POLITECNICO DI MILANO

Department CMIC

Lecture 17 – FR17





Free-Radicals: Chemistry and Biology

Prof. Attilio Citterio

Dipartimento CMIC “Giulio Natta”

http://iscamap.chem.polimi.it/citterio/education/free-radical-chemistry/

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Reactive Oxygen Species (ROS) Oxygen Centered Radicals

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Reactive Oxygen Species (ROS)

Radicals:	
$O_2^{\bullet -}$	Superoxide
$HO^{\bullet}$	Hydroxyl
$RO_2^{\bullet}$	Peroxyl
$RO^{\bullet}$	Alkoxyl
$HO_2^{\bullet}$	Hydroperoxyl

Non-Radicals:	
H_2O_2	Hydrogen peroxide
$HOCl$	Hypochlorous acid
O_3	Ozone
$^1O_2$	Singlet oxygen
$ONOO^-$	Peroxynitrite

ROS

Hydroxyl radical and high valent metal oxo species

- **Highly reactive, indiscriminant oxidants**
- **Redox metal ions usually involved in generation**

Superoxide

- ***Reactive but highly selective***
- ***Most vulnerable are labile Fe-S clusters***

Hydrogen peroxide

- **Relatively unreactive except as precursor to hydroxyl radicals**

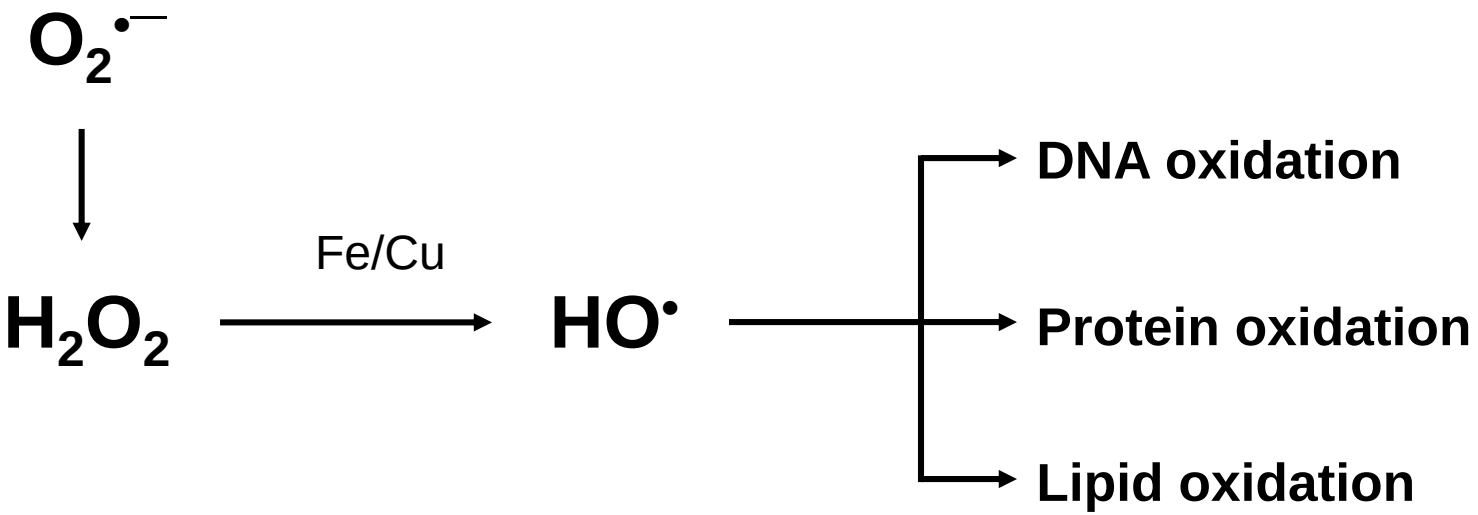
(Peroxynitrite)

- ***Controversial---not dealt with here***

Dioxygen itself is not the primary agent of oxidative stress.

- ***It is the precursor of all of the ROS and reacts extremely rapidly with organic free radicals, when they are present.***

Cytotoxicity of ROS



The role of ROS as a signal molecules has gained increasing attention. The cytotoxicity of ROS may be associated with the ability of ROS to signal distinct pathways, such as the NFkB pathway, to induce pathology.

Oxidant Sources and Antioxidant defense

Sources of ROS

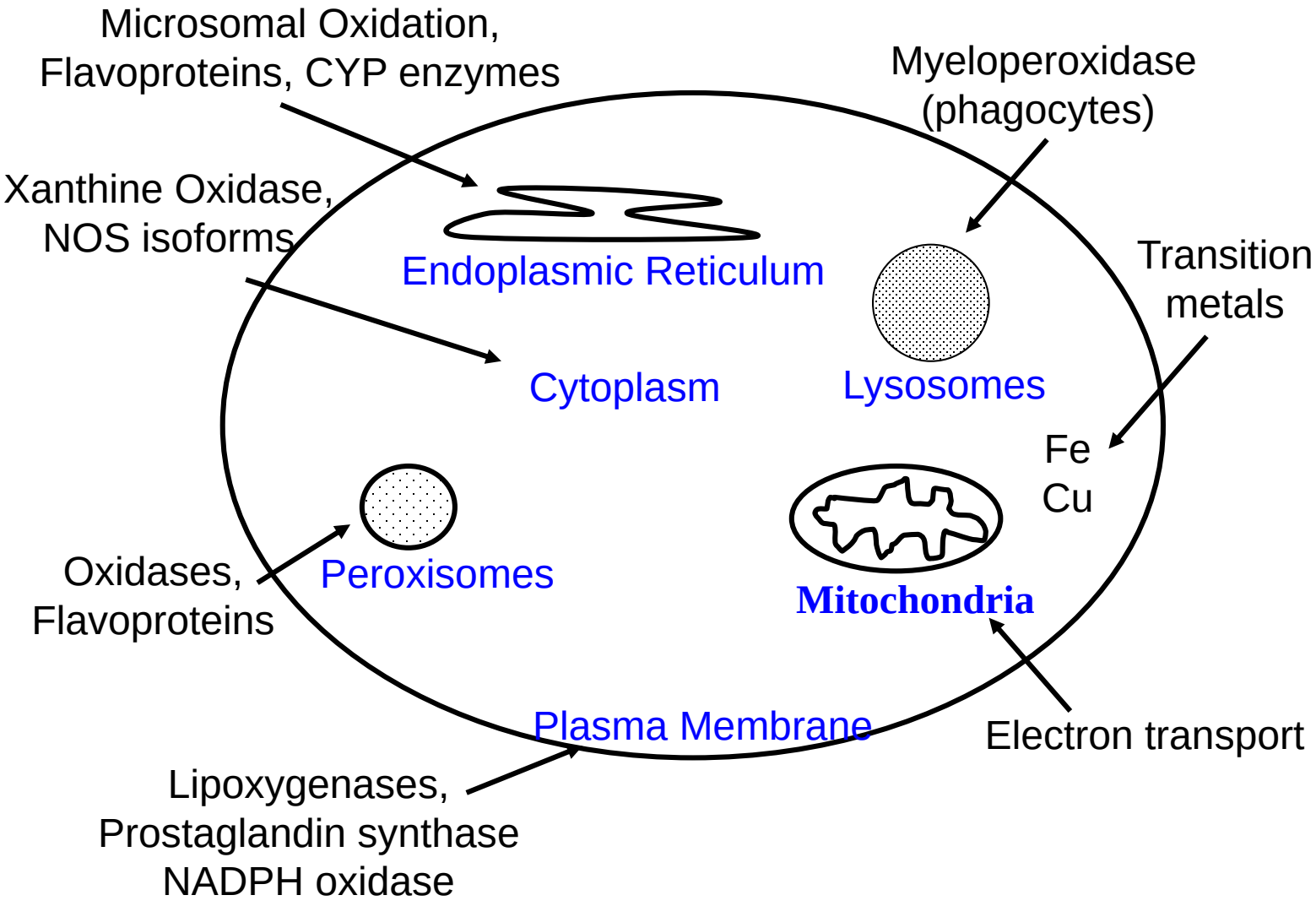
Endogenous	*Exogenous*
NAD(P)H oxidase	NAD(P)H oxidase
Mitocondria	Mitocondria
Peroxisomes	Peroxisomes
Cytochrome P450	Cytochrome P450
Xanthine oxidase	Xanthine oxidase
Cyclooxygenase	Cyclooxygenase
Lipoxygenases	Lipoxygenase
γ-Glutamyl transpeptidase	γ-Glutamyl transpeptidase

ROS

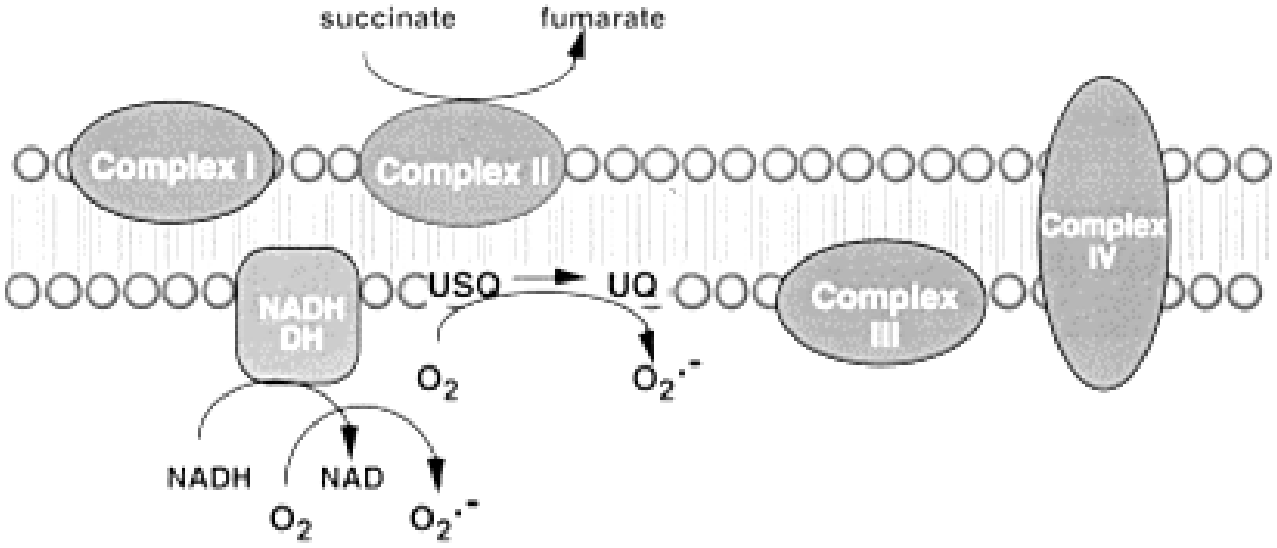
Antioxidant defense

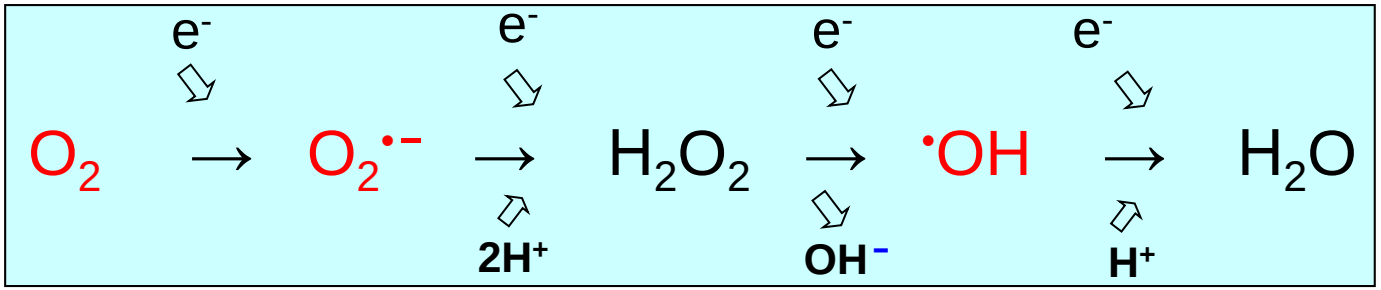
Enzymatic	*Non-enzymatic*
Superoxide dismutase	Glutathione
Catalase	Thioredoxin
Glutathione peroxidase	Glutaredoxin
Prion protein (PrP')	Vitamins C, A, E
	Lipoate
	Urate
	Ubiquinone
	Pyruvate

