

Review

A Comprehensive Review on Eryptosis

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Key Words

Erythrocytes • Eryptosis • Membrane changes • Flow cytometry • Confocal microscopy • Scanning electron microscopy

Abstract

Erythrocytes (RBCs) are extremely sensitive cells, and although they do not have nuclei and mitochondria, are important health indicators. This is particularly true because, during inflammation, whether it is systemic or chronic, the haematological system is constantly exposed to circulating inflammatory mediators. RBCs have a highly specialized and organized membrane structure, which interacts and reacts to inflammatory molecule insults, and undergo programmed cell death, similar to apoptosis, known as eryptosis. Over the past years, eryptosis studies have focussed on determining if membrane changes have occurred, particularly whether a phosphatidylserine (PS) flip, Ca^{2+} leakage into the cell, changes to ceramide and cell shrinkage have occurred. Mostly, flow cytometry is used, but confocal microscopy and ultrastructural studies also confirm eryptosis. Here, we provide a comprehensive overview of eryptosis, where we revisit the biochemical process of the process, review all literature in PUBMED, that is shown under the search word, "eryptosis", and also discuss current methodologies to determine the presence of eryptosis; included in the discussion of the methodologies, we discuss a pitfalls section for each method. This paper is therefore a comprehensive synopsis of current knowledge of eryptosis and discusses how RBCs may provide an essential *in vivo* cell model system to study not only inflammation in disease, but also track disease progression and treatment regimes.

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Introduction

Erythrocytes (RBCs) are extremely sensitive cells, and although they do not have nuclei and mitochondria, form an important component of the human body as health indicator. This is particularly true because, during inflammation, whether it is systemic or chronic the haematological system is constantly exposed to circulating inflammatory mediators. RBCs have a highly specialized and organized membrane structure, which interacts and reacts to inflammatory molecule insults. Although it does not have a nucleus or mitochondria, it undergoes programmed cell death, similar to apoptosis, and this is called eryptosis. The basis for eryptosis is discussed in various publications by the Lang group e.g. [1-9], and it is known

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to contribute to conditions like anaemia, metabolic syndrome, diabetes, malignancy, hepatic failure, heart failure, uraemia, hemolytic uremic syndrome, sepsis, fever, dehydration, mycoplasma infection, malaria, iron deficiency, sickle cell anaemia, thalassemia, glucose-6-phosphate dehydrogenase deficiency and Wilson's disease [10].

In this paper, we provide a comprehensive overview of eryptosis, revisit the biochemical process of eryptosis, review all literature in PUBMED that is shown under the search word, "eryptosis" and also discuss current methodologies to determine the presence of eryptosis. We conclude by providing a comprehensive synopsis of current knowledge of eryptosis and discuss how RBCs may provide an essential *in vivo* cell model system to study not only inflammation in disease, but also track disease progression and treatment regimes. The next paragraphs will review the structure of the RBC membrane, and how it is changed during eryptosis.

The RBC membrane

The mature RBC consists of a complex plasma membrane, containing a specialized lipid bilayer that interacts through protein interactions with integral membrane proteins. The membrane lipids that form the double-layered surface of all cells are categorized as phospholipids, glycolipids, and cholesterol. Cholesterol is commonly spread equally throughout the 2 leaflets, while the 4 major phospholipids are asymmetrically arranged. The preservation of this asymmetric arrangement of phospholipids, especially the localization of PS and phosphoinositides to the inner leaflet, plays a major role on the integrity of the RBC. Disruption of this lipid asymmetry leads to displaying of PS on the outer leaflet and its externalization has been suggested to play a major role in premature destruction of RBCs [11]. For a detailed review see [12]. The cytosol contains highly enriched haemoglobin, and molecules like cytosolic Ca^{2+} . The phospholipid bilayer facilitates enzymatic reactions, is involved in membrane transport and signal transduction pathways and these are all involved in maintaining of lipid homeostasis. There are hundreds of different phospholipid molecular species that differ with regards to their polar, hydrophilic heads and non-polar hydrophobic hydrocarbon tails. Glycerophospholipids have a glycerol backbone, while sphingomyelin has a sphingosine backbone.

Lipid species and proteins aggregate in domains in the membrane, called rafts that form specialized areas that act together and are involved in individual physiologic processes such as signal transduction. Membrane cholesterol is unesterified and lies between the two layers of the lipid bilayer [13, 14]. Lipid rafts enriched in cholesterol and sphingolipids are also in association with specific membrane proteins that include flotillins, stomatin, G-proteins, and β -adrenegic receptors [15].

Phospholipids are asymmetrically distributed across the lipid bilayer with the amino phospholipids, phosphatidylserine and phosphatidylethanolamine (PS and PE) on the inside and choline containing phospholipids, phosphatidylcholine and sphingomyelin (PC and SM) on the outer leaflet. SM is the most abundant sphingolipid, constituting a class of structural lipids with ceramide as the hydrophobic backbone [13, 16-22]. See Fig. 1 for an overview of the RBC membrane (adapted from [14, 23]).

Three integral types of proteins facilitate the transmembrane passage, as well as the structural arrangement of lipids in the RBC membrane, and they are flippases, floppases and scramblases. Floppase controls the reverse transfer of choline-containing phospholipids, PS and SL and cholesterol (against concentration gradients) from the inner leaflet to the outer leaflet. Flippase (aminophospholipidtranslocase) pumps the amino-containing phospholipids from the outer to the inner leaflet [24]. Scramblase initiates non-specific bidirectional transport of phospholipids down their concentration gradients in an energy independent manner across the RBC membrane. The activation of the phospholipid scramblase is known to be involved in loss and disruption of the asymmetry of the membrane phospholipids that is essential for maintaining lipid homeostasis in RBCs [14, 25].

