Heliyon 10 (2024) e23507

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Review article

The application of approaches in detecting ferroptosis

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ARTICLE INFO

Keywords:
Ferroptosis
Cell death
Detection methods

ABSTRACT

Ferroptosis is a regulatory cell death (RCD) caused by iron-dependent lipid peroxidation, which is the backbone of regulating various diseases such as tumor, nervous system diseases and so on. Despite ferroptosis without specific detection methods currently, there are numerous types of detection technology commonly used, including flow cytometry, cell activity assay, microscopic imaging, western blotting, quantitative polymerase chain reaction (qPCR). In addition, ferroptosis could be detected by quantifying oxygen-free radicals reactive oxygen species (ROS), the lipid metabolite (malondialdehyde ((MDA)), related pathways and observing mitochondrial damage. In the face of numerous detection methods, how to choose appropriate detection methods based on experimental purposes has become a problem that needs to be solved at present. In this review, we summarized the commonly used detection methods of the critical substances in the process of ferroptosis, in the hope of facilitating the comprehensive study of ferroptosis, with a view to providing a guidance for subsequent related research.

1. Introduction

Cell death has been identified and classified as accidental death (uncontrolled passive process) and regulated cell death (active process) over the past decades [1,2]. Compared with accidental death, regulated cell death has been widely studied [3,4]. Ferroptosis is a form of nonapoptotic cell death, that is driven by iron-dependent lipid peroxidation [5]. Ferroptosis emerged from Dolma et al. identification in 2003 as a small molecule that induced a nonapoptotic form of cell death [6], which they found to be regulated in an iron-dependent manner [7]. In subsequent studies, scholars discovered a cell death method that is different from apoptosis in many aspects, such as morphology, biochemistry, and genetics [8]. For example, the morphological features of mitochondria are observed as include swelling, dense electrons, and the reduction or disappearance of mitochondrial cristae in ferroptosis [9]. In 2012, this special way of regulating cell death was named “"ferroptosis"” [5]. We will show the characteristics of ferroptosis, pyroptosis, necroptosis, autophagy, and apoptosis in Table 1. Although the history of research on ferroptosis is not so long, the research field of ferroptosis has been growing exponentially in the past 20 years and has been proven to play a vital role in the development of various organisms and

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https://doi.org/10.1016/j.heliyon.2023.e23507
Received 2 August 2023; Received in revised form 14 November 2023; Accepted 5 December 2023
Available online 13 December 2023

diseases. The presence of ferroptosis has thus far been observed in different systems, revealing the universality of this mechanism. Ferroptosis is closely related to various diseases and even plays a key role. The study of ferroptosis in relation to cancer has received much attention because it is closely related to the development and metastasis of cancers [10,11], such as colorectal cancer [12], liver cancer [13], and gastric cancer [14]. The expression of ferroptosis-related genes can serve as a prognostic indicator for cancer patients. Ferroptosis may become a potential strategy for preventing cancer metastasis and drug resistance. In addition, research on ferroptosis in some inflammatory diseases, such as ischaemia-reperfusion injury [15], neuroinflammation [16], and acute kidney injury, has also received widespread attention [17]. As an independent form of cell death, ferroptosis is important in both regulatory pathways and its relationship with various diseases. Currently, research on ferroptosis is still in its early stages; therefore, exploring the pathogenesis of ferroptosis and its role in various diseases, and proposing effective and targeted treatment methods have significant theoretical significance and practical value.

Ferroptosis is part of a bodily defence that also includes by metabolism, iron regulation and ROS [23–25]. Therefore, the regulation of metabolism, iron homeostasis and ROS levels is crucial way to regulate the susceptibility of cells to ferroptosis. In recent decades, it has been reported that a variety of pharmacological or natural compounds and intrinsic cellular proteins can regulate the process and function of ferroptosis [1,26]. Therefore, a full understanding of the effective components in ferroptosis and their regulatory network might pave the way for the development of research on ferroptosis.

To demonstrate the role of the crucial effective components, detection approaches have been used and even invented, such as flow cytometry, cell activity assays, microscopic imaging, western blotting, and real-time qPCR. In this review, we provide a framework to summarize the latest advances and challenges in assays of genes, proteins and processes that have played a key role in ferroptosis in the past 5 years, thereby enhancing the development of research on ferroptosis.

2. Mechanisms of ferroptosis

Ferroptosis is at the intersection of metabolism, ROS biology and iron regulation, which are the mechanisms governing ferroptosis [5]. The preconditions for ferroptosis are the oxidation of specific lipids and damage to natural mechanisms that could block the accumulation of oxidized lipids [27–30]. In addition, the decrease in iron utilization may increase the sensitivity of ferroptosis and genes related to iron metabolism are generally upregulated during ferroptosis [31–33]. When ferroptosis occurs, the morphological, biochemical and genetic characteristics related to ferroptosis should be reflected. The morphological changes include loss of plasma membrane integrity, swelling of cytoplasmic and cytoplasmic organelles, and moderate chromatin condensation [6,34,35]. As an ROS-dependent form of cell death, ferroptosis mainly is associated with the biochemical process of iron accumulation and lipid peroxidation [23,24,36]. In addition, the overexpression of some genes and proteins has also been considered a biomarker of ferroptosis [37] (Fig. 1).

The figure briefly shows the representative pathways of ferroptosis. The GSH-GPX4, FSP1-CoQ10, and GCH1-BH4 pathways are considered the three major stand-alone mechanisms modulating ferroptosis. The antiporter System Xc^- containing SLC7A11 and SLC3A2 mediates the uptake of cystine, which is consumed during the synthesis of intracellular GSH. Next, the antioxidant enzyme GPX4 reduces lipid hydroperoxides into lipid alcohols via GSH to protect cells from ferroptosis. The micronutrient selenium is needed for the biosynthesis of GPX4. Gpx4 also catalyses the reduction of oxidized biolipids, which convert toxic lipid hydroperoxides into nontoxic lipid alcohols through its cofactor GSH. CoQ10 is another important antioxidant molecule that can be reduced to CoQ10H2 by FSP1 and hence protect the cells from ferroptosis. The GCH1-BH4 axis suppresses ferroptosis by regulating the antioxidants BH4 and CoQ10, and lipid peroxidation. The production of PUFAs and subsequent lipid peroxidation play a major role in promoting ferroptosis.

Table 1
Biochemical features, morphological features and regulatory pathways of ferroptosis, apoptosis, necroptosis, autophagy and pyroptosis.