Endogenous Sources of ROS and RNS



Mitochondria as a source of ROS

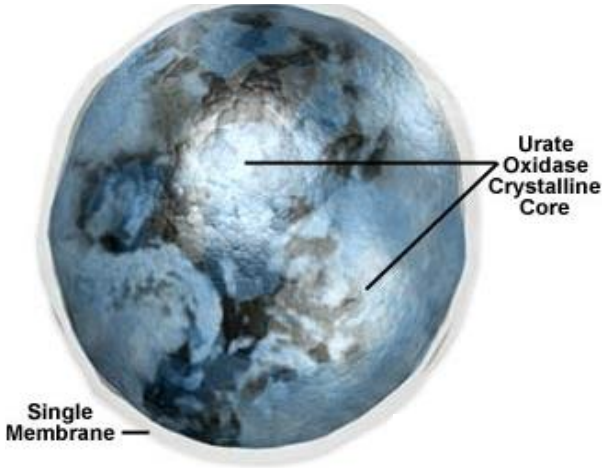




The source of mitochondrial ROS appears to involve a non-heme iron protein that transfers electrons to oxygen. This occurs primarily at Complex I (NADH-coenzyme Q) and, to a lesser extent, following the auto-oxidation of coenzyme Q from the Complex II (succinate-coenzyme Q) and/or Complex III (coenzyme QH2-cytochrome c reductases) sites. The precise contribution of each site to total mitochondrial ROS production is probably determined by local conditions including chemical/physical damage to the mitochondria, O_2 availability, presence of xenobiotics.

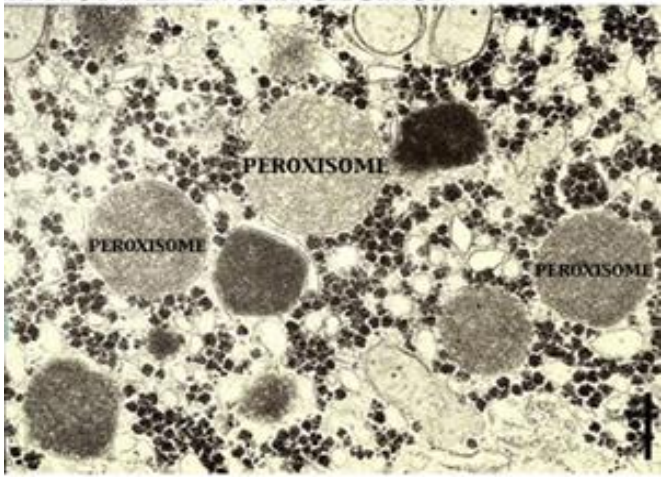
From Kehrer JP (2000) Toxicology 149: 43-50

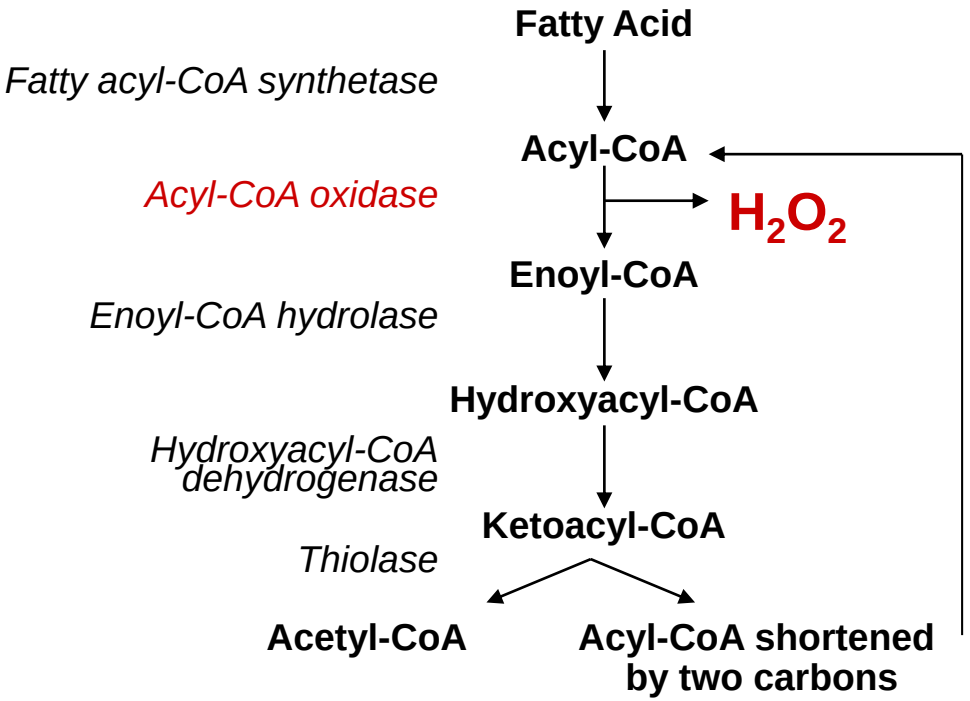
PEROXISOME



- **β-oxidation of fatty acids**
- **bile acid synthesis**
- **purine and polyamine catabolism**
- **amino acid catabolism**
- **oxygen metabolism**