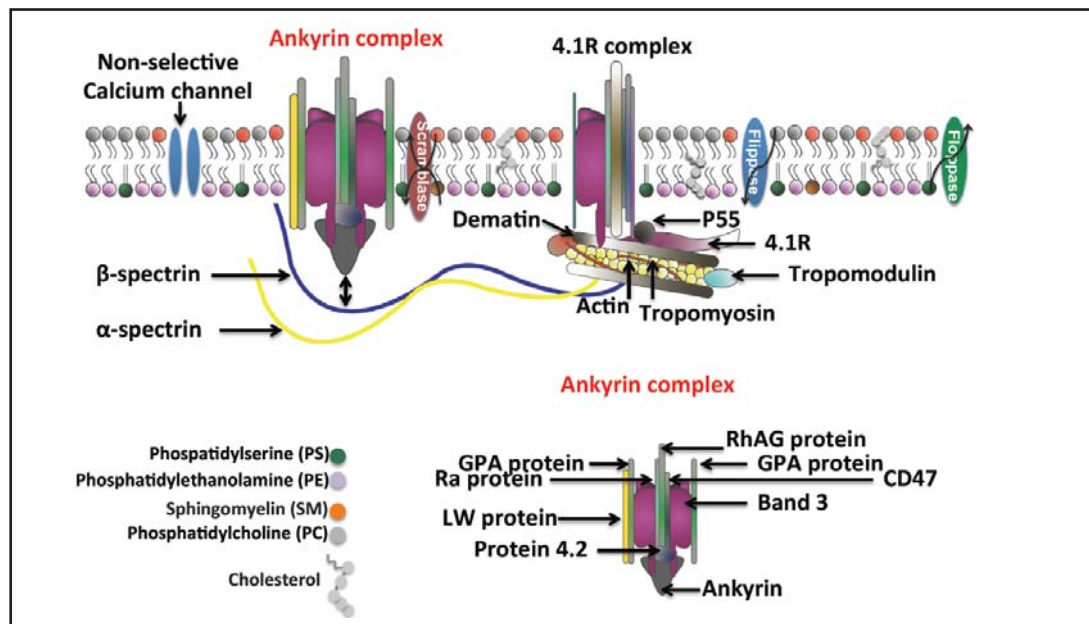


Fig. 1. RBC membrane structure.

The magnesium ATP dependent flippase, which transfers PS and PE from outer to inner monolayer at the expense of ATP, maintains the correct distribution of the phospholipid layer. Loss of phospholipid asymmetry and the exposure of PS are triggered by a phospholipid scrambling activity. PS is usually confined to the inner leaflet of the RBC and its externalization requires both inhibition of the flippase and activation of the scramblase and both may follow from elevation of intracellular Ca^{2+} [26]. Energy-independent flippases therefore allow common phospholipids to equilibrate rapidly between the two monolayers and also play a role in the biosynthesis of a variety of glycoconjugates such as glycosphingolipids, N-glycoproteins, and glycosylphosphatidylinositol (GPI)-anchored proteins [27]. Scramblase facilitates the flip-flop of lipids in a non-selective fashion from the inner, as well as the outer leaflet [28] – this process is a bidirectional process, down a concentration gradient. In the presence of calcium, scramblase behaves like a channel for lipids, allowing them to diffuse from one monolayer to the other, according solely to the concentration gradient. Structural proteins like Band 3 regulates the structure and function of the RBCs, as it facilitates anion transport via the RBC membrane and it is an important binding site for cytoskeletal and other RBCs proteins [29] [30] [31, 32]. Band 3 is a multi-spanning ion transport channel or trans-membrane protein, and the tetramers tether the bilayer to the skeleton via an interaction between its cytoplasmic domain and ankyrin, which is associated with spectrin (see [12] for a detailed discussion).

What happens in the RBC membrane during eryptosis?

Eryptosis is characterized by cell shrinkage and cell membrane scrambling and is stimulated by calcium entry through Ca^{2+} -permeable, PGE_2 -activated cation channels, by ceramide, caspases, calpain, complement, hyperosmotic shock, energy depletion, oxidative stress, and deranged activity of several kinases (e.g. AMPK, GK, PAK2, CK1 α , JAK3, PKC, p38-MAPK) [33-39]. This process happens as follows: hyperosmotic shock, energy depletion, or removal of extracellular Cl^- activates the non-selective Ca^{2+} -permeable cation channels in the membrane. This causes Ca^{2+} to enter and leak into the cell followed by the PS-flip to expose PS at the erythrocyte surface [40]. Also subnanomolar concentrations of prostaglandin E_2 (PGE_2) lead to activation of erythrocyte cation channels and influx of Ca^{2+} , calpain activation, and ankyrin-R degradation [40]. PGE_2 is produced from membrane phospholipids by the sequential action of Ca^{2+} -independent

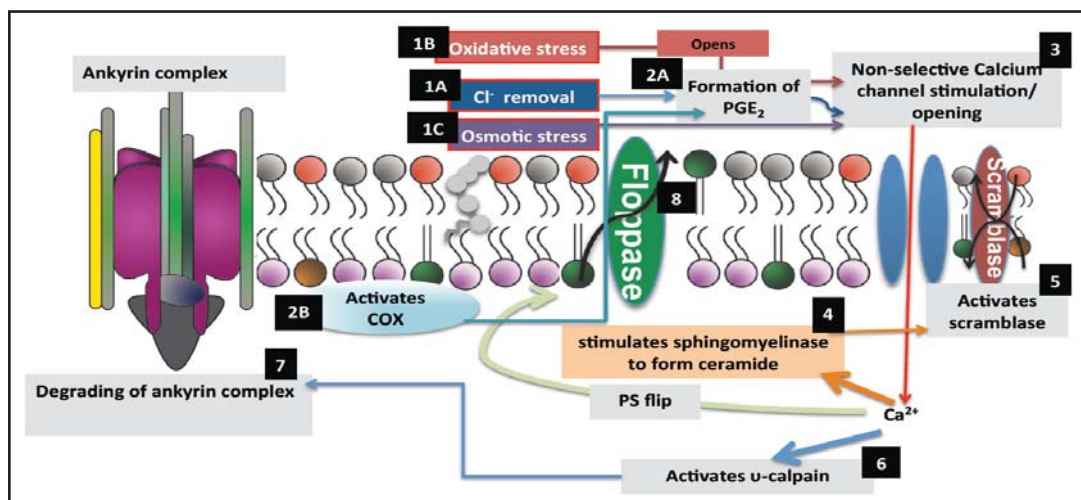


Fig. 2. Membrane scrambling resulting in blebbing and shrinkage, characteristics of eryptosis, due to action of oxidative stress, osmotic stress and Cl^- removal followed by Ca^{2+} influx.

phospholipase A_2 (PLA_2), cyclooxygenase (COX) and PGE-synthase (see Fig. 2, adapted from [35, 40, 41]).