Type	Biochemical features	Morphological features	Regulatory pathways	Ref.
Ferroptosis	Iron accumulation and lipid peroxidation	Small mitochondria with increased mitochondrial membrane densities, reduction or vanishing of mitochondria crista, outer mitochondrial membrane rupture and normal nucleus	Positive: TFRC, ALOX, ACSL4, LPCAT3 Negative:GPX4, AIFM2, ESCRT-III	[9]
Apoptosis	DNA fragmentation	Cellular and nuclear volume reduction chromatin agglutination, nuclear fragmentation, formation of apoptotic bodies and cytoskeletal disintegration, no significant changes in mitochondrial structure	Positive: Caspase Bax, Fas Negative: Bcl-2, PI3K	[18]
Necroptosis	Drop in ATP levels	Plasma membrane breakdown, generalized swelling of the cytoplasm and organelles, moderate chromatin condensation, spillage of cellular constituents into the microen	Positive: RIPK1, RIPK3 and MLKL Negative: AURK4, ESCRT-III	[19, 20]
Autophagy	Formation of autophagosome, elevated autophagic flux, and lysosomal activity	Formation of double membraned autolysosomes, including macroautophagy, mircroautophagy and chaperone-mediated autophagy	Positive: AMKP, ULK, VP34 Negative: mTOR	[21]
Pyroptosis	Caspase-dependent, gasdermin D cleavage, formation of inflammasome, IL-18 and IL-1β release	Cells swelling, formation of membrane proes and membrane rupture, peculiar from of chromatin condensation and intact nuclei, intact and swollen mitochondria with reduced matrix density and collapsed cristae	Positive: CASP1, CASP4, CASP5, CASP11, Gasdermin D Negative: ESCRT-III, GPX4	[22]

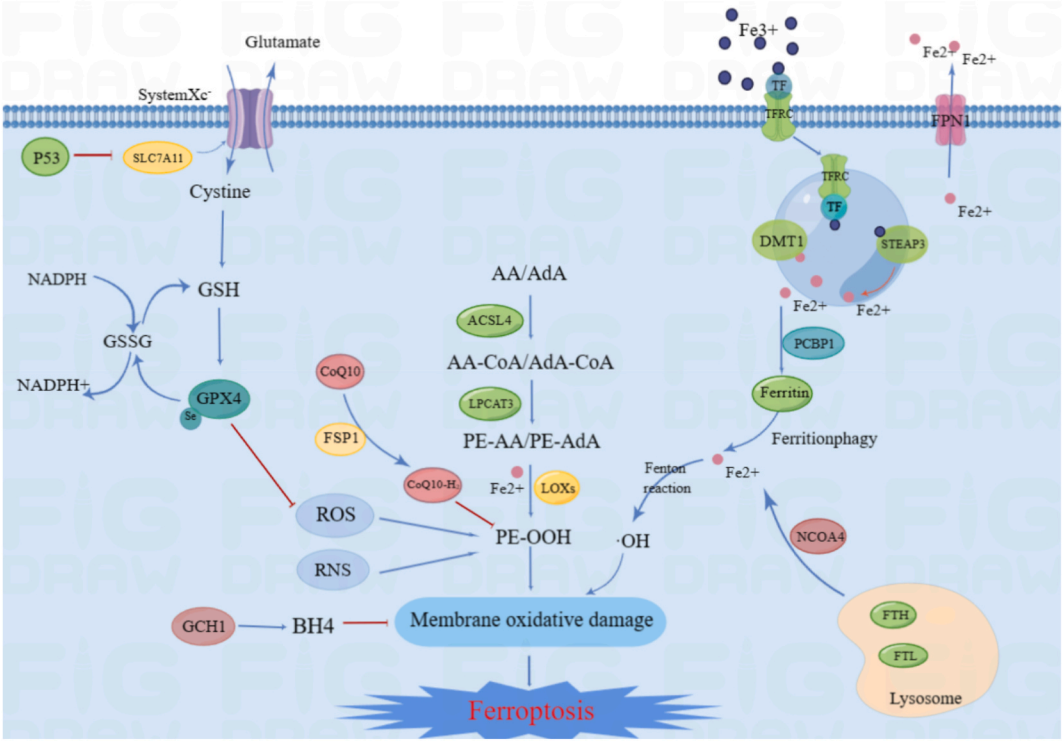

Fig. 1. The mechanisms related to ferroptosis.

$Fe3^+$ is imported into cells by Tf through TfR1, after which it is reduced to $Fe2^+$ and imported via DMT1. Iron is stored as Fe3+ in ferritin, where it is not available to promote ferroptosis. The regulation of iron-abundance through controlling the level of the iron-storage protein ferritin via ferritinophagy dictates sensitivity to ferroptosis.

2.1. Regulation of ferroptosis

2.1.1. Lipid metabolism

Ferroptosis is driven by the peroxidation of specific membrane lipids. It is important to identify the specific lipids and enzymes that play critical roles in ferroptosis. It has been reported that polyunsaturated fatty acids (PUFAs) serve as essential peroxidation substrates for ferroptosis [38,39]. Acylcoenzyme A (CoA) synthetase long-chain family member 4 (ACSL4) and lysophosphatidylcholine acyltransferase 3 (LPCAT3) were identified to facilitate PUFAs incorporation into membrane lipids [40–43]. This is a critical step in the occurrence of ferroptosis. The arachidonic acid lipoxygenase (ALOX) family exists in mammals and plays a crucial role in PUFA mediated peroxidation [44]. In the ALOX family, ALOX15 was identified as the main mediator of phospholipid peroxidation caused by ischaemia, which was further confirmed by chemical genetics [45]. On the other hand, the live or death of cells in response to ferroptotic stimuli depends on the balance of injury and the anti-injury response [46]. Furthermore, ALOX12 is critical for p53-mediated ferroptosis [44]. In addition, the activity of PUFAs in ferroptosis is competitively affected by monounsaturated fatty acids (MUFAs), and MUFA-induced ferroptosis depends on acylcoenzyme A (CoA) synthetase long-chain family member 3 (ACSL3) or stearoyl-CoA desaturase 1 (SCD1) [43,47]. Prostaglandin-endoperoxide synthase 2 (PTGS2) encodes cyclooxygenase-2 (COX-2), is the key enzyme in prostaglandin biosynthesis, and acts both as a peroxidase and as a dioxygenase [48]. PTGS2 is usually considered as a biomarker, but not a driver, of ferroptosis [37]. However, PTGS2 may mediate ferroptosis in neural cells after traumatic injury in the brain [49].

2.1.2. Iron metabolism

Ferroptosis is an RCD caused by iron-dependent lipid peroxidation. Fe^{3+} and Fe^{2+} are the two oxidation states of iron, which affect the sensitivity of ferroptosis [50,51]. Fe^{2+} plays an important role in metabolism and biochemical processes, and the abnormal increase in Fe^{2+} in the endoplasmic reticulum (ER) and lysosomes could enhance ferroptosis [52,53]. Transferrin receptor protein (TFRC) is located in the cell membrane, which and can mediate Holo-transferrin uptake and increase intracellular iron content [54]. After being absorbed by TFRC, the six transmembrane epithelial antigen 3 (STEAP3) metal reductase in the endosome reduces Fe^{3+} to Fe^{2+}, and then solute carrier family 11 member 2/divalent metal-ion transporter-1 (SLC11A2/DMT1) releases Fe^{2+} from the endosome into the cytoplasm [55]. The iron-storage protein ferritin includes ferritin light chain (FTL) and ferritin heavy chain 1 (FTH1), which can be degraded by lysosomes to increase free iron levels. The labile iron pool (LIP) is a storage place for intracellular iron, which

provides raw material for the synthesis, assembly and insertion of iron cofactors. Generally, LIP is composed of Fe2+, while some scholars consider LIP to be a buffer system composed of small and large molecules [56,57]. LIP exists not only in the cytoplasm, but also in mitochondria, lysosomes and the nucleus [58]. Inhibiting nuclear receptor coactivator 4 (NCOA4)-mediated ferritinophagy, a type of selective autophagy for the degradation of ferritin by lysosomes, decreases iron storage and limits ferroptosis in cancer cells [59,60]. Poly (RC)-binding proteins (PCBPs) act as iron chaperones, PCBP1 and PCBP2 are the major iron chaperones that play integral roles in intracellular iron trafficking [56]. Ferroportin (FPN) is a transporter protein, that can excrete excessive cellular iron to maintain the balance of iron homeostasis.