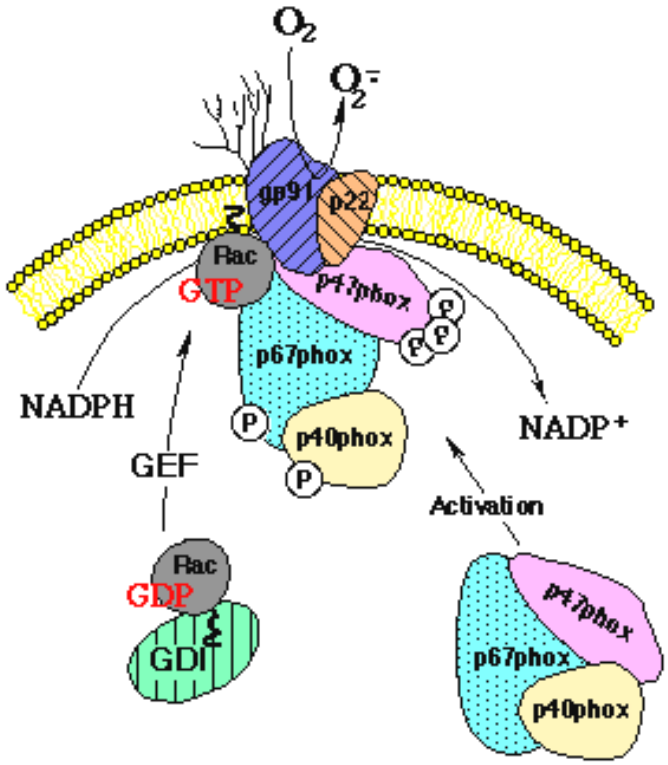
PEROXISOMES AS SEEN IN A HUMAN LIVER SECTION

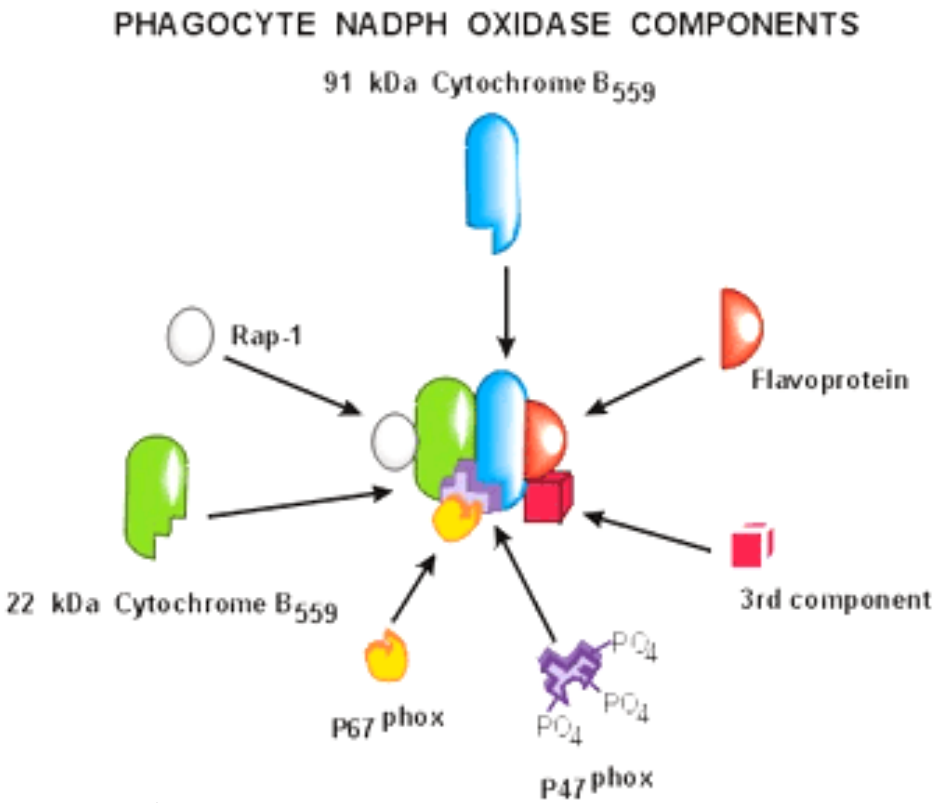


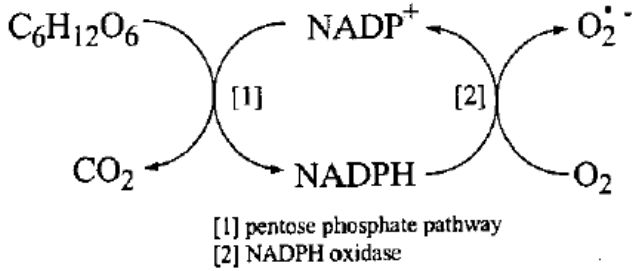


NADPH oxidase as a source of ROS

Mainly in neutrophils (oxidative burst), but also in many other cell types

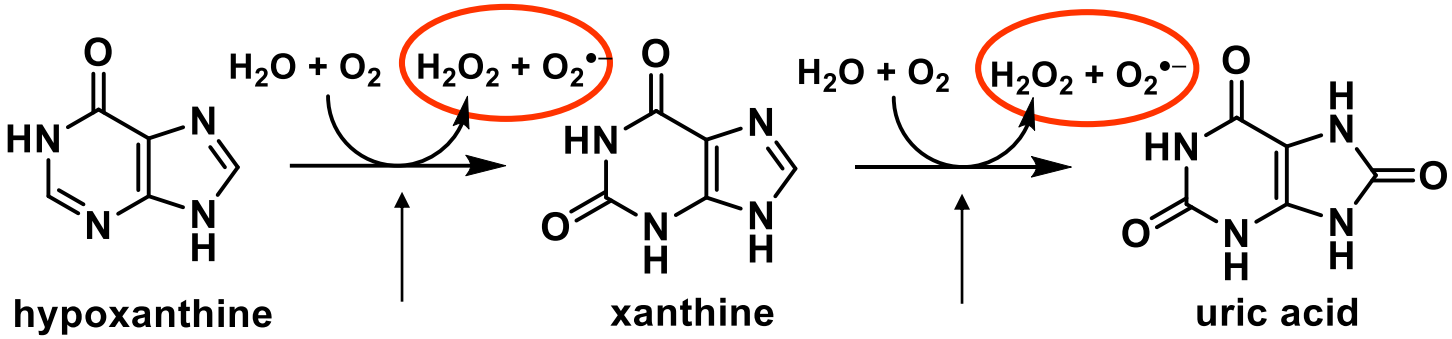






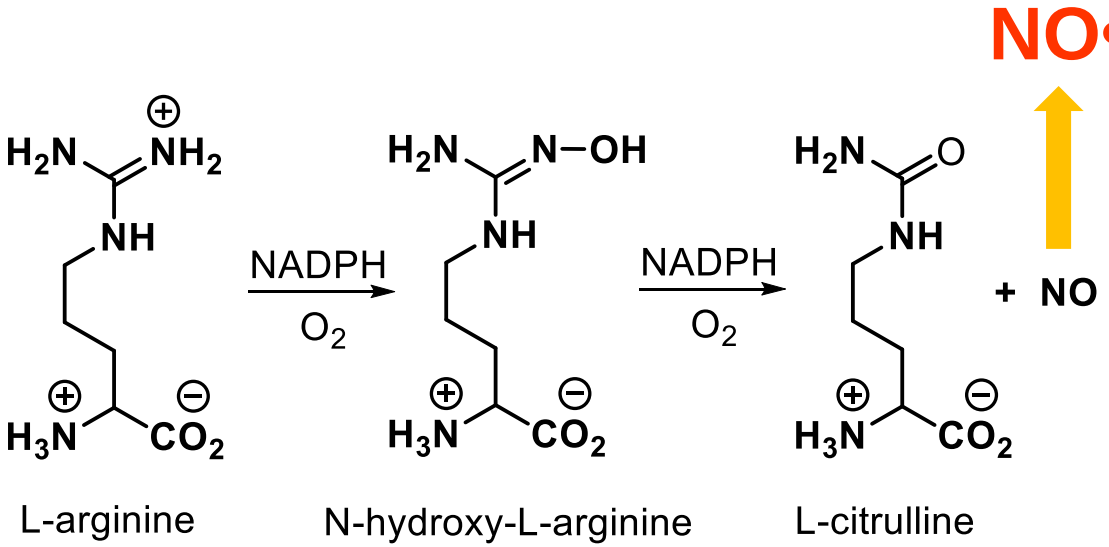
[1] pentose phosphate pathway
[2] NADPH oxidase

Cytoplasmic sources of ROS

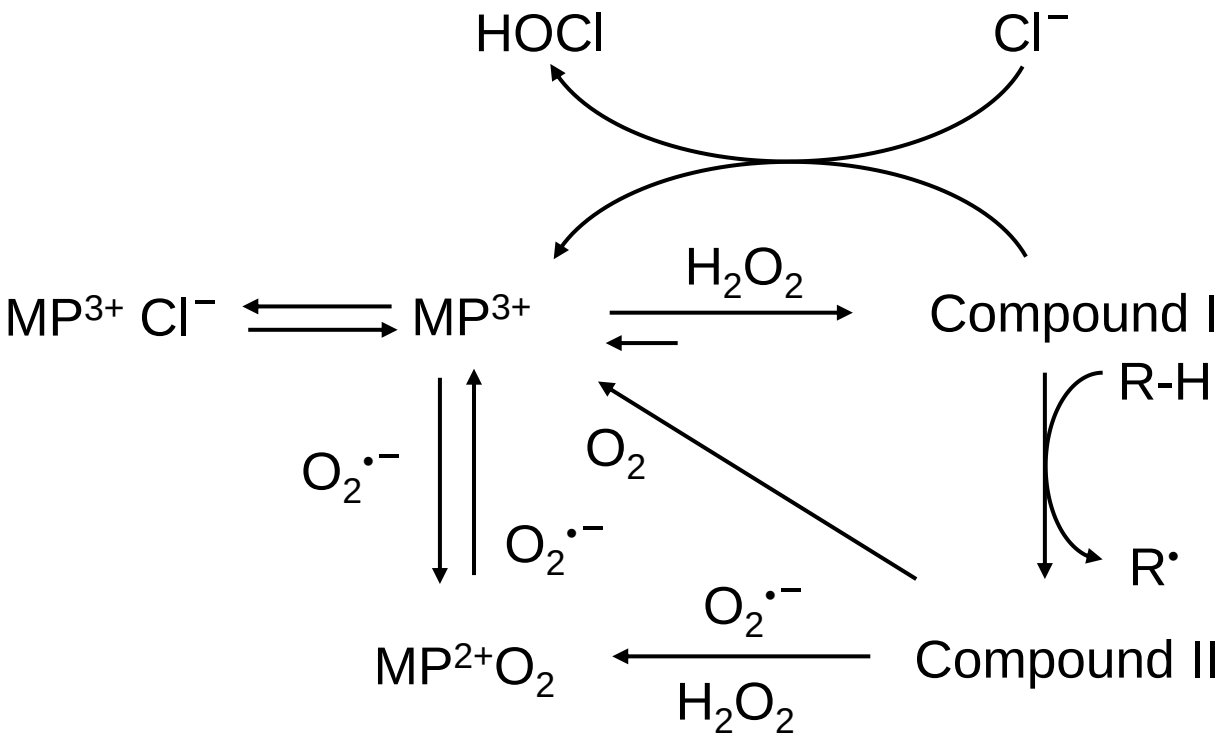


Nitric Oxide Synthases (NOS):

neuronal	nNOS (I)
endothelial	eNOS (III)
inducible	iNOS (II)

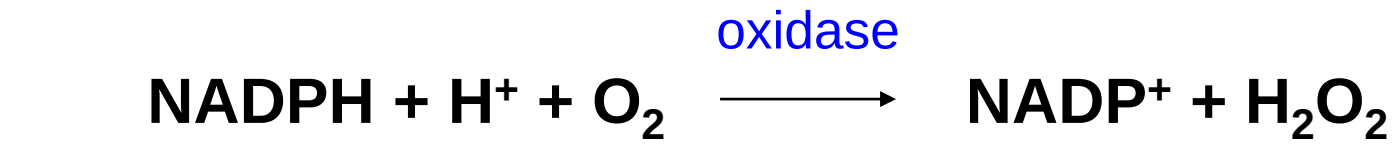


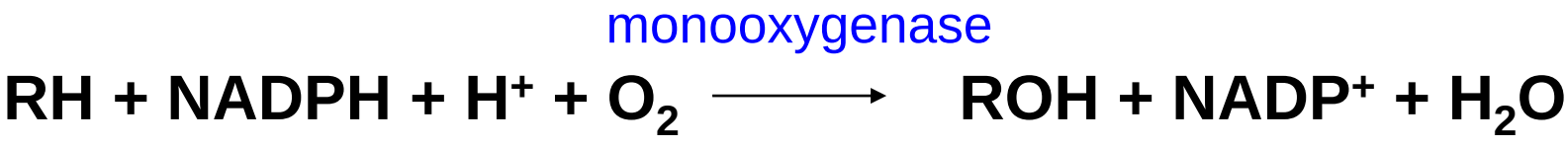
Lysosome as a source of ROS (Clorox!)

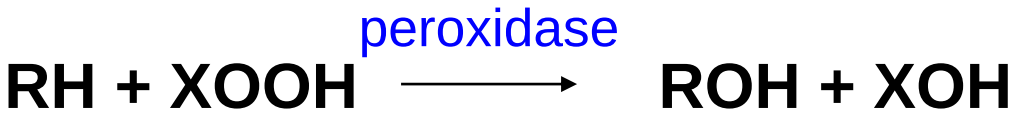


Myeloperoxidase undergoes a complex array of redox transformations and produces HOCl, degrades H_2O_2 to oxygen and water, converts tyrosine and other phenols and anilines to free radicals, and hydroxylates aromatic substrates via a cytochrome P450 type activity.

