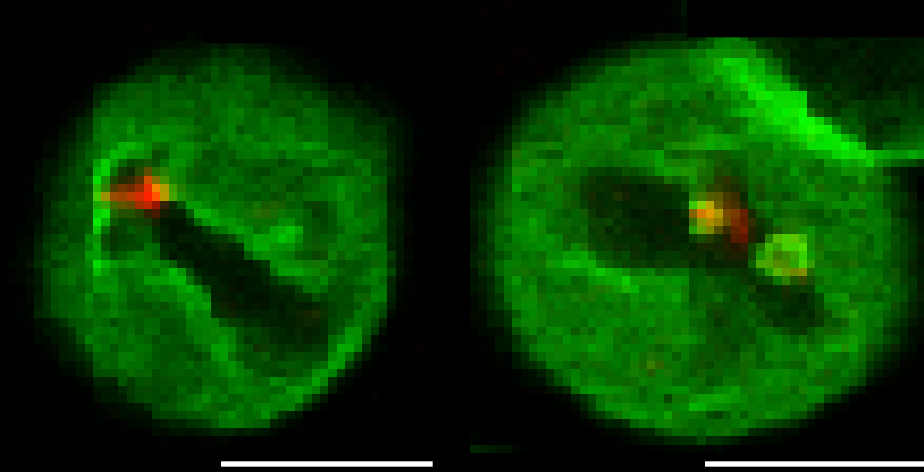
GPA & ER marker calreticulin



GPA & golgi marker giantin



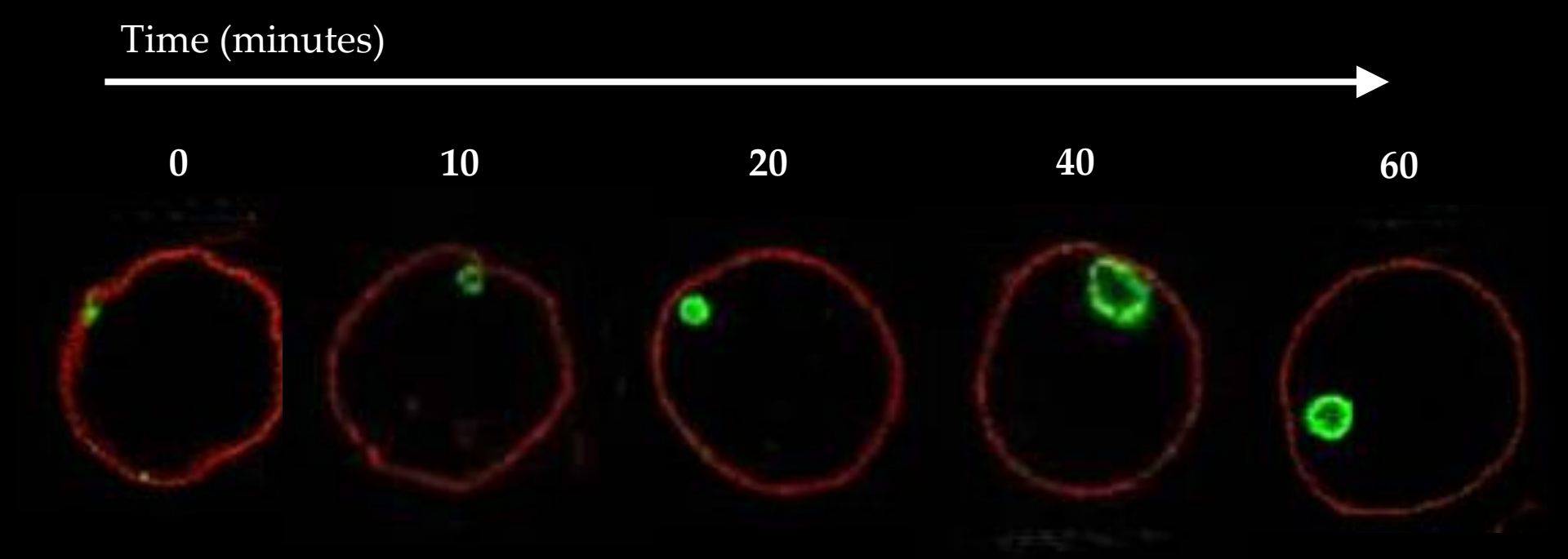
GPA & lysosome marker LAMP1



scale bars 5μm