2.1.3. Oxidant system

ROS and reactive nitrogen species (RNS) are considered to be markers of ferroptosis and take part in the regulation of cell survival and death [61,62]. On the other hand, the live or death of cells in response to ferroptotic stimuli depend on the balance of injury and the anti-injury response. Glutathione peroxidase 4 (GPX4) has been demonstrated to be a glutathione (GSH)-dependent peroxidase, that can resist the oxidation of lipids in membranes [63]. Suppresses solute carrier family 7 member 11 (SLC7A11) has also been reported as a ferroptosis inhibitor, which is a unit of the glutamate-cystine antiporter x_c^- [64]. Ferroptosis-suppressor-protein 1 (FSP1) regenerates reductive ubiquinol (CoQ10H2) by oxidizing ubiquinone (CoQ10) with nicotinamide adenine dinucleotide phosphate (NADPH) [40,63,65]. CoQ10H2 plays a key role in inhibiting ferroptosis and lipid peroxide production. Tetrahydrobiopterin (BH4), the leading figure in the GTP cyclohydrolase 1 (GCH1)/BH4 pathway, works as an antioxidant for trapping free radicals and supports the synthesis of CoQ10 by converting phenylalanine into tyrosine [66,67]. Erythroid 2-related Factor 2 (NRF2) is a stress-inducible transcription factor, and the target genes of NRF2 are involved in preventing lipid peroxidation and ferroptosis [68]. NRF2 plays a key role in mediating iron not only by regulating solute carrier family 40 member 1 (SLC40A1) but also by regulating SLC7A11 [69,70]. Hypoxic inducible factor (HIF) is a transcriptional complex, and HIF subunits can be divided into three types: HIF-1α, HIF-2α, and HIF-3α [71]. Current studies have reported that HIF-1α can limit ferroptosis by storing lipids in droplets and influencing lipid metabolism, which can attenuate peroxidation-mediated damage [72,73].

2.2. The hallmarks of ferroptosis

2.2.1. Morphological features

Cells undergoing ferroptosis usually show features such as the loss of plasma membrane integrity, cytoplasmic oncosis, swelling of cytoplasmic organelles and moderate chromatin condensation [74]. At the ultrastructural level, mitochondria-induced cysteine starvation, ER-related oxidative stress, lysosomal dysfunction and Golgi stress-related lipid peroxidation all contribute to induction of ferroptosis [75]. Recent evidence suggests that mitochondria-mediated ROS production, deoxyribonucleic acid (DNA) stress and metabolic reprogramming are necessary for the induction of lipid peroxidation and ferroptosis [9,76]. Mitochondria in ferroptotic cells usually exhibit swelling, increased membrane density, reduced or missing crests, and rupture of the outer membrane [77]. The ER in ferroptotic cells is the most critical site of lipid peroxidation during ferroptosis, in which the compounds that regulate ferroptosis by affecting lipid peroxidation are mainly located [78]. Lysosomes are reservoirs of iron, which can cause ferroptosis in principle, and the abnormal increase in Fe^{2+} in lysosomes may promote ferroptosis [79]. There is a close interaction between the excessive accumulation of lipid peroxidation and Golgi dysfunction, which is involved in the regulation of oxidative stress, lipid peroxidation and ferroptosis [80,81].

2.2.2. Biochemical features

Lipid peroxidation, as a marker of ferroptosis, has undergone extensive research. Lipid peroxidation is a reaction of oxidative degradation of lipids caused by oxidative fracture and shortening of lipid carbon chains under the effect of lipid peroxidase or free radicals, resulting in cytotoxic substances such as lipid free radicals, lipid hydroperoxides and reactive aldehydes, and eventually causing cell damage [82]. Lipid peroxidation mainly affects unsaturated fatty acids in the cell membrane. Lipid peroxidation produces lipid hydroperoxides (LOOHs) and reactive aldehydes (e.g., 4-hydroxynonenal (4HNE) and MDA), which increase during ferroptosis [1]. Hydroperoxides are not stable, so for the products of lipid oxidation, 4HNE and MDA are mainly detected [83].

Fe^{2+} is important for metabolic and biochemical processes, such as oxygen transport, energy metabolism and iron-sulfur protein production in mitochondria. As a cofactor, iron regulates the activity of iron-requiring enzymes by forming redox-active (loosely-bound) and redox-silent complexes, thereby playing a complex role in oxidative stress [84]. However, the exact effect of iron on ferroptosis remains unclear [85]. As we know, Iron is mainly stored as a complexe with ferritin (FT) or stored in LIPs in cells [40]. FPN is a transporter protein that can excrete excessive cellular iron to maintain the balance of iron homeostasis. Once iron homeostasis is disrupted, excessive amounts of Fe^{2+} can trigger the Fenton reaction and produce large amounts ROS [86], followed by lipid peroxidation and the induction of ferroptosis [87]. In summary, excess iron ions, can be one of the indicators for the occurrence of iron death in cells.

Ferroptosis is a peroxidation-induced nonapoptotic cell death, and its process is strongly associated with iron and ROS content [83]. ROS are produced by normal physiological processes and are crucial to cell signalling and tissue homeostasis [88]. ROS are oxygen-containing molecules with incomplete low valence, including superoxide (O2•-) peroxides (H_2O_2 and ROOH) and free radicals (HO• and RO•). Excess ROS can be neutralized by reactions catalysed by antioxidants, superoxide dismutase (SOD), GPX4 and catalase. When the balance between the generation and neutralization rates of ROS is broken, oxidative stress leads to the generation of free radicals, impairing DNA proteins and lipids [89]. Elevated levels of intracellular iron and the consumption of the antioxidant GSH are now believed to increase ROS, which in turn triggers lipid peroxidation and ultimately leads to ferroptosis [90].

3. Detection approaches for ferroptosis

As an independent RCD, ferroptosis has a unique cell morphology, which is different from that of other RCDs, such as autophagy, pyroptosis and necroptosis [91]. The process of ferroptosis is complex and involves a great number of metabolites and enzymes. Although there are no characteristic testing methods for ferroptosis yet, many approaches are commonly used in ferroptosis research. We summarized the detection approaches to provide guidance for follow-up research.

3.1. Detection of the cellular state in ferroptosis

At the cellular level, researchers often use these reagents to detect reductions in cell number or changes in cell state, for instance,3-(4, 5-dimethylthiazole-2)-2, 5-diphenyltetrazolium bromide (MMT), cell counting kit-8 (CCK-8), propidium iodide (PI), lactate dehydrogenase (LDH) and adenosine triphosphate (ATP) kits. The MMT, CCK8, LDH and APT kits are colorimetric or stain, and PI can be used with flow cytometry or fluorescence microscopy. The reason for using these methods is that, as an RCD, cell death and cell state changes occur as a consequence of ferroptosis. However, cell viability assays are not specific. Cell viability testing is essential in studies that prove the presence of ferroptosis in the discovery process of disease. The detection approaches are summarized in Table 2.