Microsomes as a source of ROS



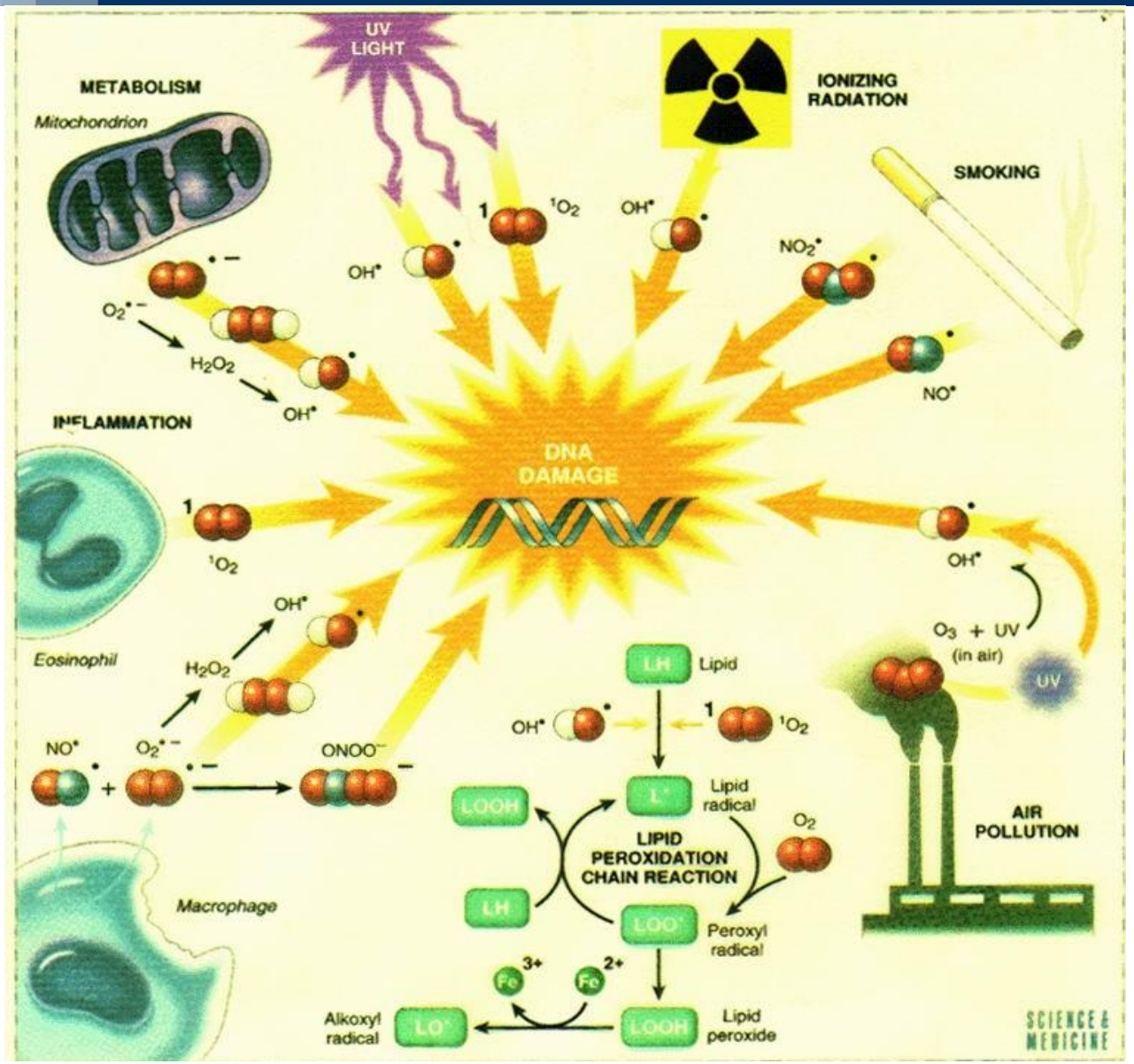




Exogenous sources of free radicals

- **Radiation**
  - UV light, x-rays, gamma rays
- **Chemicals that react to form peroxides**
  - Ozone and singlet oxygen
- **Chemicals that promote superoxide formation**
  - Quinones, nitro aromatics, bipyrimidiulium herbicides
- **Chemicals that are metabolized to radicals**
  - e.g., polyhalogenated alkanes, phenols, amino phenols
- **Chemicals that release iron**
  - ferritin

Reactive Oxygen Species (ROS)



Free Radicals can work as Second Messengers

Second messengers should be:

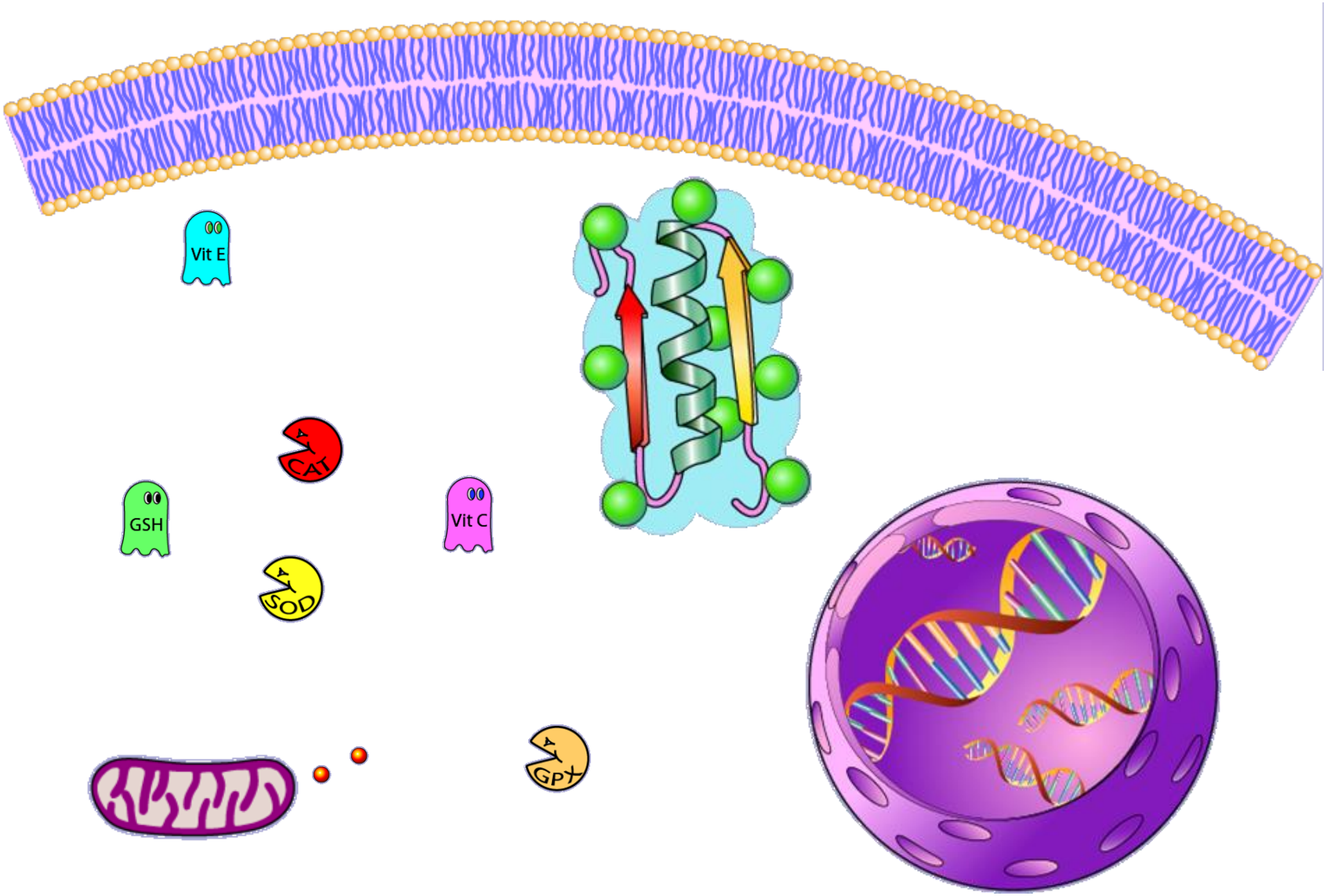
- Short lived (concentrations can change rapidly)
- Enzymatically generated in response to stimulant
- Enzymatically degraded
- Specific in action (?)

Some free radicals fit these criteria!

- $O_2^{\bullet -}$, H_2O_2 , $NO^{\bullet}$, $ONOO^-$

How Free Radicals can be Involved in Signaling?