When Cl^- is removed (1A), it causes the formation of PGE_2 (2A), and COX activation (2B) leading to arachidonic acid formation and PGE_2 efflux, followed by stimulation and opening of the membrane Ca^{2+} -channels (3). Oxidative stress (1B) also causes the Ca^{2+} channel to open [41]. During osmotic stress (1C) RBC shrinkage also happens due to Ca^{2+} entry [42]. Increased cytosolic Ca^{2+} activates Ca^{2+} -sensitive K^+ channels resulting in the subsequent exit of KCl and cell shrinkage [35]. Energy depletion also causes Ca^{2+} entry [43] [44]. Ca^{2+} entry also stimulates sphingomyelinase to form ceramide (4) [42]; Ca^{2+} and ceramide then activate a scramblase (5) and this leads to the exposure of PS and breakdown asymmetry of the cell membrane [35]. As mentioned previously, PS is usually confined to the inner leaflet of the RBC and its externalization requires both inhibition of the flippase and activation of the scramblase and both may follow from elevation of intracellular Ca^{2+} [26]. Elevated cytosolic free Ca^{2+} concentrations also activate μ -calpain (6), which degrades components of the cytoskeleton, like the ankyrin R complex (7), leading to membrane blebbing [40]. PS exposure (involvement of flippase) (8) may not necessarily be regulated by PGE_2 that is formed by stressed RBCs but also may be triggered by PGE_2 from other sources [40].

Compounds tested that prevent or induce eryptosis

The following section comprehensively reviews literature where researchers tested the effects of various molecules and compounds on eryptosis formation or inhibition. In various comprehensive investigations, the Lang group is the leading eryptosis researchers (see Table 1). Table 1 lists compounds that induce or inhibit eryptosis. The main methods used to determine this process were:

- Annexin V binding for PS exposure
- Fluo3 fluorescence marker for cytosolic calcium
- DCF fluorescence for ROS
- Anti-ceramide antibody for ceramide formation/ anti-ceramide-FITC antibody and radioactive labelling
- Cell volume by forward scatter
- Cytosolic ATP levels using luciferin-luciferase-based assay
- Glutathione by CMF fluorescence/ mercury orange fluorescence
- ELISA methods

The individual methods will be briefly discussed in the next section. Table 2 lists literature that includes research by other groups and the Lang group, but utilizes also additional novel methods, while Table 3 lists literature where animal models were used and Table 4 lists literature where eryptosis were noted in specific diseases.

Table 1. Investigations by the Lang group, suggesting that products cause eryptosis in human RBCs

Compound causing eryptosis		References
1,4-naphthoquinone derivative	Salinomycin	Cyclosporine [34, 37, 41, 43, 45-179]
naphthazarin	Topotecan	Adenosine
Sulforaphane	Phloretin	Retinoic acid
Phosphoprotein: phosphatase inhibitor Cantharidin	Cryptotanshinone	Aluminum ions
Antiviral drug: Elvitegravir	Baicalein	Ciglitazone and PPARgamma ligand 15deoxy-delta12,14-prostaglandin J2 (15d-PGJ2)
Reverse transcriptase inhibitor: efavirenz	Salinomycin	Clofazimine
K ⁽⁺⁾ , H ⁽⁺⁾ ionophore and antibiotic nigericin	Piperlongumine	Ribavirin
Protease inhibitor: lopinavir	Saponin	Treosulfan
Ellipticine	Estramustine	Sphingosine and sphingosine-1-phosphate
Edelfosine (1-O-octadecyl-2-O-methylglycero-3-phosphocholine)	Narasin	Vitamin K(3)
Juglone	Zopolrestat	Resveratrol
P-glycoprotein inhibitor: zosuquidar (LY335979)	Amitriptyline: mice model	Beauvericin
Farnesyltransferase inhibitor: Manumycin A	Bismuth	Dicoumarol
Oxaliplatin	Ruxolitinib	Xanthohumol
Glycochenodesoxycholic and taurochenodesoxycholic	Boswellic acid	Thymoquinone
Nystatin	FTY720: anti-inflammatory drug	Valinomycin
Gramicidin	Alpha-lipoic acid Monensin	lpratropium bromide
Hydroxytyrosol	Phytic acid	Sunitinib
Triparanol	Licochalcone	Sulindac sulfide
Bromfenvinphos	PRIMA-1: anticancer drug	Zinc
Nonactin	Garcinol	Zidovudine
Anidulafungin	Caffeine	Pyruvium pamoate
Pseudomonas aeruginosa virulence factor pyocyanin	Protease inhibitor: nelfinavi	RBCs artificially aged by glycation
Combretastatin A4	Peptidoglycans (PGNs) from bacterial cell walls belong to 'pathogen-associated molecular patterns' (PAMP)	Benzethonium
Phosphate Disodium	Probucol	Thioridazine
Terfenadine	Naringin	Carbon monoxide
Piceatannol	Lipoxygenase inhibitor bay-y5884	Blebbistatin
Ceranib-2	Cadmium ions	Paclitaxel
Pazopanib Ritonavir	Selenium compounds	Novobiocin
Regorafenib	Silver ions	Celecoxib
Lapatinib	Gold	Shikonin
Tannic acid Rifampicin	Carnosic acid	Gedunin
Dermaseptin	Trans-cinnamaldehyde	Plumbagin
Uremic toxin, indoxyl sulfate	Extracellular phosphate	Rotenone
Uremic toxin, acrolein	Saquinavir	Patulin
Lumefantrine	Synthetic lipopeptide Pam3CSK4	Hexavalent (VI) chromium
Artesunate	Honokiol	Gambogic acid
Alantolactone	Apigenin	Sorafenib
Auranofin	Fumagillin	Cisplatin
Penta-o-galloyl-beta-d-glucose	Gossypol	Methyl dopa
Mitoxantrone	Nitazoxanide	Arsenic
Mefloquine	Anti-A IgG antibodies	Thymol
Fluoxetine	Curcumin	Amphotericin B
Carmustine	Anandamide	Aristolochic acid
Mycotoxin ochratoxin A	Vanadate	Miltefosine
Withaferin	Radiocontrast agents	Tin
Enniatin A	Mushroom tyrosinase	Amiodarone
NFKappaB-inhibitors: Bay 11-7082 and parthenolide	Hemin-exposure	Lithium
Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)	Phlorhizin	Amyloid peptides Geldanamycin
Baicalein	Azathioprine	Oridonin
	Dimethylfumarate	The effect of temperature
	Mitotane	Amantadine
	Ursolic acid	RBC age
	Topotecan	Plufenamic acid effect on intraerythrocytic growth of <i>P. falciparum</i>
	Phloretin	
	Cryptotanshinone	
	Nitric Oxide	
	Methylglyoxal	

Table 2. Papers on eryptosis using similar techniques as in Table 1, but with additional methods