MMT is reduced to blue purple crystal methyl black by succinate dehydrogenase in the mitochondria of living cells, depositing in cells. A microplate plate analyzer measured the optical density (OD) value at 570 nm. With more viable cells, more deposits are generated, and the OD value is higher. However, MTT is not suitable for suspended cells because the culture medium needs to be removed before dissolving formazan. This step can easily cause the loss of formazan, leading to deviations in the experimental results. However if the culture medium is not removed, the serum and phenol red will affect the experimental results. The main advantage of CCK-8 is that the reaction product is water-soluble and does not require the use of lysis buffer to dissolve the precipitate or the need to absorb the supernatant. It is suitable for both adherent and suspended cells. The detection time and the number of processing steps are reduced, which also greatly improves the detection efficiency and experimental sensitivity. The disadvantage is that the cost of CCK-8 is high, and the color of CCK-8 is light red. cck8 is close to the color of the culture medium containing phenol red, and it is easy to miss or add too much. As a substance similar to MMT, CCK-8 has a parallel application principle, whereas the product can be directly dissolved in water, making it easier to operate. When a ferroptosis inducer is applied to the cells, the OD value will decrease using CCK-8 or MMT to detect cell viability [92]. Trypan blue dye enters cells that are inactivated or have incomplete cell membranes, and blue cells were observed with the aid of a microscope. However, trypan blue staining is generally not used alone but is used together with CCK-8 and MMT [93,94]. In addition, trypan blue acts as an indicator of membrane permeability in combination with flow cytometry, and red fluorescence is tested under the red filter to assess cell survival [95].

Cell death can also be assessed using Hoechst 33,342 and SYTOX Green dual staining. It was then detected using a microscope and the percentage of dead cells was calculated by ImageJ software [54]. SYTOX Green can label the DNA of dead cells to produce green fluorescence, while Hoechst 33,342 can label the DNA of living cells to produce blue fluorescence. Fluorescence microscopes, fluorescence microplate readers, and flow cytometry can all be used to examine these fluorescent probes.

PI, which is a small flurescent molecule, can be used to discriminate dead cells from live cells with intact membranes. PI enters cells that do not possess an intact plasma membrane and bind to DNA [96]. Using PI staining in the study of ferroptosis, the more fluorescence produced, the more cells died [97].

LDH is a stable cytoplasmic enzyme that is present in all cells. When the plasma membrane is damaged, LDH is rapidly released into the cell culture supernatant, which is an important manifestation of apoptosis, necrosis and other forms of cellular damage. The LDH kit is based on the principle that the amount of formazan is proportional to the amount of lactate dehydrogenase in the culture, which in turn is proportional to the number of dead or damaged cells [98]. Therefore, when ferroptosis occurs in cells, the results of the assay will show higher levels of LDH [99,100]. Comparing LDH and MTT, the results show that LDH is simpler, more sensitive, and faster to measure. Since endogenous ATP, a source of energy metabolism for cells, is rapidly hydrolysed upon cell death, measuring its content can reflect cell activity and the number of viable cells [101]. The higher the fluorescence intensity, the stronger the cell activity.

Table 2
Detection of cellular state in ferroptosis.

Reagent	Reaction principle	Detection method	Variation	Ref.
CCK8/MMT	Reagent is reduced to blue purple crystal methyl black by succinate dehydrogenase in the mitochondria of living cells	Microplate plate analyzer, Microplate reader	Decline	[92]
Trypan blue dye	Trypan blue dye can enter inactivated cells or incompleted cell membranes	Microscope	Increase	[93–95]
Hoechst33342 and SYTOX Green	SYTOX Green marks the nucleic acid of dead cells, and Hoechst33342 marks the DNA of living cells	Fluorescence microscope, fluorescence microplate reader, flow cytometer	Green fluorescent increase, blue fluorescent decline	[54]
PI	PI is a small flurescent molecule, can enter the cell and bind to DNA	Fluorescence microscope, Flow cytometer	Increase	[96,97]
LDH	The plasma membrane ruptures and LDH is released	Microplate reader	Increase	[98–100]
CellTiter-Glo Luminescent Assay Kit	Content of ATP can reflect cell activity	Luminometer, Liquid scintillation meter	Decline	[101]

Overall, the detection of cell status or cell activity is commonly used in the study of ferroptosis. CCK-8 should be the most widely used method at present. This may be because the reagent is simple to operate and researchers mainly focus on studying regulatory pathways and mechanisms. In terms of instrument selection, microplate readers and fluorescence microscopes are relatively common in laboratories and are simple to operate, so we recommend using colorimetric methods or fluorescence detection to detect the cellular state. Flow cytometry is an ideal method to study the effects of different factors on the cell cycle and cell activity. Its disadvantage is that the sample must be single cells, and must be processed and detected immediately after staining. At the same time, the operation process of flow cytometry is more complicated than that of ordinary microscopes, microplate readers, etc., requiring dedicated personnel to operate and maintain.

3.2. Detection of subcellular organelles in ferroptosis

The accumulation of lipid peroxide leads to ferroptosis. In recent years, researchershave studied the subcellular membrane containing these oxidized lipids and clarified the contribution of different organelles to ferroptosis. Therefore, detecting changes insubcellular organelles in the process of ferroptosis is helpful to prompte the occurrence of ferroptosis. The detection approaches are summarized in Table 3.

Although the mechanism of the role of mitochondria in ferroptosis is not well defined, we cannot ignore the importance of mitochondria in ferroptosis [112]. Mitochondrial ROS, mitochondrial iron, mitochondrial DNA (mtDNA) and the tricarboxylic acid (TCA) cycle have been found to be strongly associated with ferroptosis [80]. The morphological features of mitochondria are observed as swelling, dense electrons, and reduction or disappearance of mitochondrial cristae [113]. Transmission electron microscopy (TEM) is an ideal method for studying the internal structure of cells. Therefore, using TEM to observe changes in mitochondrial structure is one of the most commonly used methods in ferroptosis research [102,103]. Mitochondria are an essential source of ROS in most of the mammalian cells, and increased mitochondrial ROS promote ferroptosis [114]. Mitochondria-associated antioxidant proteins protect mitochondria from oxidative damage in ferroptosis such as SOD2 [115], GPX4 [114], and microsomal glutathione *S*-transferase 1 (MGST1) [116]. Mito-Tracker Green and MitoSox Red were used to label mitochondria and mitochondrial ROS, and the mitochondria and mitochondrial ROS were observed under a confocal microscope [80]. MitoSOX Red treats cells, and the relative level of mitochondrial fluorescence was quantified by flow cytometry [74,104]. Intracellular iron is transported into the mitochondria to synthesize haem and Fe–S. Haem and Fe–S have been shown to be involved in ferroptosis [117,118]. In research, mtDNA stress activates GAS-STING1 pathway-dependent autophagy to mediate ferroptosis [76]. Increased mitochondrial membrane permeability and decreased mitochondrial membrane potential are alterations characteristic of mitochondrial membranes in ferroptosis [119]. Therefore, the occurrence of ferroptosis can be verified not only by observing the changes in mitochondrial morphology, but also by evaluating the change of mitochondrial membrane potential and permeability. 3,3' -Diethyloxyheterocarbonylcyanine iodine (DIOC6) is a green fluorescent lipophilic dye with cell membrane permeability. It can selectively label mitochondria in living cells, and green fluorescence is weakened when the mitochondrial membrane potential decreases due to ferroptosis [95]. Mito Tracker Deep Red FM

Table 3
Detection of subcellular orgenells in ferroptosis.