- Heme oxidation
- Oxidation of iron-sulfur centers in proteins
- Changes in thiol/disulfide redox state of the cell
- Change in conformation → change in activity
- Oxidative modification of proteins: degradation, loss of function, or gain of function
- Oxidative modification of DNA: activation of repair, and/or apoptosis
- Oxidative modification of lipids: disruption of membrane-associated signaling, DNA damage, and formation of protein adducts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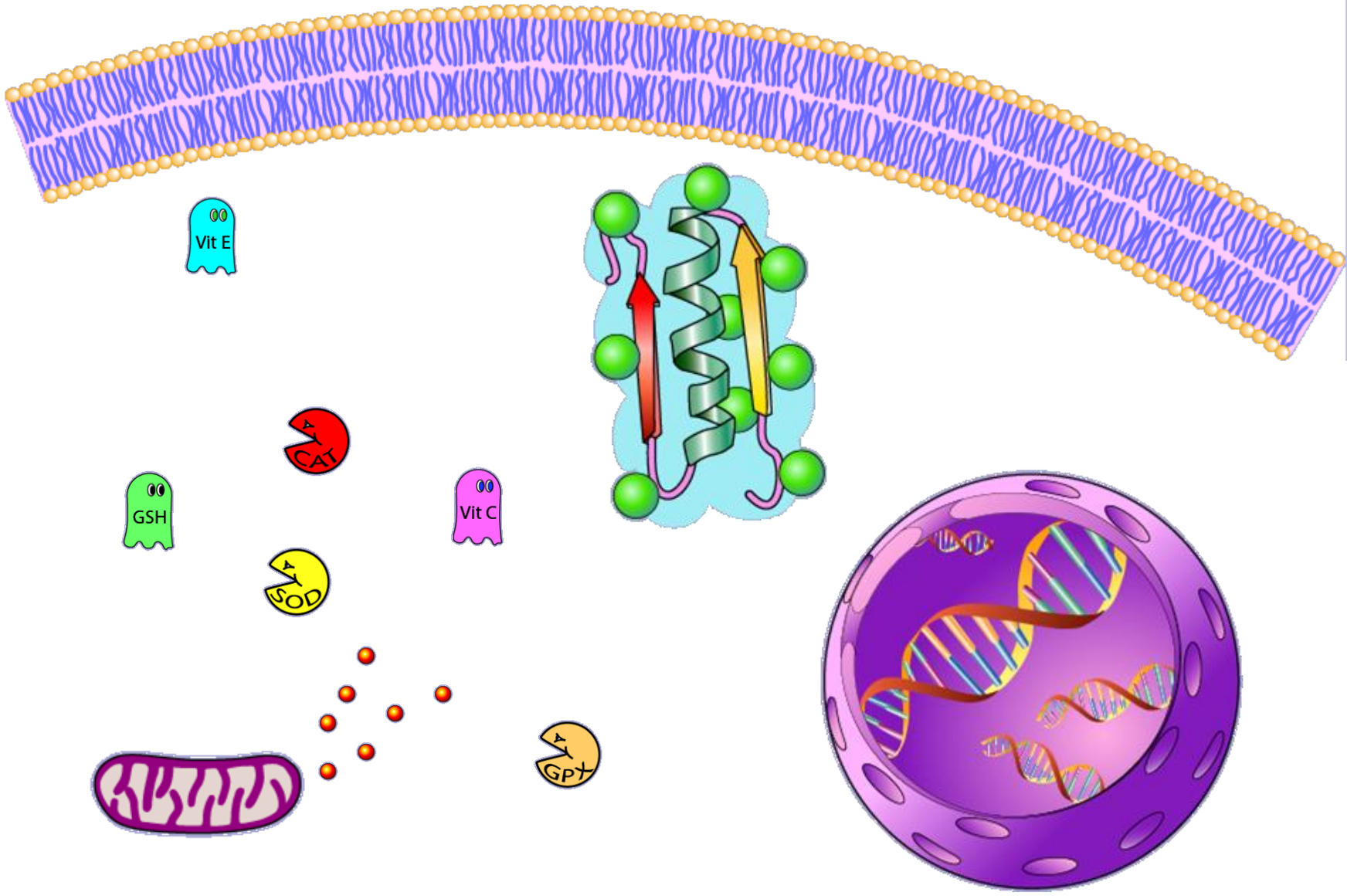
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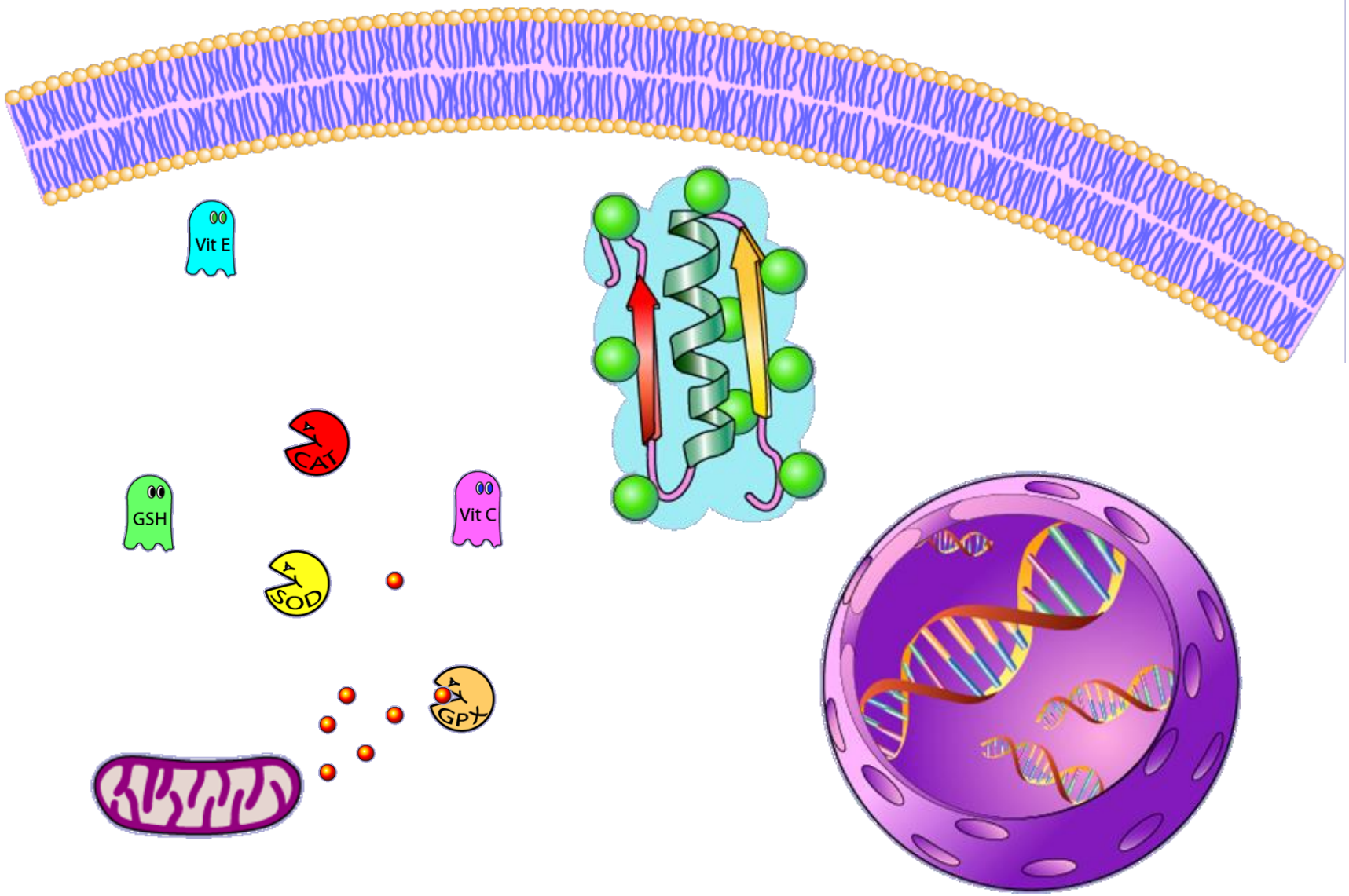
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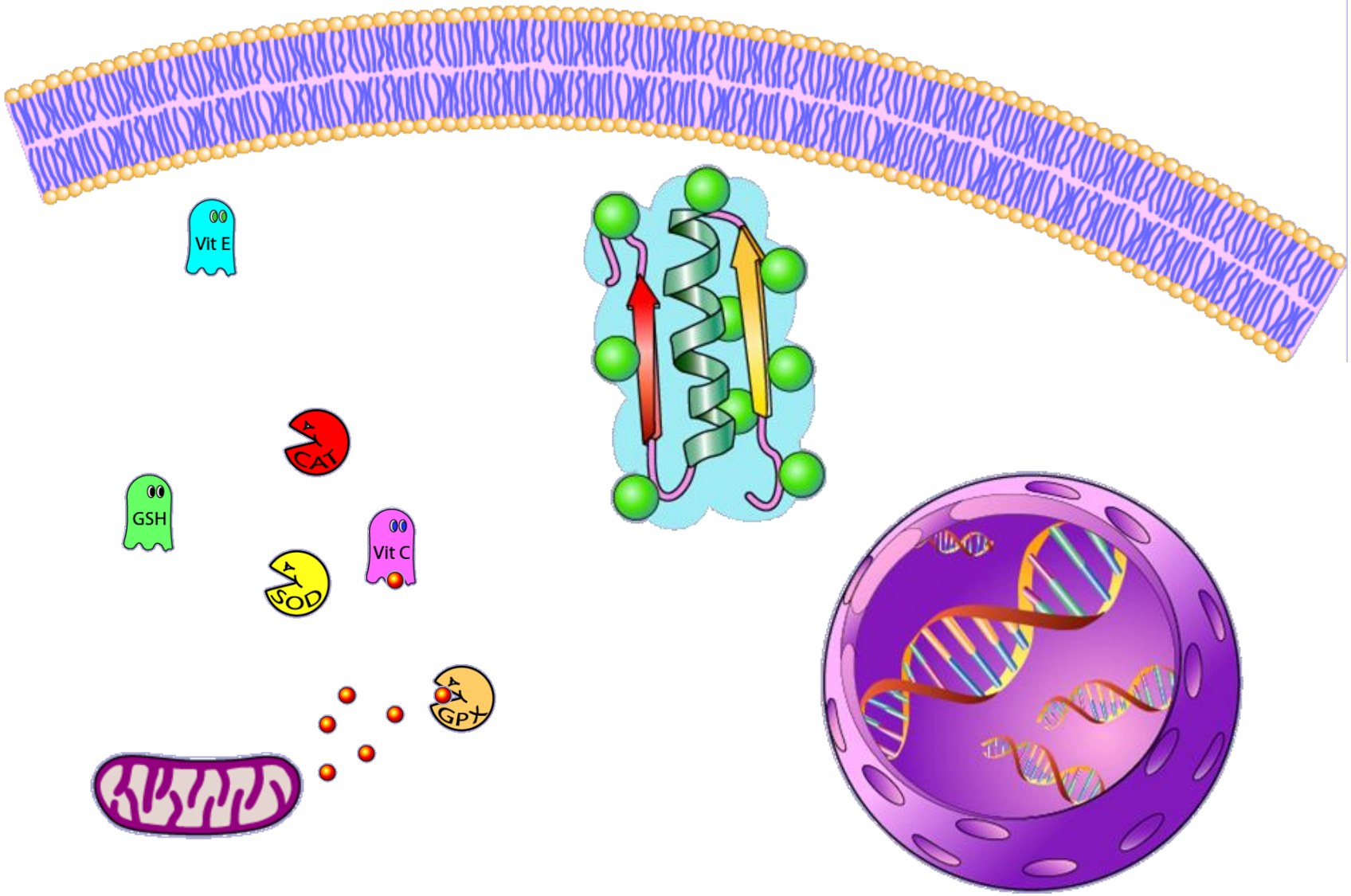
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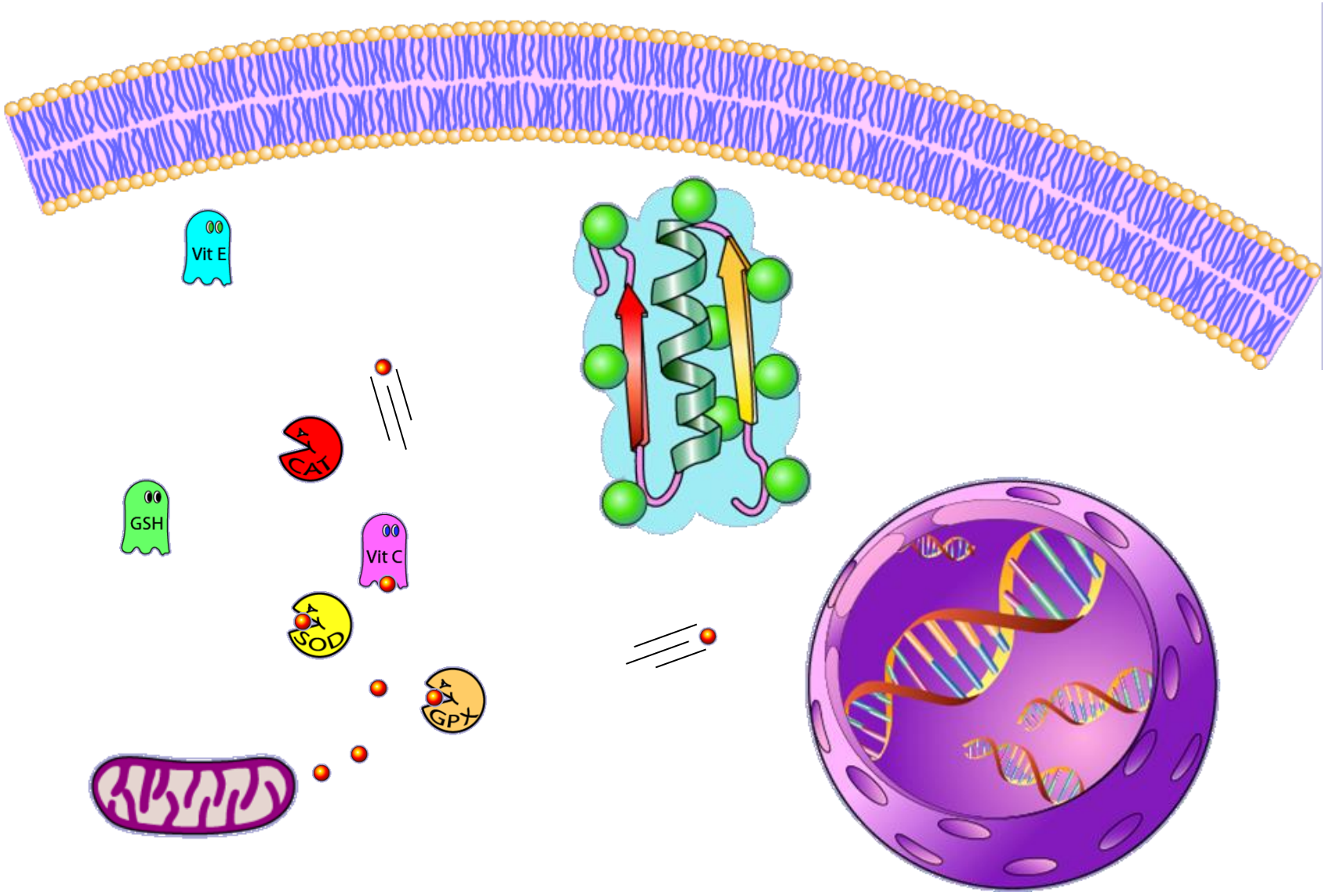