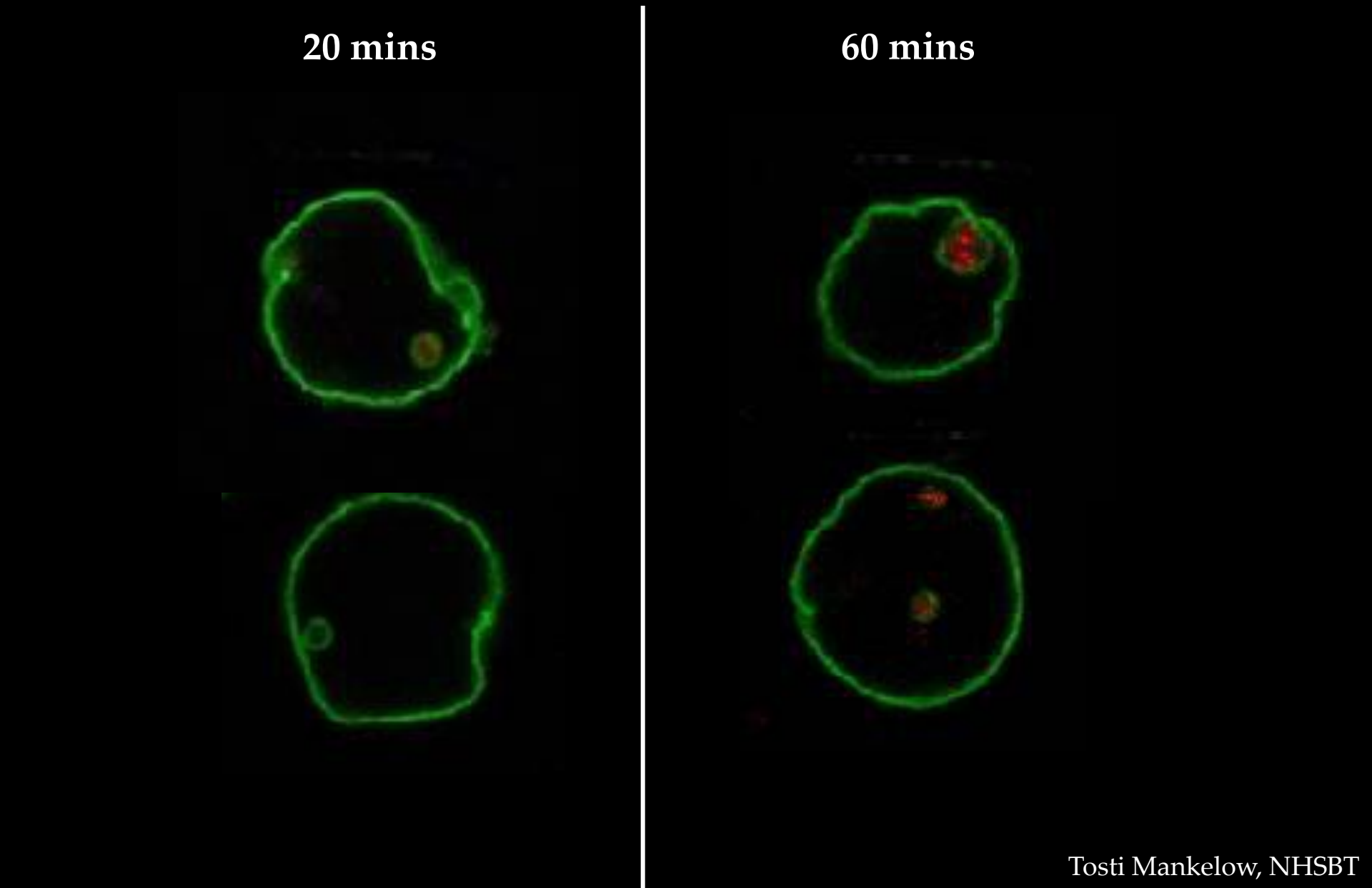
# GPA internalisation in Reticulocytes

Internal GPA
External GPA




Tosti Mankelow, NHSBT

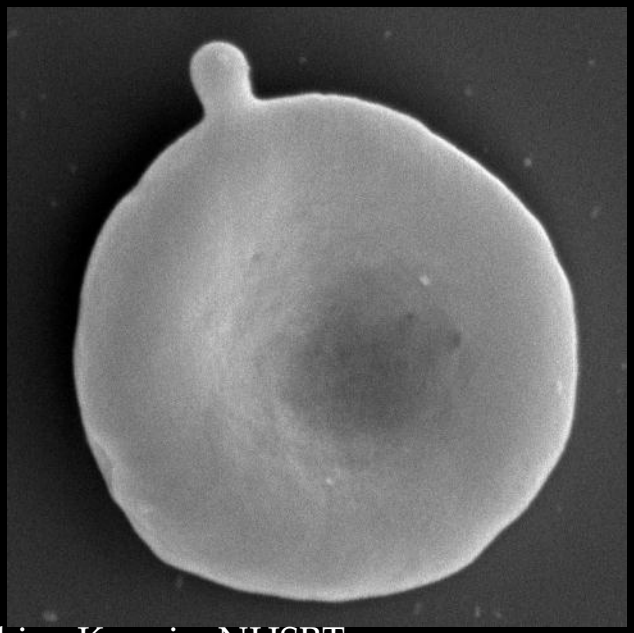
# Internalised GPA and autophagosome marker LC-3