	Reagent	Reaction principle	Detection method	Variation	Ref.
Mitochondrial	/	The morphological features of mitochondria are changed.	TEM	Swelling, dense electron, reduction or disappearance of cristae	[102, 103]
	Mito-Tracker Green, MitoSox Red	Increased mitochondrial ROS promote ferroptosis	Confocal microscope	Fluorescence weaken	[80]
	MitoSox Red	Increased mitochondrial ROS promote ferroptosis	Fluorescence microscope, Flow cytometer	Fluorescence weaken	[74, 104]
	DIOC6/MitoTracker Deep Red FM	Increased mitochondrial membrane permeability	Confocal microscope, Fluorescence microscope, Flow cytometer	Fluorescence weaken	[95]
	Tetramethylrhodamine methyl ester (TMRM)/JC-1	Decreased mitochondrial membrane potential	Flow cytometer, fluorescence microscope, fluorescent microplate reader	Fluorescence weaken	[105, 106]
Endoplasmic reticulum	ER-targeting fluorescent probe (PV1)	Endoplasmic reticulum viscosity increase	two-photon phosphorescence lifetime imaging	Viscosity increase	[78]
			Confocal microscope, Fluorescence microscope, Flow cytometer	Fluorescence enhancement	[107]
	ATF4, eukaryotic initiation factor 2, α subunit (eIF2α)	Endoplasmic reticulum stress	western blotting	Increased expression	[108]
Lysosomes	LysoTracker Green	Iron accumulate in lysosomal	Confocal microscope, Fluorescence microscope, Flow cytometer	Fluorescence enhancement	[109]
	–	Cathepsin B (CTSB) is considered as an executioner of ferroptosis	Western blotting	Increased expression	[110]
Golgi	Cis-Golgi marker GM130	Golgi dispersal diminish	Fluorescence microscopy.	Fluorescence weaken	[111]

can also text the changes in mitochondrial membrane permeability. The principle is that the probe enters cells and gathers on active mitochondria, emitting red fluorescence. When mitochondrial permeability is increased and red fluorescence weakens, the possibility of ferroptosis is further proven. Tetramethylrhodamine methyl ester (TMRM) is a permeable dye that accumulates in active mitochondria with intact membrane potential, which is a common method to detect changes in mitochondrial membrane potential [105]. When ferroptosis occurs in cells and mitochondrial membrane potential is lost, TMRM no longer accumulates, and the fluorescence signal becomes weak or disappears. The fluorescence probe JC-1 can determine the mitochondrial membrane potential △Ψm [120, 106].

The ER is an important organelle in eukaryotes and plays a key role in numerous cellular functions. Previous studies have found that the use of ferroptosis inducers can increase ER viscosity, lipid peroxidation and ER stress, and reduce MUFAs. Zinc is transported from the ER to the cytosol, which is also one of the manifestations of ferroptosis [80]. Quantitative measurement of viscosity increase in the ER during ferroptosis using two-photon phosphorescence lifetime imaging [78]. An alternative method to detect viscosity changes in the ER is the ER-targeting fluorescent probe (PV1) [107]. Activating transcription Factor 4 (ATF4) is a key factor in ER stress, and the diversity of ATF4 target genes leads toe multiple biological functions in ferroptosis. Detecting the expression of ER stress-related proteins, such as ATF4, eukaryotic initiation Factor 2, and α subunit (eIF2α), by western blotting is a common way to demonstrate ER stress [108].

Lysosomes are signalling hubs and degradation centresin cells and play significant roles in cellular homeostasis, development, and ageing [121]. Stimulation of cells with erastin or glutamate, a ferroptosis inducer, increases lipid peroxidation, nitric oxide (NO), chthepsins, and iron within lysosomes [80]. Currently, lysosomes act through three mechanisms: (i) accumulation of lysosomal iron or NO; (ii) release of lysosomal cathepsins; and (iii) activation of autophagy [122,123]. The lysosome is a master regulator of iron metabolism. The accumulation of lysosomal NO or iron can promote lysosome-dependent ferroptosis by inducing lipid peroxidation. We will describe later how to detect iron or NO using reagents or probing. Cathepsin B (CTSB) is a lysosomal cathepsin that is considered an executioner of ferroptosis [110]. At present, western blotting is widely used to detect the expression of CTSB [110]. Among several selective autophagy pathways associated with lysosomes, lysosomes reduce ferroptosis through GPX4, SLC40A1, Recombinant Aryl Hydrocarbon Receptor Nuclear Translocator Like Protein (ARNTL), ferritin and lipid droplet (LDs) reduction, etc [80]. These ferroptosis-related genes and proteins can be detected by–qPCR or western blotting.

There is a close interaction between excessive accumulation of lipid peroxidation and Golgi dysfunction, although the mechanism is not yet clear. Several Golgi-dispersing compounds, including AMF-26/M-COPA, brefeldin A, and golgicide A, have also been shown to induce ferroptosis [111]. Golgi morphology was analysed by immunofluorescence staining using the cis-Golgi marker GM130 followed by fluorescence microscopy. Golgi dispersal was substantially diminished during ferroptosis [111] (Fig. 2).

In the current study, mitochondria played a very important role in ferroptosis. In studies on detecting ferroptosis, researchers have prioritizedto detecting changes in mitochondria, whether using TEM to observe changes in mitochondrial biomorphology or detecting changes in mitochondrial membrane potential. Although TEM technology is the most direct technology, it also has shortcomings. During the process of making slices, it is easy to change the membrane structure due to human factors, and other double-membrane organelles will also interfere with the results, leaving great deficiencies in quantitative analysis. However, which organelle changes to focus on is closely related to the researcher's research content.

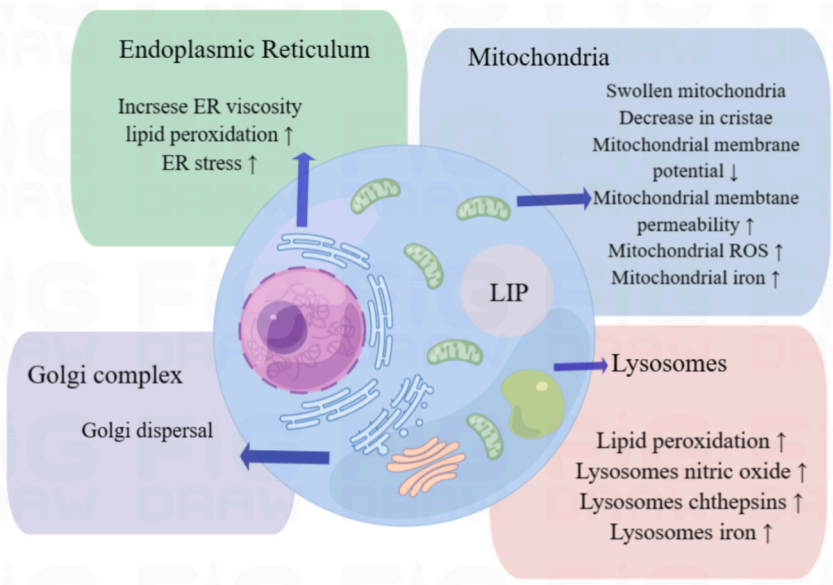

Fig. 2. Detection of subcellular organelles in ferroptosis.