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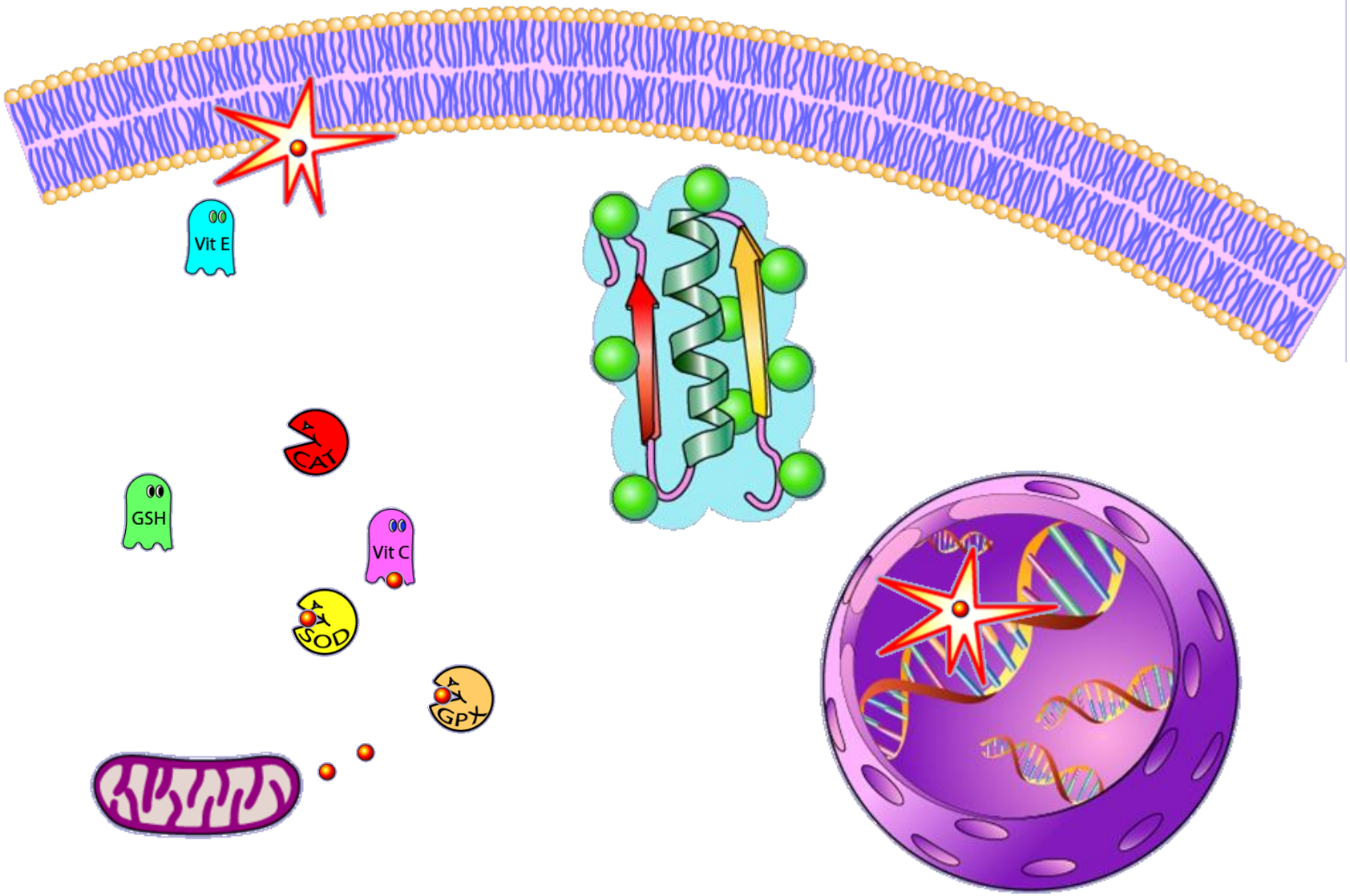
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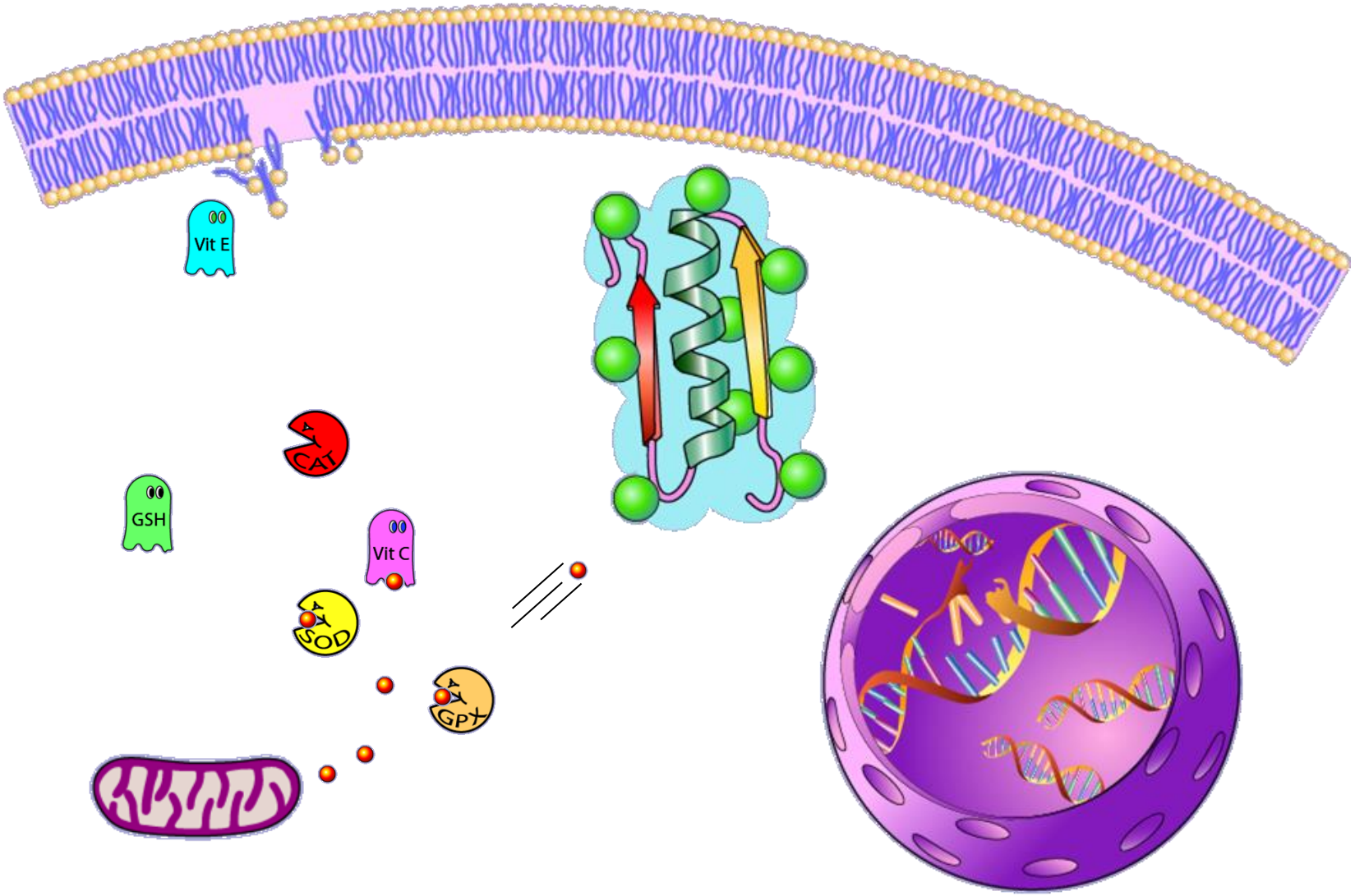
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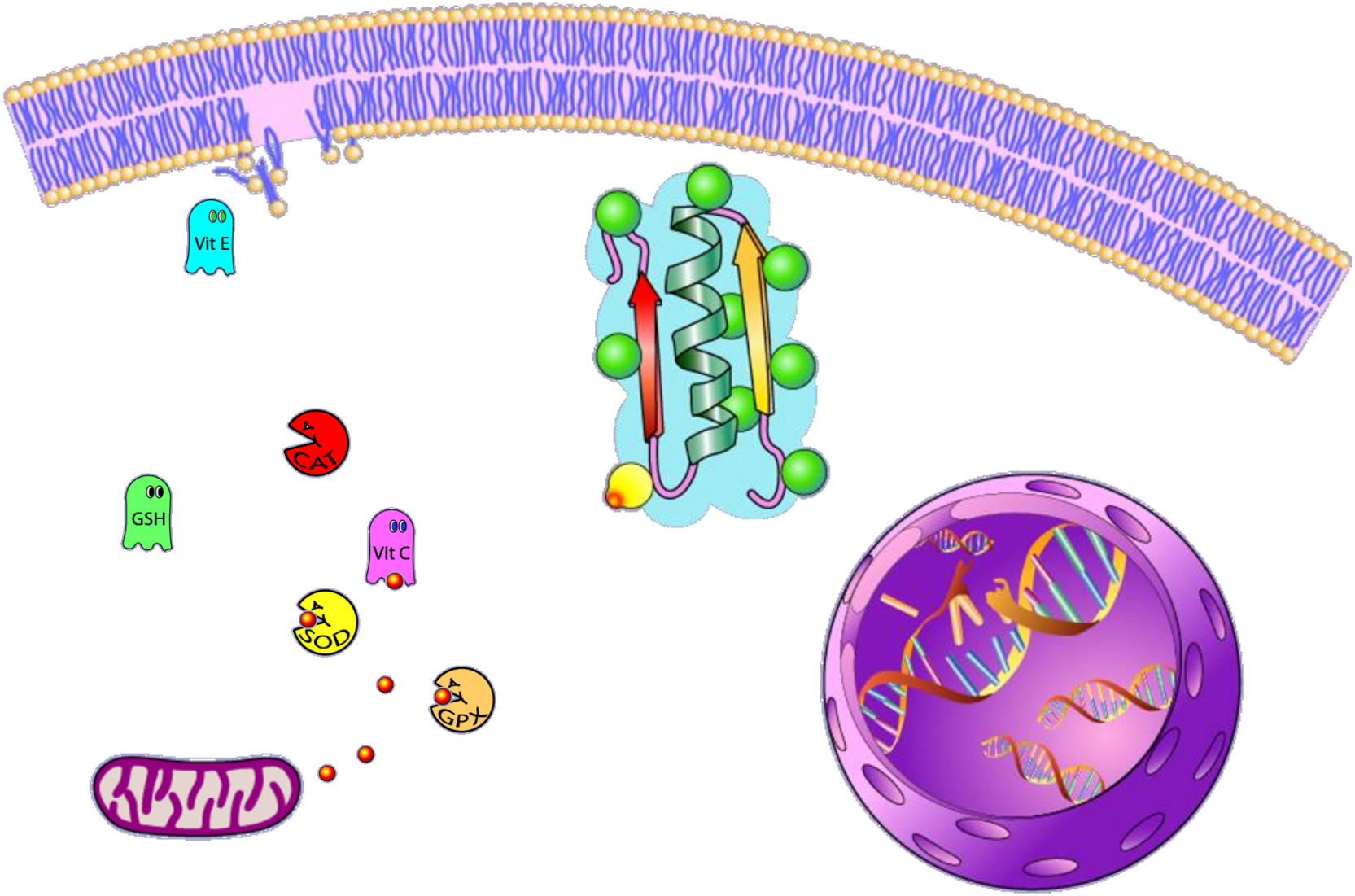
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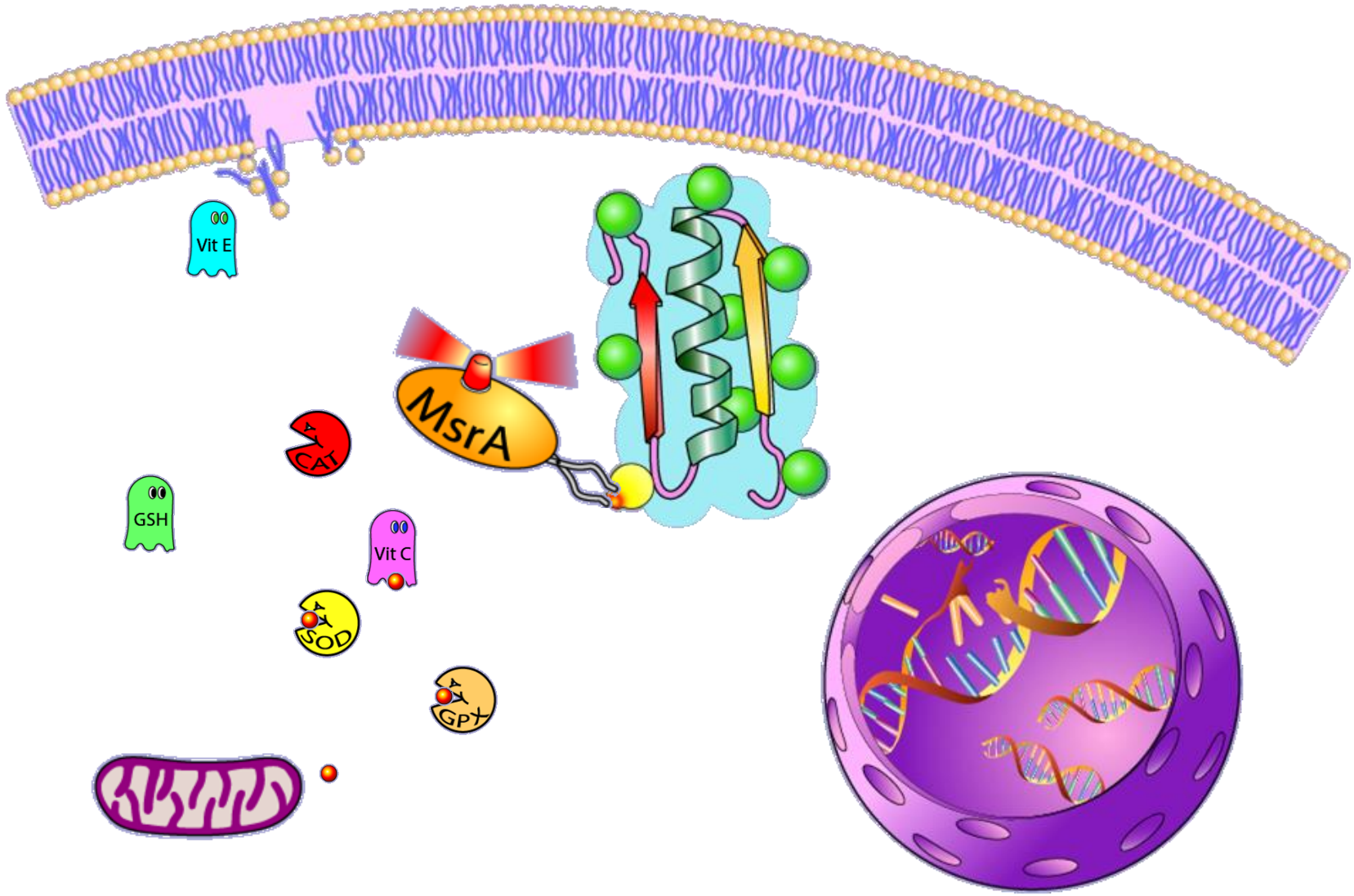
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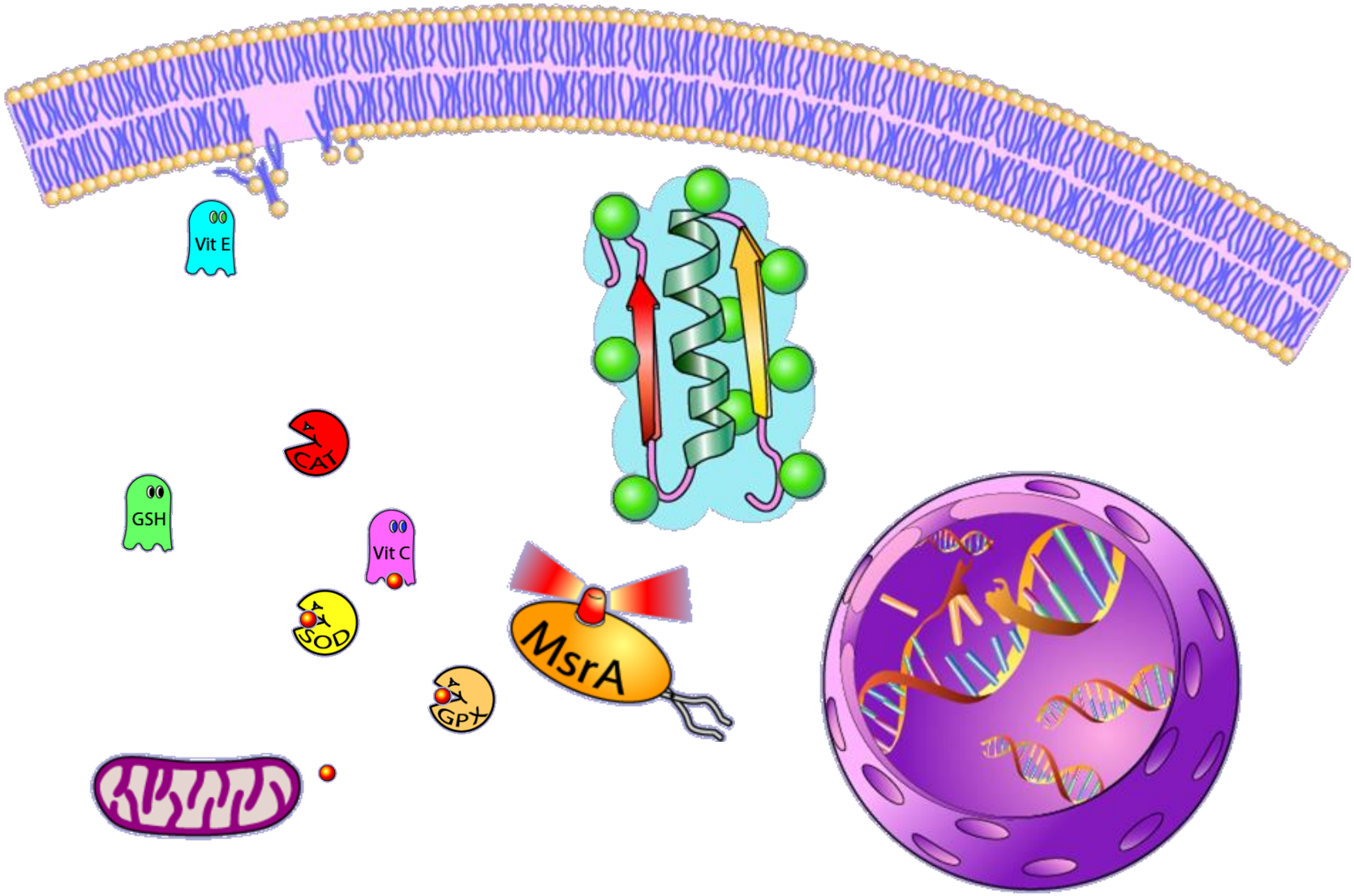