Compound	Technique	References
Fucosanthin	PS exposure; ROS; Calcium measurement; Hemolysis; Lipid peroxidation using BODIPY fluorescence	[180]
Polyphyllin D	Quantitative tunable resistive pulse sensing (TRPS) technology determined PS externalization with microbeads functionalized with annexin-V for PS binding	[181]
Uremic solutes indoxyl sulfate and indole-3-acetic acid; Lactadherin	PS exposure labeled with Alexa Fluor 488-lactadherin; Procoagulant activity was assessed by clotting time and purified coagulation complex assays	[182]
Oleic acid with milk alpha-lactalbumin	Calcein leakage; PS exposure; Morphological changes and hemolysis were studied by confocal microscopy; FACS analysis; Scanning electron microscopy; Hemolysis	[183]
Bisphenol A and its analogs	Calcium / Ceramide measurement; PS exposure; Calpain and caspase-3 activities	[184]
Fe ³⁺ magnetic nanoparticles	PS exposure; Hemolysis; Hemorheology indices; Calcium / Ceramide measurement	[185]
Evaluation of small noncoding RNAs in ex vivo stored human mature red blood cells	ncRNA expression analysis; Bioinformatics analysis	[186]
L-Carnitine inhibits eryptosis induced by uremic serum	PS exposure; Glutathione; ROS	[187]
Lonomycin and PS specific peptide (PSP)-conjugated liposomes	PS exposure	[188]
Indicaxanthin	PS exposure; ROS; Glutathione (spectrophotometric analysis); PGE ₂ release (PGE ₂ Enzyme Immunoassay Kit); Calcium measurement	[189]
Low concentrations of <i>E. coli</i> alpha-hemolysin	SDS-PAGE of ghost RBCs; Calcium measurement; Calpain activation; Mass spectrometric analysis	[23]
Glucose	A comprehensive analysis using various methods e.g.: Enzymatic activities; Glutathione S-transferase and glutathione reductase; Glycated hemoglobin (spectrophotometrically); Calcium / Ceramide measurement; Superoxide production (labeling cells with dihydroethidine and flow cytometry); Lipid peroxidation (fluorophore BODIPY (581/591) C ₁₁ and flow cytometry); ATPase activity (absorbance measurement)	[190]
Sphingomyelinase-induced adhesion of eryptotic erythrocytes to endothelial cells	PS exposure; Cell volume; Adhesion of RBCs to HUVEC using a flow chamber; CXCL16 protein abundance measured by Western blotting and FACS analysis of fluorescent antibody binding	[35]
Bacterial peptidoglycan	Adhesion of RBCs to HUVEC using a flow chamber; PS exposure; Cell volume; CXCL16 abundance determined by confocal microscopy and FACS analysis	[191]
RBC age	Flow cytometry and live cell imaging (fluorescence microscopy); Calcium measurement; PS exposure	[192]
Nocodazole	PS exposure; Cell volume; Calcium measurement; Ceramide measurement; ROS; Tubulin abundance was quantified by TubulinTracker Green reagent and visualized by confocal microscopy	[193]
HOE642, recently characterised as an inhibitor of the K ⁺ [(Na ⁺)/H ⁺] exchanger	Radioisotope flux measurements and conductance determinations	[194]
Early immune response to <i>Plasmodium falciparum</i> -infected RBCs	PS exposure	[195]
Janus kinase 3 expression in RBCs	PS exposure; Cell volume; Immunohistochemistry and confocal microscopy to test for JAK3	[196]
RBC adherence to endothelial cells	Adhesion test: flow chamber model; PS exposure; Cell volume	[197]
Hemotrophic mycoplasmas (uncultivable bacteria)	PS exposure	[198]
Amp-activated protein kinase	Western blotting and confocal microscopy for AMPK expression; Calcium measurement; PS exposure; Cell volume	[199]
Listeriolysin from <i>Listeria monocytogenes</i>	Patch-clamp technique; PS exposure; Cell volume; Calcium measurement	[200]
Leukotriene	Western blotting was employed to detect the cysteinyl-leukotriene receptor cysLT ₁ , competitive immune assay to determine leukotriene release from RBCs; Calcium measurement; Cell volume; PS exposure	[201]
Primaquine	Microsomal metabolism-linked assay; PS-exposure	[202]
Polyphyllin	PS-exposure; Flow cytometry and confocal microscopy	[203]
Betulonic acid	PS exposure; Calcium measurement	[204]
Ferutinin	PS-exposure; Calcium measurement; Caspase-3 activity; Calcein leakage; Flow cytometry and confocal microscopy	[205]
p38 kinase expression in RBCs	Western blotting was utilized to determine phosphorylation of p38 kinase; Calcium measurement; Cell volume; PS exposure	[206]
Hemin treatment	RBCs labelled with biotin prior to hemin treatment and <i>Plasmodium chabaudi adami</i> infection: fluorescent microscopy	[207]
NFkappaB-inhibitors: Bay 11-7082 and parthenolide	Western blotting to search for NFkappaB and IkappaB-alpha expression in erythrocyte	[208]
Comparison of cation channel activity and susceptibility to eryptosis in neonatal and adult erythrocytes	Prostaglandin E ₂ (PGE ₂) formation Cation channel activity; Ca ²⁺ entry; Cell volume; PS exposure	[209]
Furosemide on non-selective voltage-independent cation channels in RBCs	Cation channel activity was determined by whole-cell patch clamp; Calcium measurement; PS exposure	[210]
Cyclin-dependent kinase CDK4 inhibition on eryptosis	Western blotting for determination of the presence of CDK4 protein in RBCs; Cell volume; Calcium measurement; PS exposure	[211]
Gold nanorods coated with cetyltrimethylammonium bromide or polyethylene glycol	Hemolysis	[212]
Salidroside	PS exposure; Calcein leakage; Caspase-3 activation; Calcium measurement	[213]
CH11-anti-CD95 exposure and CD95 activation	PS exposure	[38]
Oxysterol	PS exposure; Calcium measurement; ROS; Cell volume; Amino-phospholipid translocase (APLT) activity evaluated by cytofluorimetric assays; Prostaglandin PGE ₂ measured by ELISA; GSH-adducts and lipoperoxides measured by spectrophotometry; Involvement of protein kinase C and caspase was investigated by inhibitors staurosporin, calphostin C, and Z-DEVD-FMK	[214]
Schizont parasitized RBC concentrated by magnetic separation were cultured with eryptotic RBC obtained by ionomycin treatment	Parasite growth evaluated in Giemsa-stained thin blood smears	[215]
Monitoring or eryptosis	Photothermal response, a label-free photothermal non-invasive technique	[216]
Aluminum exposure induces eryptosis which is prevented by erythropoietin	ROS measurement; Glutathione measurement	[217]
Protein kinase CK1	Western blotting was utilized to verify that erythrocytes express the protein kinase CK1alpha; PS exposure; Cell volume; Calcium measurement	[218]
Tanshinone IIA	PS exposure; Cell volume; Calcium measurement; Hemolysis; ATP; Ceramide measurement; Clearance of circulating erythrocytes was estimated by CFSE-labelling	[219]
Cr ⁽⁶⁺⁾	Hemolytic rate and blood gas assays; PS-exposure; Cell volume; ATP; Calcium measurement; ROS; Morphology of RBCs	[220]