3.3. Detection of genes, proteins and transcription factors in ferroptosis

There are many genes, transcription factors, and proteins involved in ferroptosis, which is a complex metabolic process. Real-time quantitative reverse transcription-PCR (RT qPCR) and western blotting are widely used in ferroptosis research as conventional detection techniques. RT qPCR adds fluorophores to the PCR and analyses the initial amount of target gene by continuously monitoring the fluorescence signal and signal strength. By binding specific antibodies to particular proteins in cell or biological tissue samples, Western blot analyses the colouring position and depth to obtain the expression of these special proteins [124]. Ferroptosis is caused by iron-dependent lipid peroxidation. The genes and proteins involved in ferroptosis are primarily related to lipid metabolism, iron metabolism, and the antioxidant system, as listed in Table 4.

ACSL4 and LPCAT3, as the first identified promoters of ferroptosis, have been extensively studied in ferroptosis experiments. ACSL4 is a key enzyme that catalyses the activation of long-chain fatty acids [138]. ACSL4 determines ferroptosis sensitivity by altering the cellular lipid composition and acts as a specific biomarker and driver of ferroptosis. ACSL4 is more biased towards PUFAs such as adrenaline (ADA) and arachidonic acid (AA) and catalyses them into ADA-CoA and AA-CoA [139]. PE-AA and PE-AdA mainly catalysed FUFA-CoA through LPCAT3. These products can trigger lipid peroxidation under the action of LOXs or free ferrous iron. However, the contribution of LOXs to ferroptosis and lipid peroxidation is still controversial [140]. ACSL3 is a negative regulatory gene of ferroptosis that can reduce the sensitivity of cancer cells to ferroptosis [85]. This may be related to ACSL3 promoting LD formation and maturation and activating MUFAs [43,141]. SCD1 is a lipid-modifying enzyme that is upregulated in many malignancies, especially ovarian cancer [47,142]. In tumours, high expression of SCD1 increases tumor resistance to ferroptosis inducers and may promote tumor recurrence [127]. In summary, the increased expression of ACSL4, LPCAT3 and LOXs is a favourable factor for promoting ferroptosis, among which ACSL4 has been studied the most. Increased expression of ACSL3 and SCD1 can inhibit ferroptosis, and research on SCD1 has mainly focused on cancer. PTGS2 is usually considered a biomarker but not a driver of ferroptosis [37]. PTGS2 expression increases when ferroptosis occurs. Commonly used detection methods are mainly qPCR and western blotting, and some scholars use immunohistochemistry.

Transferrin receptor 1 (TFR1), a carrier protein that transports Fe3+ from the extracellular space to the intracellular space, is the first step in causing iron overload. Currently, TFR1 is mainly used as a marker of ferroptosis. Scholars have made an important discovery in their research: the accumulation of TfR1 on the cell surface is a feature of ferroptosis [143]. When ferroptosis occurs, there is usually an increase in TFR1, and the most common detection method is western blotting, followed by qPCR [144,145]. NCOA4 is a selective cargo receptor that mediates autophagic degradation of ferritin to maintain intracellular iron homeostasis. In NCOA4-mediated ferritinophagy, NCOA4 increases, accompanied by decreases in FTL and FTH1 [132]. FPN is a protein that maintains intracellular iron balance by transporting Fe2+ out of the cell. Ferroptosis can be induced by inhibiting the expression of FPN [146]. Regarding the genes and proteins related to iron metabolism, the most studied are FTH1 and NCOA4, followed by TFR1 and FTL.

The system x_c^-/GSH/GPX4 pathway is the earliest and most important regulatory pathway. The source of cysteine, the biosynthesis of GSH, and the function of GPX4 are central to controlling ferroptosis [147]. The system x_c^- exchanges the extracellular and intracellular cystine and glutamate in a1:1 ratio, in which solute carrier family 3 member 2 (SLC3A2) transports glutamate out of the cell, and SLC7A11 transports cystine into the cell for GSH biosynthesis [148,149]. Then, under the action of glutamylcysteine synthetase (GCS) and glutathione synthetase (GSS), glutamate, cysteine and glycine are synthesized into GSH. GSH is the primary cofactor for GPX4 synthesis. GSH can improve the reaction of GPX4 with intracellular phospholipid hydroperoxides (PLOOHs) to reduce the corresponding phospholipid alcohols (PLOHs), thereby reducing the occurrence of ferroptosis [150]. The study found that the expression or activity of GPX4 is controlled by selenium and GSH. Therefore, researchers most commonly use qPCR, western blotting

Table 4
Detection of gene and protein in ferroptosis.

Gene/Protein	Function	Variation	Ref.
Gene and protein of lipid metabolism			
ACSL4	Key enzyme of catalyses the activation of long-chain fatty acids	Increase	[125]
LPCAT3	Catalysed FUFA-CoA produce PE-AA and PE-AdA	Increase	[126]
LOXs	Assist lipid peroxidation	Increase	[126]
SCD1	Increases tumor resistance to ferroptosis inducers	Increase	[127]
ACSL3	Promoting LD formation and maturation and activating MUFA	Decline	[128]
PTGS2	Marker of ferroptosis	Increase	[129]
Gene and protein of iron metabolism			
TFR1	Transports Fe 3+ from extracellular to intracellular	Increase	[130]
NCOA4	Mediates autophagic degradation of ferritin to maintain intracellular iron homeostasis	Increase	[131]
FTL/FTH1	Store iron ions	Decline	[132]
FPN	Transports Fe 2+ from intracellular to extracellular	Decline	[133]
Gene and protein of antioxidant system			
SLC7A11	Transport cystine	Decline	[126]
GPX4	Important antioxidant	Decline	[125]
GSH	Primary cofactor for GPX4 synthesis	Decline	[134,135]
FSP1	Regenerate the reduced form of CoQ10	Decline	[136]
CoQ10	Protecting against the lipid peroxidation that drives ferroptosis.	Decline	[63]
GCH1	Influence initiation and rate-limiting of BH4 synthesis	Decline	[66,137]
BH4	Inhibit lipid peroxidation	Decline	[66]

and immunohistochemistry to demonstrate that ferroptosis may have occurred, by verifying decreased expression of GPX4 or SLC7A11 [151]. Quantification of GSH using the GSH-GSH/Glutathione, Oxydized (GSSG) Ratio Detection Assay Kit allows assessment of intracellular GSH levels [134,135]. NADPH is an essential electron donor in all living organisms and is a biomarker of ferroptosis sensitivity. NADP is the oxidized form of NADPH. The NADP/NADPH system is used by many ferroptosis regulators to regulate electron transfer, such as GPX4 and NOX, [152]. The available evidence suggests that a lower NADP/NADPH ratio or higher levels of NADPH promote resistance to ferroptosis. For the research of NADPH, scholars mainly use the NADP/NADPH-Glo Assay Kit to detect the ratio of NADP/NADPH [135,153]. The FSP1/CoQ10 pathway and GCH1/BH4 pathway are pathways that inhibit ferroptosis and are independent of the GPX4 pathway [66,154]. At present, their regulatory mechanism for ferroptosis still needs to be further studied, and ferroptosis is detected by western blotting and qPCR [34,155]. Determination of BH4 levels was measured using high-performance liquid chromatography (HPLC) [156].