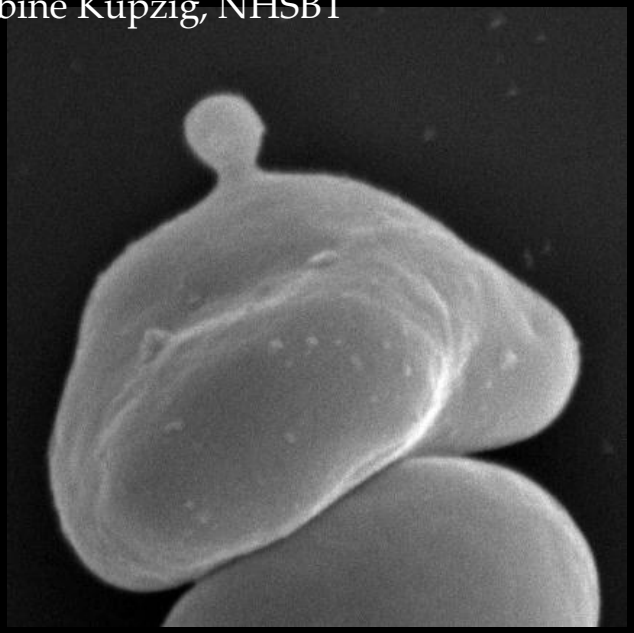

Tosti Mankelow, NHSBT

# Blebbing

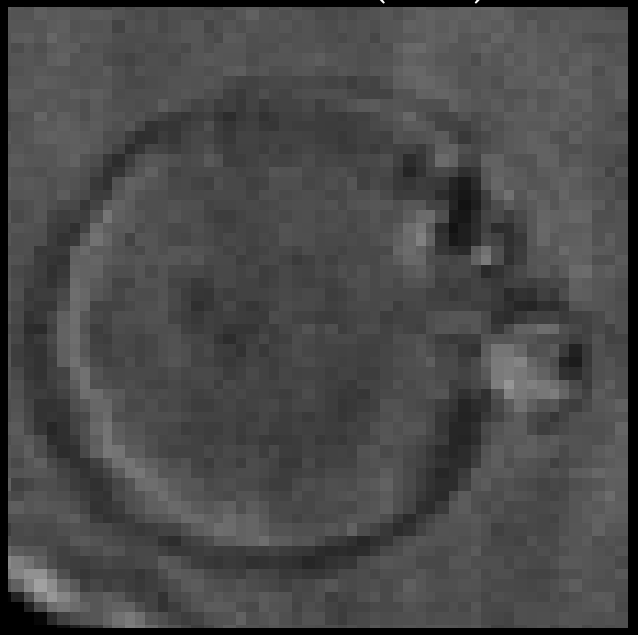
SEM

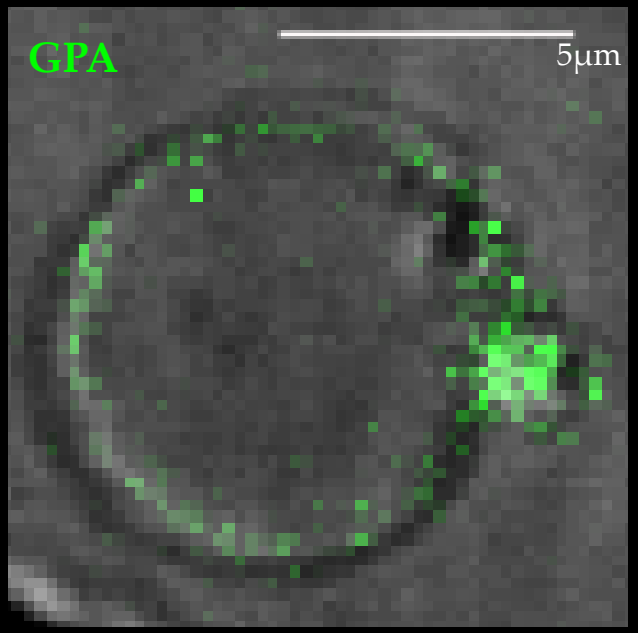


Sabine Kupzig, NHSBT



Confocal (live)





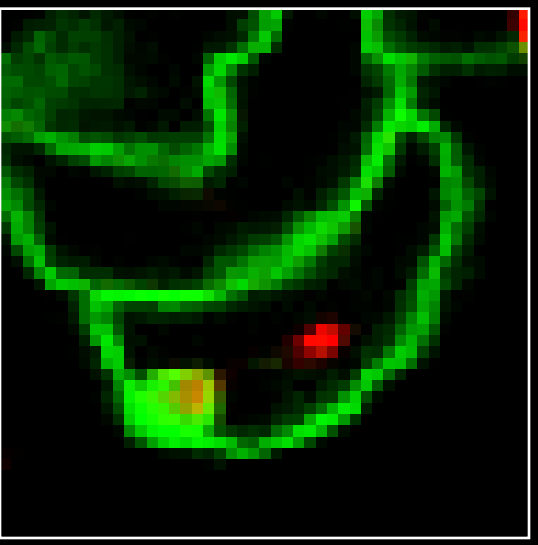
# Splenic maturation *in vivo*

Evidence that mature reticulocytes released the bone marrow circulate with autophagic vacuoles and that the final maturation step involving the removal of these vacuoles occurs in the spleen during the first 48-72 hours after release.

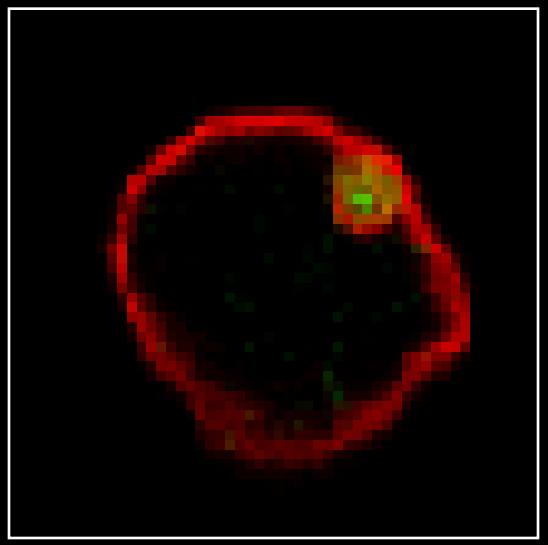
# *In vivo* evidence of GPA positive vesicles