Table 3. Eryptosis in animal RBCs

Compound	Technique	References
Adhesion of erythrocytes from <i>anx7(-/-)</i> and <i>anx7(+/-)</i> -mice exposed to ionomycin	PS exposure; Cell volume; Adhesion to human umbilical vein endothelial cells (HUVEC) utilizing a flow chamber	[221]
Water deprivation of RBCs: mice exposed to ionomycin	Measuring plasma 1,25(OH) ₂ D ₃ and aldosterone concentrations using ELISA, and plasma calcium utilizing photometry; PS exposure	[222]
cGMP-dependent protein kinase type I (cGKI) in the regulation of erythrocyte survival, gene-targeted mice lacking cGKI	PS exposure	[223]
Adhesion of klotho-deficient eryptotic erythrocytes to endothelial cells: mouse study	PS exposure after binding and adhesion of RBCs to HUVEC from trapping of labelled erythrocytes in a flow chamber	[224]
Accelerated suicidal erythrocyte death in klotho-deficient mice	PS exposure; Cell volume; Calcium measurement	[225]
Paclitaxel on parasitemia and survival of <i>Plasmodium berghei</i> infected mice	Intra-erythrocytic DNA/RNA content; PS exposure; Cell volume	[226]
Lead effect on parasitemia and enhances survival of <i>Plasmodium berghei</i> -infected mice	PS exposure	[227]
Chlorpromazine effect on eryptosis, parasitemia and survival of <i>Plasmodium berghei</i> -infected mice	PS exposure; Cell volume; Intra-erythrocytic DNA/RNA content	[228]
NO-synthase inhibitor l-name on parasitemia and survival of <i>Plasmodium berghei</i> -infected mice	PS exposure	[223]
Diabetic rat model	Caspase-3 immuno-reactivity	[229]
Murine model: effect of storage on RBCs	Calcium measurement	[230]
Phosphoinositide-dependent kinase PDK1: mice study	PS exposure; Cell volume; Calcium measurement	[231]
Endothelin B receptor stimulation: mice study	Number of phosphatidylserine-exposing RBCs, reticulocytes and spleen size	[232]
Vitamin D-rich diet in mice	Plasma concentrations of erythropoietin were estimated using an immunoassay kit; Blood count using an electronic hematology particle counter; Relative reticulocyte numbers using Retic-COUNT(R) reagent; PS exposure; Calcium measurement	[233]
Gene-targeted mice lacking Annexin A7	PGE ₂ formation was determined utilizing an immunoassay; Ion channel activity by whole-cell patch clamp recording; PS exposure; Cell numbers; Calcium measurement	[234]
Cordycepin effect: mice study	Caspase-3 activation; Calcium measurement	[235]
Heart failure-associated anaemia: rat model	PS exposure; Cell numbers; Calcium measurement	[236]
Human and Sheep RBCs	Flow cytometry, single cell fluorescence video imaging, or confocal microscopy; PS exposure; Calcium measurement	[21]
Amphotericin B effect on parasitemia and survival of <i>Plasmodium berghei</i> -infected mice and infected human RBCs	<i>In vitro</i> growth and DNA amplification assays	[237]
Mitogen- and stress-activated kinase MSK1/2: Mice study	Blood count; Haematocrit; Hemolysis; Mean RBC volume	[238]
Loss-of-function mutation of the adenomatous polyposis coli gene: mice study	PS exposure; Cell size; cytosolic ATP	[239]
Anandamide in <i>Plasmodium berghei</i> -infected mice	PS exposure	[240]
Amiodarone: mice study	PS exposure	[241]
Azathioprine: mice study	PS exposure	[242]
Cyclosporine: mice study	PS exposure; Cell volume	[243]
Link between AMP-activated protein kinase (AMPK) and the eryptotic phenotype: mice study	Global proteome analysis of erythrocytes from AMPK(-/-) mice and wild-type mice using high-accuracy mass spectrometry and label-free quantitation and measured changes of expression levels of 812 proteins	[244]

Methods to study eryptosis

From the above-mentioned tables, it is noted that there are a few basic techniques used in the study of eryptosis, and they mainly include flow cytometry with some researchers using confocal microscopy. The techniques are combined and summarized from the papers listed in the tables and particularly the Bissinger, Qadri and Lang papers give extensive method overviews [52, 246, 266]. We also looked at the instructions or standard protocols from the manufacturers.

Externalization of PS

Flow cytometry

PS is externalized during eryptosis and this externalization can be measured using Annexin-V binding. In order to determine Annexin-V-binding, a small amount (2-5ul) of

Table 4. Diseases associated with eryptosis

Disease	Methods	References
Acute cardiac failure	Phosphatidylserine exposure was estimated from Annexin-V-binding, calcium measured with Fluo3-fluorescence, ceramide abundance tested with antibodies and ROS with 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) as determined by flow cytometry.	[245]
Lung cancer	Annexin-V binding for PS exposure, Fluo3 fluorescence marker for cytosolic calcium, DCFDA fluorescence for ROS and anti-ceramide antibody for ceramide formation: flow cytometry.	[246]
RBC from chronic kidney disease); hemodialysis; patients and healthy controls	Flow cytometry	[247]
Hyperthermia in hereditary spherocytosis	PS exposure	[248]
Obesity	CD47 expression and Caspase-3 and Caspase-8 activity (flow cytometry); PS exposure	[249]
Anaemia in end-stage renal disease	PS exposure; Calcium measurement; Cell volume; Ceramide; ROS	[250]
Lead exposed individuals	(ATP) concentration; Calpain; Reduced/oxidized glutathione; Thiobarbituric acid-reactive substance (TBARS) concentration; PS exposure; Calcium measurement; δ -aminolevulinic acid dehydratase (δ ALAD) activity	[251]
Hemolytic anemia	Transmission electron microscopy; PS exposure; Wheat germ agglutinin (WGA)-FITC	[252]
Hereditary spherocytosis	PS exposure; Cell surface glycoproteins using wheat germ agglutinin using flow cytometry	[253]
Paroxysmal nocturnal hemoglobinuria and myelodysplastic syndrome.	PS exposure; Cell surface glycoproteins using wheat germ agglutinin using flow cytometry	[254]
Human population exposed to arsenic through drinking water	RBC membrane micro-viscosity ; Cholesterol/phospholipid ratio	[255]
Type II diabetes with chronic kidney disease	PS exposure; Cell volume	[255]
Sickle cell disease	GSH/GSSG ratio; Lipoperoxidation; PS exposure	[256]
Autoimmune hemolytic anemia	ROS	[257]
Beta-thalassemia: splenectomized and nonsplenectomized individuals	PS exposure	[258]
+) -sj733, a clinical candidate for malaria	PS exposure; Calcium measurement	[259]
RBCs from healthy volunteers were exposed to plasma of patients suffering from sepsis, or to supernatants from sepsis producing pathogens	PS exposure	[260]
RBCs from healthy volunteers were exposed to plasma from a patient with severe idiopathic recurrent hemolytic uremic syndrome secondary to factor H depletion	PS exposure; Calcium measurement; Ceramide measurement; Cell volume	[261]
Anaemia in the elderly	Ps exposure; Calcium measurement	[262]
Type II diabetes	PS exposure; Calcium measurement; ROS; Ceramide measurement; Cell volume; Glutathione measurement	[263]
Parkinson's disease	Non-protein-bound iron, methemoglobin and glutathione were determined in RBCs; Non-protein-bound iron was determined in plasma; RBC caspase-3 activation was evaluated by an immunosorbent enzyme assay	[36]
Autoimmune hemolytic anaemia treated with erythropoiesis-stimulating agents	Ultrastructural analysis	[264]
	Serological tests	[265]