3.4. Detection of various reaction products in ferroptosis

When ferroptosis occurs, reaction products, such as ROS, Fe, and lipid peroxidation, usually change in the cytoplasm. The detection approaches for these products are summarized in Table 5.

The increase in intracellular iron ions will trigger ferroptosis, and excessive ROS will be generated through the Fenton reaction [23]. Part of the iron ions in cells is stored in ferritin in the form of compounds, and the other part is stored in LIP. We have briefly described the metabolism of iron in cells. Now we will mainly describe the detection method and reagents used for intracellular iron ions. In research on ferroptosis, FerroOrange or Phen green SK (PGSK) are commonly used to detect Fe^{2+}, as observed by fluorescence microscopy or flow cytometry [157–159]. FeRhoNox-1 is a probe that combines with Fe^{2+} to produce an irreversible orange-red fluorescent substance, and the change in intracellular iron is observed by flow cytometry or microscopy [54]. The Iron Assay Kit detects ferrous iron in tissues and cells, and the content of iron ions is reflected by the od value of the microplate reader [145]. And LysoTracker Green is a probe that labels iron ions in lysosomes. The cell photographs were captured by a confocal microscope to acquire green fluorescent lysosomes [109]. In other studies, ferrous ions can be detected using these probes, such as FIP-1, IP-1, Probe 3, RhoNox-1, SiRhoNox-1, and ICL-1. CP655, FD1, Sensor 1, FS1, BOD-NHOH, etc., can be used to detect ferric ions [58].

Ferroptosis is a peroxidation-induced nonapoptotic cell death, and its process is strongly associated with iron and ROS content [83]. ROS are produced by normal physiological processes and are essential for cell signalling and tissue homeostasis [88]. ROS detection is chiefly divided into three categories, namely, intracellular ROS, lipid ROS and mitochondrial ROS. Intracellular ROS are often detected by 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), a probe specific for hydrogen peroxide (H_2O_2), and green fluorescence is observed when ROS increase [109,166]. Another method to test cellular ROS is dihydroethidium (DHE), which can freely enter the cell through the living cell membrane and is oxidized by intracellular ROS to produce ethidium oxide. Then, ethidium oxide is incorporated into chromosomal DNA to produce red fluorescence [167,160].

In addition to ROS, RNS also cause ferroptosis [168,169]. Peroxynitrite (ONOO-) and NO can react with unsaturated fatty acids to form nitrated oxidation products [170]. ONOO- and NO are representative RNS [171]. RNS can attack PUFAs, which in intracellular organelles and plasma membranes produce lipid peroxides [68]. 4-Amino-5-Methylamino-2′,7′-Difluorofluorescein Diacetate (DAF-FM Diacetate) can combine with NO production to produce a fluorescent product visualizing the fluorescent imaging by using inverted confocal microscopy [61]. HKYellow-1 and BTMO-PN are newly developed highly selective and sensitive ONOO- probes that which were used to detect the intracellular ONOO- level. Using instruments such as confocal microscopy, flow cytometry and fluorescence microscopy, the intracellular ONOO- level can be detected [61,162].

Ferroptosis is a form of regulated cell death that relies on iron and is characterized by the accumulation of lipid peroxides, resulting

Table 5
Detection of various reaction products in ferroptosis.

	Reagent	Reaction principle	Detection method	Variation	Ref.
Fe^{2+}	FerroOrange, PGSK, FeRhoNox-1	Increase of intracellular Fe^{2+} will trigger ferroptosis	Confocal microscope, Fluorescence microscope, Flow cytometer	Fluorescence enhancement	[157–159]
	Iron Assay Kit		Microplate reader	Increase	[145]
ROS	DCFH-DA, DHE	ROS leads to oxidative stress	Confocal microscope, Fluorescence microscope, Flow cytometer	Fluorescence enhancement	[109,160, 161]
RNS	DAF-FM Diacetate	NO mediate lipid peroxidation	Confocal microscope	Fluorescence enhancement	[61]
	HKYellow-1, BTMO-PN	ONOO- mediate lipid peroxidation and lipid peroxidation	Confocal microscope, Flow cytometry, Fluorescence microscope	Fluorescence enhancement	[61,162]
Lipid peroxidization products	C11-BODIPY	Lipid peroxidation is increased in ferroptosis	Fluorescence microscope, fluorescence microplate reader, flow cytometer	Increase	[163]
	MDA Assay Kit	MDA is main lipid peroxidization products	Microplate reader	Increase	[164]
	4-HNEAssay Kit	4-HNE is main lipid peroxidization products	Microplate reader	Increase	[164]
	TBARS	TBARS can detect MDA and some minor related compounds	Microplate reader	Increase	[165]

in oncotic cell swelling and eventual disruption of cellular membranes. Lipid peroxidation, a hallmark of ferroptosis, refers to the oxidative deterioration of lipids that contain carbon double bonds, particularly polyunsaturated fatty acids [172]. The cytotoxicity of lipid peroxidation products is manifested in two aspects: on the one hand, it influences the physical properties of the lipid bilayer, thereby changing membrane fluidity and membrane permeability; on the other hand, the degradation products naturally formed by lipid peroxidation cause toxicity to cells [1]. Currently, one of the most commonly used and effective methods is the C11-BODIPY assay, which utilizes a fluorescent probe that selectively sensitizes lipid peroxidation in cell membranes [172]. C11-BODIPY, as a lipid-soluble ratio fluorescent probe, can indicate lipid peroxidation and antioxidant properties in membrane systems and living cells, and is commonly used to measure intracellular lipid peroxides [173]. When intracellular lipid peroxides gaining, the increasing oxidation state of C11-BODIPY can be detected and the fluorescence color changes from red to green. Another way to detect lipid oxidation is to examine their products [174]. The two lipid peroxidation products investigated are 4-HNE and MDA. The reaction of MDA with thiobarbituric acid produces a chromophore whose concentration can be quantified by absorbance, and then the test kit can measure lipid peroxidation [175–177]. Moreover, the reaction of the aldehyde group of 4-HNE with 2, 4-dinitrophenylhydrazine can measure the degree of carbonylation of proteins in biological samples [164]. Some scholars use thiobarbituric acid-reactive substance (TBARS) to measure lipid peroxidation in cells. TBARS was measured at 532 nm using a microplate reader [165]. The MDA detection kit is the most widely used in the study of lipid peroxidation. The MDA kit can detect MDA not only in cells, but also in tissues. Since cells contain less MDA, the number of cells should be as large as possible so that the trend in the test results may be more obvious.

During the occurrence and development of ferroptosis, not all metabolites produced within cells are specific. For example, ROS are a common metabolites in CRD, such as apoptosis [178] and autophagy [179]. Therefore, detecting the increase in ROS alone cannot determine the occurrence of ferroptosis. Ferroptosis is an RCD caused by iron-dependent lipid peroxidation. Detection of iron ions and lipid peroxidation is important for ferroptosis, especially lipid peroxidation.