- samples  from a splenectomised patient

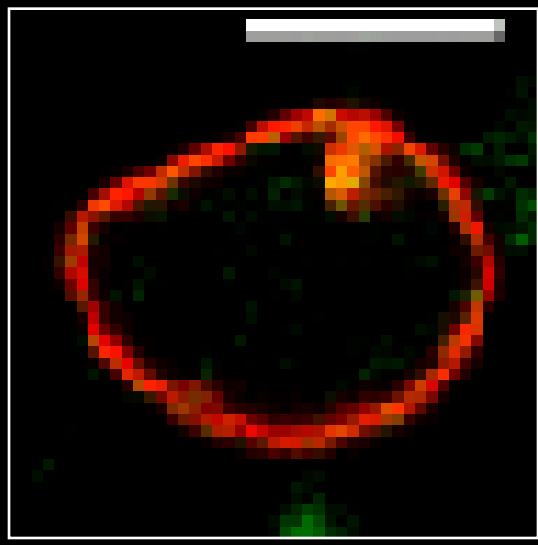
GPA and mitochondrial marker, Mitotracker™



GPA and Golgi marker, giantin



GPA and autophagosome marker, LC3



# Conclusions

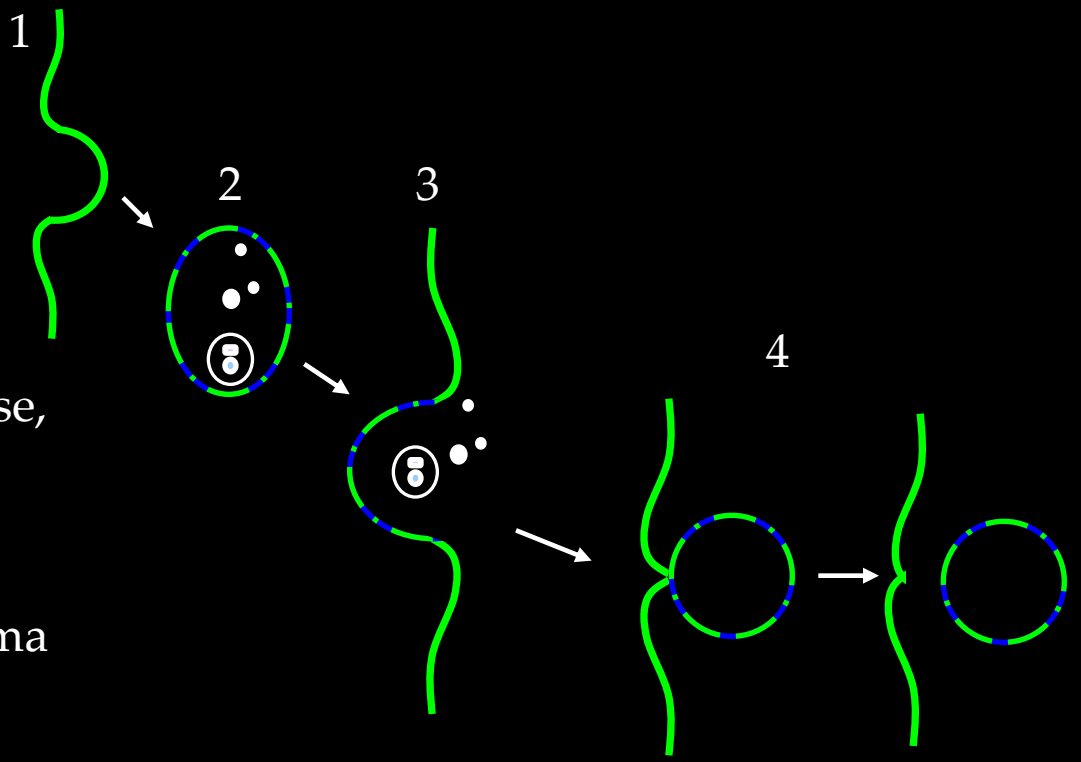
We have identified a distinct, final stage of reticulocyte maturation

1. Large GPA positive vesicles endocytose

2. and fuse with autophagosomes

3. these then fuse with the plasma membrane and the contents exocytose, eliminating unwanted organelles.

4. the membrane then blebs and is released resulting in the loss of plasma membrane surface area.




This is distinct from the exosomal release of plasma membrane proteins (β1 integrin, CD98 and tetraspanins) seen in earlier R1 reticulocytes.

Our data suggest that reticulocyte maturation occurs in two stages.

**Griffiths *et al.* 2012 Blood**

# Thanks

**NHSBT, Bristol**

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Edwin Massey

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Virginie Betin

Jon Lane