freshly drawn citrated blood should be mixed in either 500 μ l Annexin-V binding buffer or Ringer solution (containing 5 mM CaCl_2), subsequently stained with Annexin-V, conjugated with the appropriate fluorochrome according to the instrument that will be used. The Annexin-V-sample-mix should be diluted 1:200 in Ringer solution, containing 5 mM CaCl_2 or Annexin-V binding buffer at 37°C for 15-45 minutes (incubation time depends on manufacturer protocol) protected from light.

The Annexin-V abundance at the RBC surface can then be determined on a flow cytometer. Annexin-V-binding can be measured with an excitation and an emission wavelength for the specific conjugated fluorochrome that will be specified in the manufacturer datasheet. A dot plot of forward scatter (FSC) vs. side scatter (SSC) should be set to linear scale for both parameters.

Confocal microscopy

For the visualization of eryptotic RBCs, 20-30 μ l RBCs (1×10^6 cells) stained with Annexin-V (with conjugated fluorochrome of choice), taken in a 1:100 dilution, in 200 μ l Ringer solution, containing 5 mM CaCl_2 or Annexin-V binding buffer. The RBCs should be

washed twice and finally resuspended in 200 μ l Ringer solution containing 5 mM CaCl_2 or Annexin-V binding buffer. There are two ways to prepare the sample before viewing under the microscope, all the steps should be done protected from light:-

1. Spread 40 μ l of the sample onto a glass slide and dry it for 15 min at room temperature, followed by covering of the slides with PROlong Gold antifade reagent. These samples can be viewed on any confocal laser-scanning microscope with a water immersion Plan-Neofluar 40/1.3 NA DIC objective.
2. A second method is to drop 10 μ l of the sample on a glass slide and cover it with a coverslip. These samples can be viewed under any confocal microscope with a Plan-Apochromat 63x/1.4 Oil DIC objective.

Pitfalls

- RBCs are very sensitive cells and great caution should be taken not to induce damage to these cells during sample preparation.
- All the chemicals that will be used in the sample preparation should be at room temperature before use and at a pH of 7.4 to avoid osmotic shock.
- The blood sample taken should not be older than 4 hours, and should be stored at room temperature.
- The blood tube(s) should be centrifuged to obtain only the RBC fraction to avoid any possible cross reactivity from other cells (white blood cells and platelets).
- Vortexing of the cells should be avoided during sample preparation - rather re-suspend cells by agitating the solution or by tapping the tube (Eppendorf or microtube) that holds the sample.

The cells tend to move around in solution when on the glass slide, let the sample stand for 30 seconds before viewing under the microscope in order for the cells to settle. Slides can also be coated with poly-L-lysine to attach cells [267].

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Reactive Oxygen Species (ROS)

Oxidative stress is an important accelerant and inducer of eryptosis and increased ROS generation is an important indicator of oxidative stress. ROS can be determined by utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) [245]. 4 μ l RBCs should be mixed in 1 ml Ringer solution or a solution containing 5 mM CaCl_2 . 150 μ l from the resulting cell suspension should be centrifuged at 1600 rpm for 3 min at room temperature. Cells should then be stained with 10 μ M DCFDA in Ringer solution at 37°C for 30 min. Following staining, the cells should be washed three times in 150 μ l Ringer solution. These washed cells should then be re-suspended in 200 μ l Ringer solution, and the ROS-dependent fluorescence intensity measured by flow cytometry, at an excitation and an emission wavelength determined by manufacturer's instructions.

Pitfalls

- The blood should be stained as soon as possible after blood donation to avoid false readings of ROS introduced after blood donation.
- As mentioned above, these cells must be handled with extreme care.

Intracellular Ca^{2+}

An increase of intracellular Ca^{2+} is part of the initiation of eryptosis and further fuels cell membrane scrambling to externalize PS to the outer leaflet of the RBC membrane. The cell-permeable acetoxymethyl (AM) ester of the fluorescent Ca^{2+} indicator Fluo-3, is commonly used to detect changes in intracellular Ca^{2+} levels in a variety of cells. The basis of this marker, is the

fact that Fluo-3 AM is almost non-fluorescent, until it is hydrolyzed intracellularly to Fluo-3 in the presence of Ca^{2+} [114].

Flow cytometry

In order to quantify intracellular Ca^{2+} , 2 μl of freshly drawn blood should be mixed in 500 μl Ringer solution, containing 5 mM CaCl_2 or any buffer solution containing 5 mM CaCl_2 . The cells should then be stained in 5 μM Fluo-3/AM and incubated at 37°C for 30 min. Ca^{2+} -dependent fluorescence intensity can then be measured in with a flow cytometer, with an excitation and an emission wavelength that is specific to the fluorochrome used.

Ceramide Formation

Ceramides are compiled of a sphingosine and a fatty acid and is known to form part of the cell membrane of cells. Ceramide is primarily produced by means of a *de novo* synthesis pathway, or by hydrolysis of sphingomyelin, catalyzed by sphingomyelinases. When ceramide is generated in the plasma membrane, it augments membrane rigidity by stabilizing smaller lipid platforms known as lipid rafts. The amount of ceramide content is indicative of increased membrane fragility [266]. Increased ceramide content is therefore and affective indicator of eryptosis.