4. Summary and prospects

Ferroptosis is a new RCD and has been reported to be related to many diseases [12,180,181]. Therefore, it is of great significance to detect the occurrence and development of ferroptosis in diseases. Brent R. Stockwell, who is an authoritative scholar of ferroptosis, proposed the following suggestions: 1) markers should be detected prior to cell death; 2) a minimum of three markers need to be detected, and lipid peroxidation should be detected; 3) markers of lipid peroxidation and expression of TFR1 can be inhibited by ferroptosis inhibitors; and 4) expression of some gene markers should not be suppressed by ferroptosis inhibitors [5]. Ferroptosis is driven by iron-dependent lipid peroxidation, and it is critical to detect such lipid peroxidation events during ferroptosis. While some markers may be activated by these other types of stress, if at least three markers are used or a suitable combination of two markers (such as lipid peroxidation and TfR1 mobilization), ferroptosis can be distinguished from these other stress conditions [5]. The markers may mitochondria typically exhibit shrunken, dense morphologies, and specific gene expression changes can be detected, such as PTGS2 SLC7A11, ACSL4, and TfR1 upregulation and movement to the plasma membrane.

In this review, we presented ferroptosis from the perspectives of cells, subcellular organelles and gene proteins and summarized the most commonly used detection methods, principles and reagents of ferroptosis in past 5 years to provide more choices for ferroptosis studies.

Although ferroptosis has been proven to play an important role in the development of various organisms and diseases, there is no gold standard for the detection of ferroptosis. In addition, it is more challenging to monitor ferroptosis in vivo than in vitro. However, we still hope to provide some reference for scholars who are studying or want to study ferroptosis. The choice of ferroptosis detection method is not fixed and is related to the experimental purpose, equipment, resources and professional knowledge. If the purpose of the experiment is to demonstrate a disease that has not yet been shown to be associated with ferroptosis. Detection of cell viability, lipid peroxidation, mitochondrial changes,/and changes in ferroptosis-related genes or proteins is necessary. In the detection of lipid peroxidation, we hope that it is not a single detection method, but should try to detect lipid peroxidation from multiple aspects and angles, such as using C11-BODIPY and MDA kits. For changes in mitochondria, researchers who do not have the conditions to use TEM can also consider testing mitochondrial membrane potential and mitochondrial membrane permeability, both of which are often tested together. There are many genes and regulatory factors involved in ferroptosis. The best way is to complete it through transcriptomic sequencing. This method covers all genes, reduces the workload and is more convincing, but it is expensive. Researchers with limited research funds also choose representative genes or proteins as detection targets. The final point is that using inhibitors of ferroptosis can reverse the results we detected previously. Those who want to develop new treatment options by inhibiting or promoting ferroptosis need to detect relevant indicators that reflect the progression of the disease in addition to the detection methods mentioned above.

5. Conclusion

As a new route of RCD, ferroptosis plays an important role in the prevention and treatment of various diseases. The process of ferroptosis is complex and involves a great number of metabolites and enzymes. However, the gold standard marker or detection method of ferroptosis has not yet been established. Therefore, it is crucial to choose the most suitable detection approaches for ferroptosis according to the purpose and experimental conditions. In this review, we summarize the commonly used reagents and detection methods in ferroptosis research to provide some guidance for follow-up research.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Data availability statement

Data included in article/supplementary material/referenced in article.

Additional information

No additional information is available for this paper.

CRediT authorship contribution statement

Zheyi Chen: Writing – original draft. **Hongbing Lin:** Writing – original draft. **Xiaoyu Wang:** Writing – review & editing. **Guiqi Li:** Software. **Na Liu:** Software. **Manli Zhang:** Supervision. **Yuqin Shen:** Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no competing interests.

Acknowledgements

Not applicable.

Abbreviations

RCD	regulatory cell death
ROS	reactive oxygen species
MDA	malondialdehyde
qPCR	quantitative polymerase chain reaction
RT-qPCR	Real-time quantitative reverse transcription-PCR
PUFAs	polyunsaturated fatty acids
ACSL4	Acylcoenzyme A (CoA) synthetase long-chain family member 4
LPCAT3	lysophosphatidylcholine acyltransferase 3
ALOX	arachidonic acid lipoxygenase
MUFA	monounsaturated fatty acid
ACSL3	Acylcoenzyme A (CoA) synthetase long-chain family member 3
SCD1	stearoyl-CoA desaturase 1
PTGS2	Prostaglandin-endoperoxide synthase 2
COX-2	cyclooxygenase-2
ER	endoplasmic reticulum
TFRC	Transferrin receptor protein
STEAP3	six transmembrane epithelial antigen 3
SLC11A2	solute carrier family 11 member 2
DMT1	divalent metal-ion transporter-1
FTL	ferritin light chain
FTH1	ferritin heavy chain 1
NCOA4	nuclear receptor coactivator 4
PCBPs	Poly (RC)-binding proteins
FPN	Ferroportin
RNS	reactive nitrogen species
GPX4	Glutathione peroxidase 4
GSH	glutathione
SLC7A11	family 7 member 11
FSP1	Ferroptosis-suppressor-protein 1
CoQ10H2	ubiquinol

CoQ10 ubiquinone
NADPH nicotinamide adenine dinucleotide phosphate
BH4 Tetrahydrobiopterin
GCH1 GTP cyclohydrolase 1
NRF2 Erythroid 2-related factor 2
HIF Hypoxic inducible factor
DNA Deoxyribonucleic acid
LOOHs lipid hydroperoxides
4HNE 4-hydroxynonenal
FT ferritin
LIP labile iron pool
SOD superoxide dismutase (SOD)
MMT 3-(4, 5-dimethylthiazole-2)-2, 5-diphenyltetrazolium bromide
CCK-8 cell counting kit-8
PI propidium iodide
LDH lactate dehydrogenase
ATP adenosine triphosphate
OD optical density
mtDNA Mitochondrial DNA
TCA tricarboxylic acid
TEM Transmission Electron Microscopy
MGST1 microsomal glutathione *S*-transferase 1
DIOC6 3,3' -Diethyloxyheterocarbonylcyanine iodine
TMRM Tetramethylrhodamine methyl ester
ATF4 Activating transcription factor 4 (ATF4)
eIF2α eukaryotic initiation factor 2, α subunit
NO nitric oxide
CTSB Cathepsin B
ARNTL Recombinant Aryl Hydrocarbon Receptor Nuclear Translocator Like Protein
LDs lipid droplets
ADA adrenaline
AA arachidonic acid
TFR1 Transferrin Receptor 1
SLC3A2 solute carrier family 3 member 2
GSS glutathione synthetase
PLOOHs phospholipid hydroperoxides
PLOHs phospholipid alcohols
GSSG
Glutathione, Oxydized
HPLC high-performance liquid chromatography
PGSK Phen green SK
DCFH-DA 2′,7′-dichlorodihydrofluorescein diacetate
H_2O_2 hydrogen peroxide
DHE dihydroethidium
DAF-FM Diacetate 4-Amino-5-Methylamino-2′,7′-Difluorofluorescein Diacetate
TBARS thiobarbituric acid-reactive substance

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