Flow cytometry

Currently there are not many techniques available to determine ceramide expression on the RBC surface, but a monoclonal antibody-based assay is most commonly used. Also, radioactive labelling may also be used to show ceramide formation in RBCs where sphingomyelin breakdown is measured, by labelling RBCs with radioactive choline followed by measuring the incorporation of [methyl-3H] choline into RBC lipids [268].

The monoclonal antibody assay involves mixing 4 μl of RBCs in 1 ml Ringer solution, followed by taking 100 μl from the resulting cell suspension and centrifuging at 1600 rpm for 3 min, at room temperature, to form a RBC pellet. Subsequently, the cells should be stained for 1 h at 37°C with 1 $\mu\text{g}\cdot\text{ml}^{-1}$ anti-ceramide antibody in a 1:10 dilution phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). Cells should then be washed twice afterwards with 100 μl PBS-BSA, followed by staining of the cells for 30 min, with a secondary antibody conjugated with a specific fluorochrome. The unbound secondary antibody should be removed by repeated washing with 50 μl PBS-BSA. Followed by re-suspending the samples in 200 μl PBS-BSA and analysed with a flow cytometry at an excitation and an emission wavelength that are specified according to the fluorochrome used.

Pitfalls

This is a very specific monoclonal antibody, and optimal conditions must be determined individually for each application, however, it is also an expensive method.

Glutathione (GSH) Abundance

Flow cytometry

RBCs have a large amount of glutathione (GSH) that buffer the damaging effects oxidative stress, which is well known to deplete GSH and changes it into its disulfide form GSSG. Flow cytometry is a defined technique to determine the level of GSH on RBCs. GSH is known to be reduced in eryptosis therefore reduced glutathione (GSH) abundance can be determined by utilizing 5-chloromethylfluorescein diacetate (5-CMFDA). After incubation, 100 μL suspension of RBCs should be centrifuged at 1600 rpm for 3 min at 22 °C, followed by discarding of the supernatant and staining of the cells with 5-CMFDA (in PBS containing

5-CMFDA at a final concentration of 1 μM). RBCs should then be incubated at 37 °C for 45 min in the dark, and washed in PBS, followed by re-suspending the 5-CMFDA-loaded RBCs in 200 μL PBS, and measuring the 5-CMFDA-dependent fluorescence intensity at an excitation and an emission wavelength, specified in the manufacturer's instructions using a flow cytometer.

Another method used to determine reduced GSH is with mercury orange. Cells should be incubated for 30 min in PBS, containing 40 μM mercury orange. After incubation of the RBCs, they should be washed only once, and re-suspended in 200 μL PBS. The fluorescence intensity can be measured with flow cytometry according to the excitation and emission wavelength specified by the manufacturer.

Confocal microscopy.

An alternative method to mercury orange has been suggested, where RBCs are incubated with pre-warmed to 37°C with 10 μM 5-chloromethylfluorescein diacetate (CMFDA) and left for 30 min at 37°C. Cells should then be washed with PBS and fixed with 3.7% paraformaldehyde. Membrane-containing cells should then be removed, followed by mounting on a glass slide, and viewed with any typical confocal microscope [269].

Pitfalls

- The Mercury orange method requires thorough washing; therefore cell loss is an important factor to take in consideration when preparing RBCs with this method.
- Mercury orange has also been found to be less consistent and has higher background staining.

Colorimetric measurement of glutathione (GSH)

The Glutathione Assay (colorimetric) Kit provides a useful, colorimetric method for analysing either total glutathione or the reduced form glutathione alone using a microtiter plate reader. 5,5'-Dithiobis(2-nitrobenzoic acid) DTNB and glutathione (GSH) react to produce 2-nitro- 5-thiobenzoic acid which has a yellow colour that can be determined by measuring the absorbance at 412 nm using a micro-plate reader.

RBC Sample Preparation according to the above-mentioned kit: Citrated blood should be centrifuged at 1000 x g for 10 min at 4°C, followed by suspending of the supernatant. The RBCs should then be lysed with a 4x volume of Glutathione Buffer and kept on ice for 10 min. Following this, 1x volume of a 5% SSA should be added, followed by mixing and centrifuged at 8000 x g for 10 minutes. Supernatant should then be transferred to a new tube and use to measure glutathione with a micro-plate reader [214].

Measurement of membrane lipid hydroperoxides

The purpose of measuring lipid hydroperoxides is to determine the oxidative degradation of lipids in the cell membrane. RBC ghosts should be prepared by washing 3x for 30 min, washing-centrifugation cycles (20000 g, 4°C), with excess hypotonic PBS, and finally suspended in 1 mL PBS. Conjugate diene (CD) lipid hydroperoxides should be extracted from 500 μl of the suspension using 3 ml of a $\text{CHCl}_3:\text{CH}_3\text{OH}$ (2:1) mixture. The organic solvent should be evaporated using nitrogen stream, followed by re-suspending in cyclohexane, and quantify spectrophotometrically at 234 nm [214].

Measurement of APL-translocase (APLT) activity

The inhibition of APLT keeps PS at the outer side of the membrane after externalization [214]. This assay confirms eryptosis by looking at the APLT activity. RBC suspensions (0.5 mL) should be incubated at 37°C with 0.5 mM NBD-PS fluorescent probe, from a 1mmol.L⁻¹ stock solution in HEPES-buffer. After 60 min, 5 μl of the sample should be added to 250 μl HEPES buffer containing 0.1 mM EGTA and 1% bovine serum albumin that will extract the NBD-PS probe from the outer plasma- membrane. Cytofluorimetric measurements of residual fluorescence of the sample will then reveal the amount of NBD-PS localized on the inner leaflet in the plasmamembrane as a result of the APLT activity [214].

Pitfalls

- Extreme care should be taken not to haemolyse the RBCs. The cells should be fixed for only 10-15 min at room temperature.
- Exposure of 0.01% Triton-X 100 for 5 min has been found to work specifically well for RBCs.

Confocal microscopy

This method could not be validated for confocal microscopy.

Ultrastructural analysis to study eryptosis

Scanning electron microscopy (SEM) is a useful and specialized method to study physical structural changes associated with eryptosis, used in our laboratory. In the biochemical analysis methods, discussed above, are primarily used as indicator of eryptosis, however, ultrastructural analysis visually confirms these changes, by showing membrane blebbing, microparticle shedding, cell shrinkage, membrane fragility and RBC agglutination. We previously showed these ultrastructural changes in various diseases associated with RBC eryptosis. SEM can be used to study single RBCs or by adding thrombin to whole blood, to create an extensive fibrin network where RBCs are then trapped [12, 264, 270-276] In healthy blood the RBCs keep their shape and do not fold around the fibrin fibres. However, during systemic inflammation and under conditions associated with the development of eryptosis, the RBCs lose their typical shape and fold around the fibres, showing fragility of the membrane and membrane blebbing. To prepare SEM samples either 10 µl of whole blood is placed on a glass cover slip or 10 µl whole blood together with 5 µl thrombin is added to create an extensive fibrin network with trapped RBCs. A thin smear is created on the glass cover slip, and washed in PBS to remove excess proteins. The samples are then fixed in 4% formaldehyde followed by post-fixation of osmium tetroxide and standard SEM preparation methods. Samples are mounted and coated with carbon and viewed at 1 kV under a scanning electron microscope. Figure 3 shows examples of damaged RBCs showing various degrees of eryptosis, prepared for SEM analysis.

Pitfalls

The whole blood smear, with or without addition of thrombin should not be too thick, and when thrombin is added, the time between the mixing of the whole blood and the thrombin

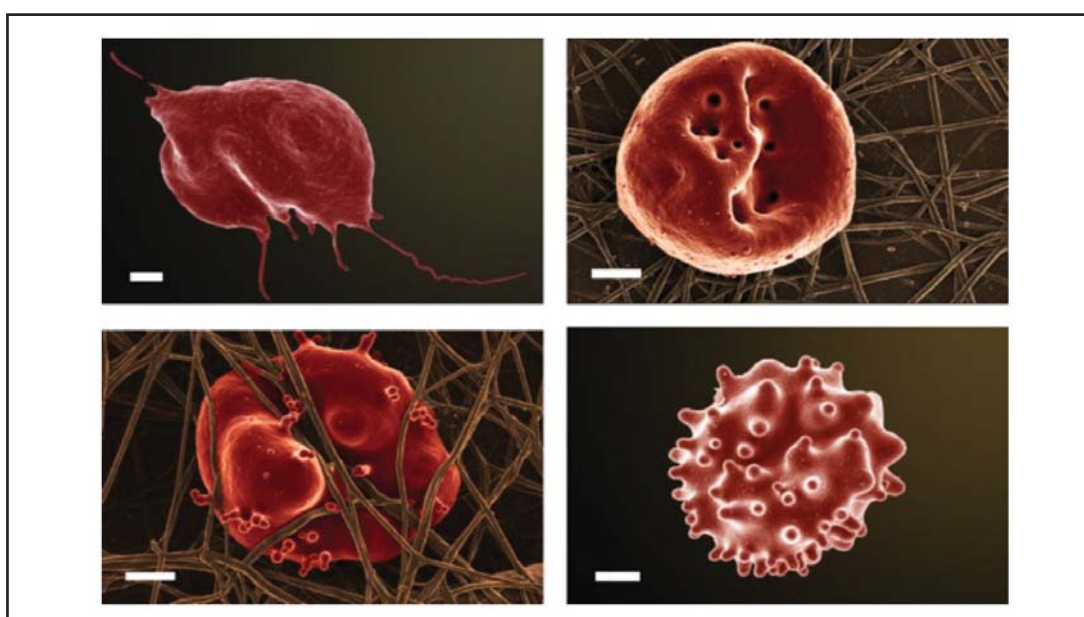


Fig. 3. RBCs showing various stages of eryptosis. Scale bar: 1 µm.

should not be too long, otherwise a coagulated layer of plasma will form and this will cover the actual clot. We determined that when thrombin is added to whole blood, PBS should be added about 30 seconds after the smear is made, to prevent the clot washing off, but also to prevent the coagulated layer on top of the clot. For whole blood smears without thrombin, the smear should be left for at least 60 seconds, before adding PBS. This is particularly helpful for healthy blood, as it washed off easier than RBCs from inflammatory conditions, due to increased agglutination of the RBCs and increased plasma proteins spontaneously associated with the RBCs. The smear should also be done gently using a bent pipette tip, and without introducing too much force onto the sample, otherwise the RBCs will be damaged.

Conclusion

When reviewing the literature, most of the papers used Annexin-V binding to identify eryptosis using the PS flip, together with selected other conformational methods. We therefore suggest that this is probably the one method that most clearly confirms eryptosis. The method can be used as a flow cytometry as well as confocal microscopy method, and is easy and versatile for even a novice. Throughout the literature, if the PS flip was shown with Annexin-V binding, all the other methods confirmed eryptosis as well, suggesting that PS is a robust and singularly proven and confirming method of the presence of eryptosis. Together with an ultrastructural method like SEM, Annexin-V binding showing the presence of PS in the outer leaflet of the membrane is an absolute confirmation of eryptosis.

RBC biophysical and shape changes may be particularly helpful in haematological changes associated with inflammatory conditions and determining the impact of medication after diagnosis. RBC healthiness can be used and is therefore extremely valuable as an indicator of inflammatory status, disease progression and/or treatment monitoring, as the biophysical and biochemical changes to RBCs are a significant accompaniment to a variety of (inflammatory) diseases, as shown in this paper. Ultrastructure and latest state-of-the-art fluorescent marker methods (e.g. confocal microscopy and flow cytometry), that represents both biochemical and biophysical clinically relevant readouts, and together cytokine profiles usually measured during inflammation, should be combined with targeted RBC membrane immunofluorescent biomarkers. We still know very little about the effects of inflammation, upregulated cytokine effects, as well as drug interactions with RBCs at the level of membrane receptor and single molecule interactions. The RBC membrane and its role in disease might provide researchers with an excellent model, particularly because it is a relatively non-invasive method to track the healthiness of an individual.

As the NIH is now driving the global process where precision medicine and individualized medicine is advised to form an integral part in the diagnosis and treatment of patients [277], eryptosis, single molecule as well as receptor interactions using both biophysical and biochemical analysis may give ultimately give practitioners an easy method to track inflammation and disease patterns before and after treatment, in a truly individualized, patient orientated precision medicine approach, where one-medication-regime-fits-all is no longer appropriate.

Ethical Considerations

Ethical clearance was obtained from the University of Pretoria Human Ethics Committee for the use of blood from patients with inflammatory conditions and healthy volunteers. All participants filled in informed consent forms.

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There are no conflicts of interest to declare by any of the authors. EP: study leader, paper concept and writing of paper; JB and JdP: preparation of samples and methods descriptions. All authors approved of the submitted paper.

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