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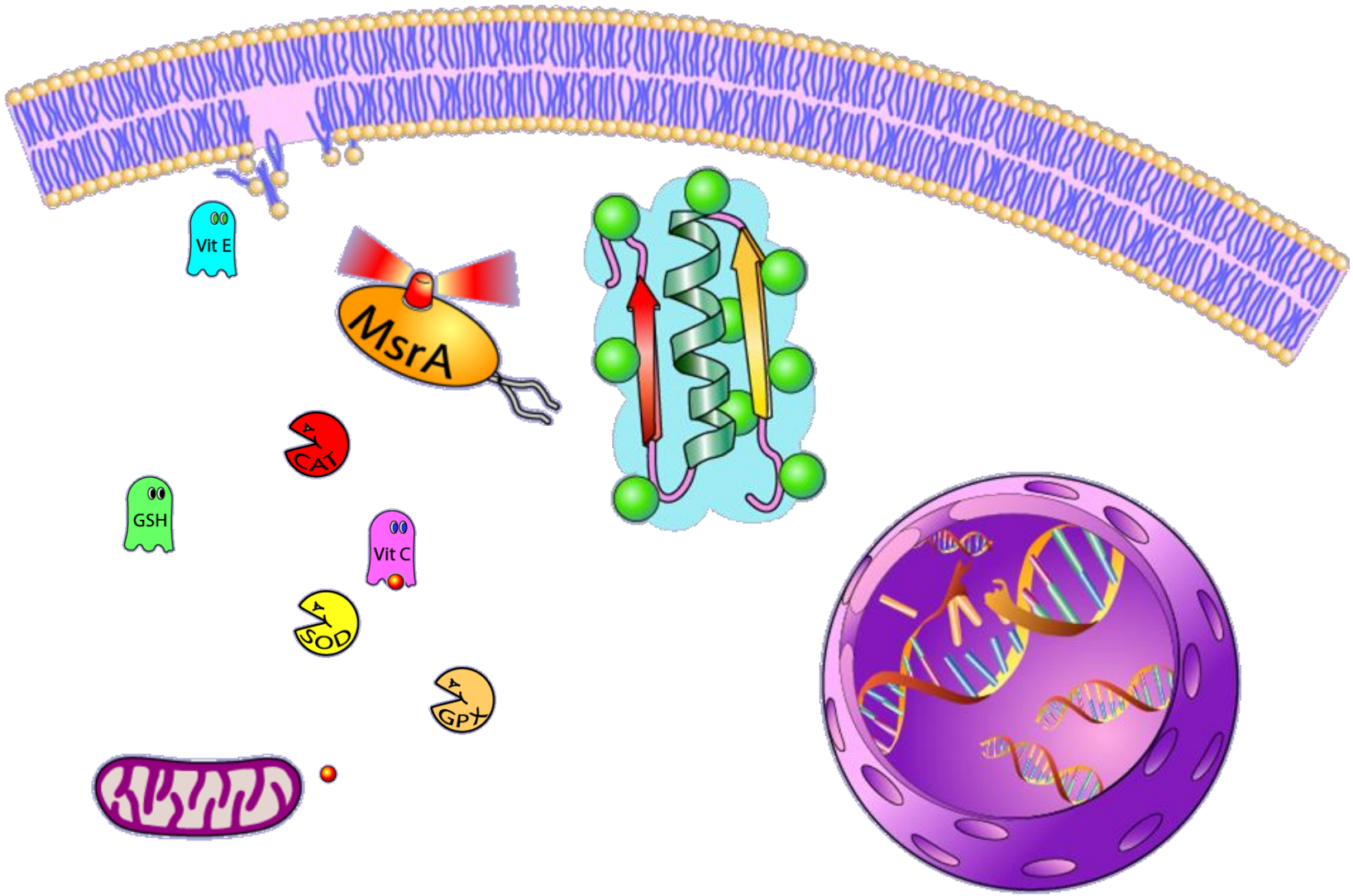
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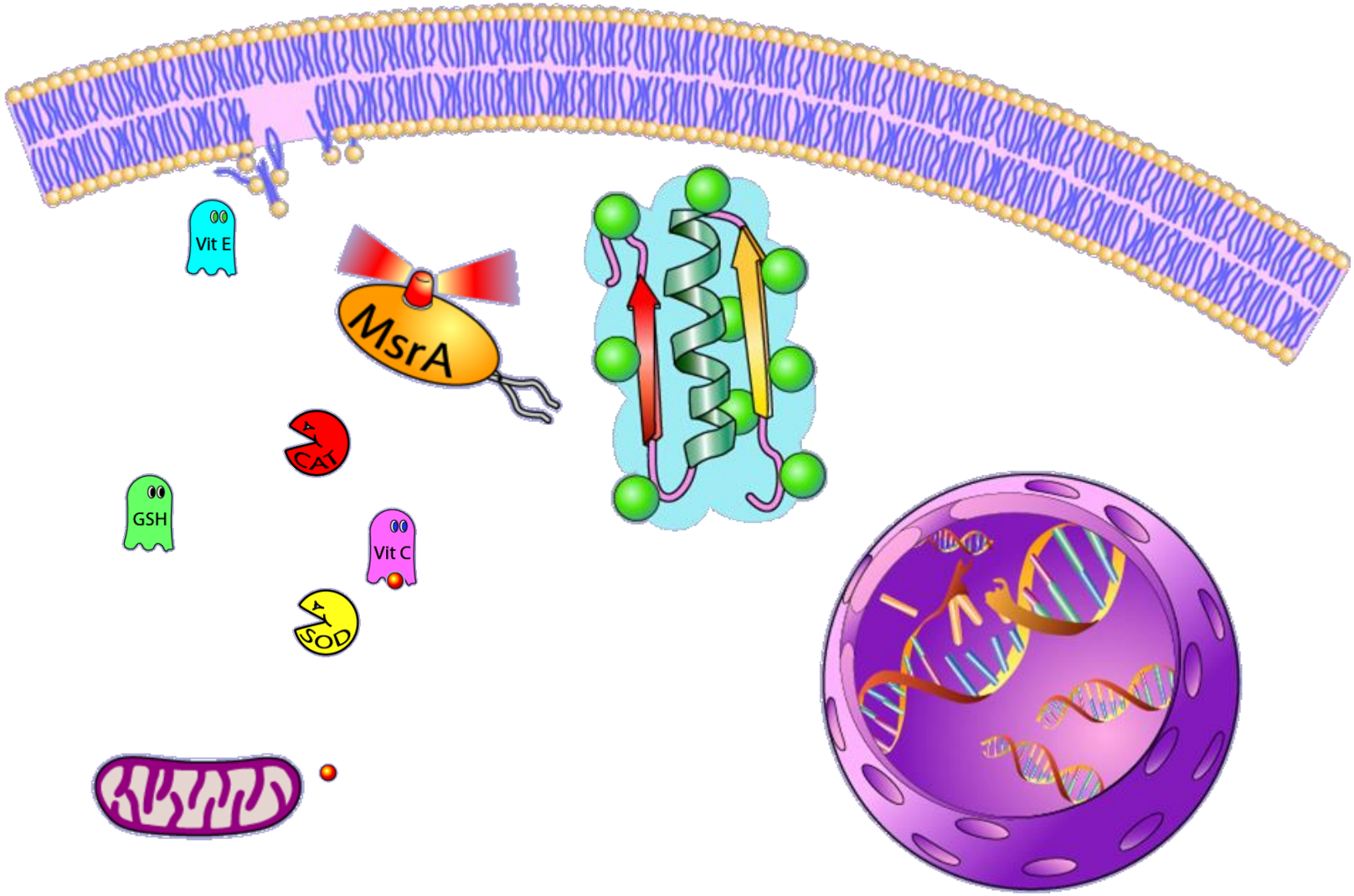
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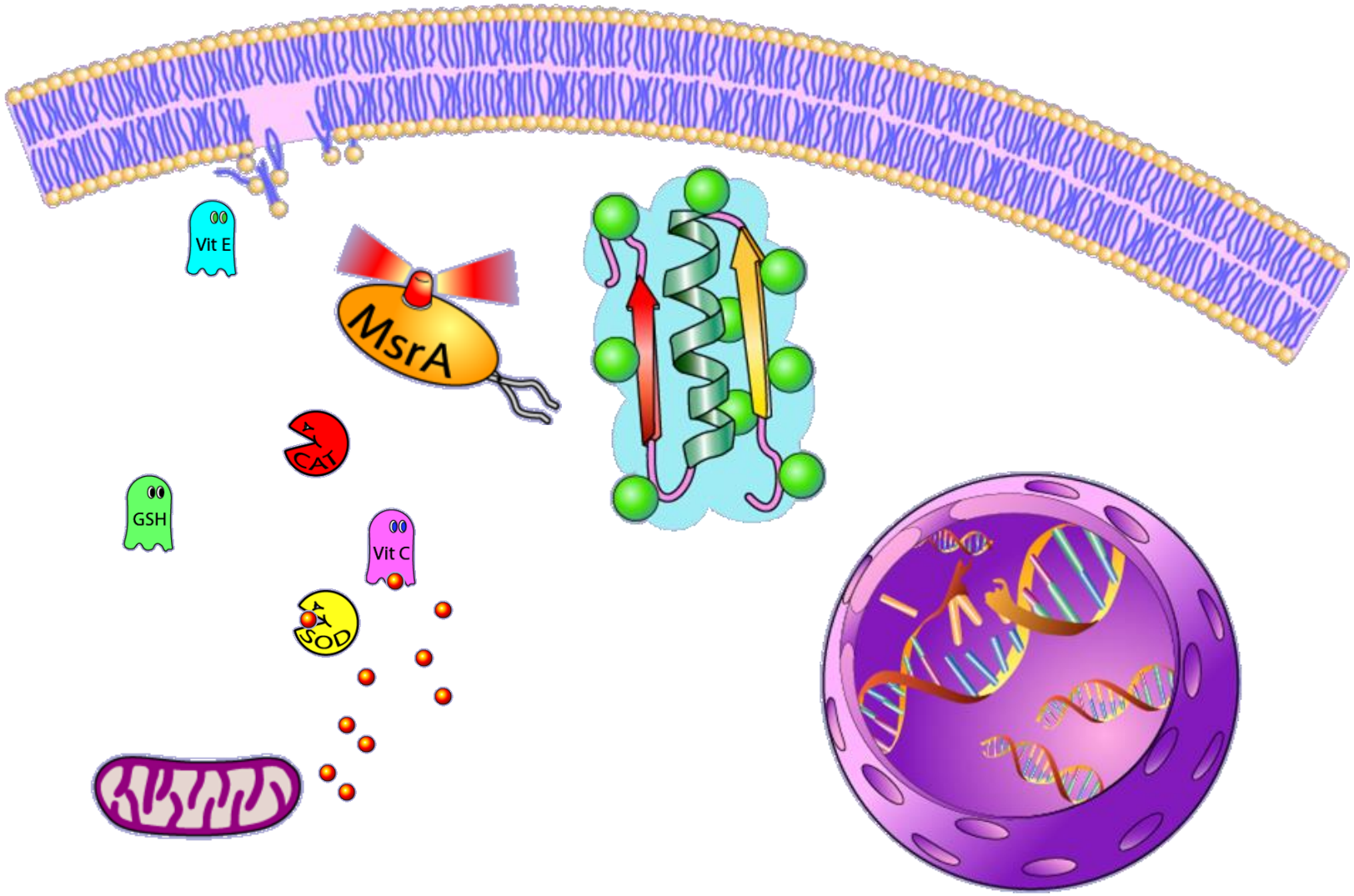
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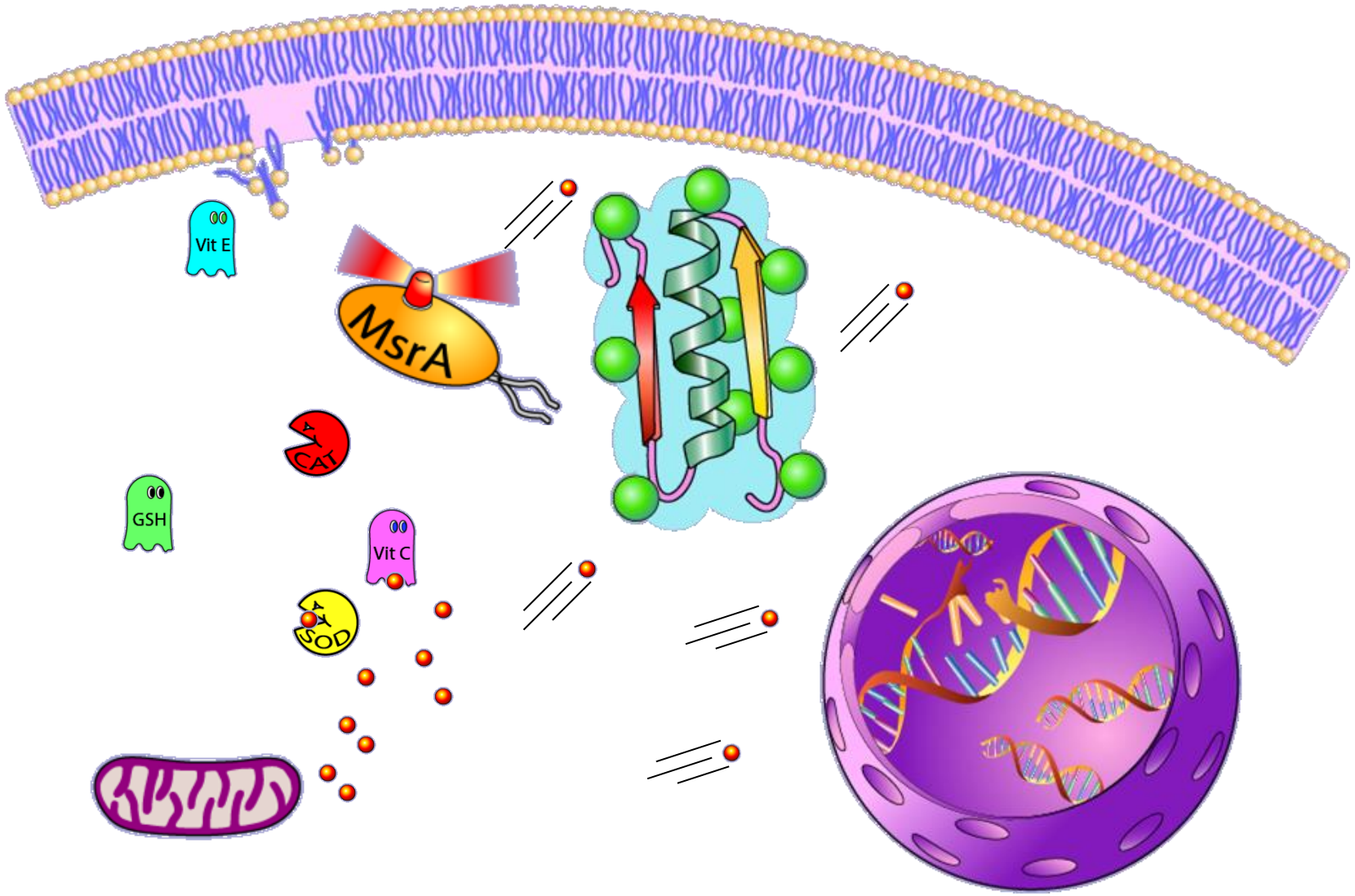
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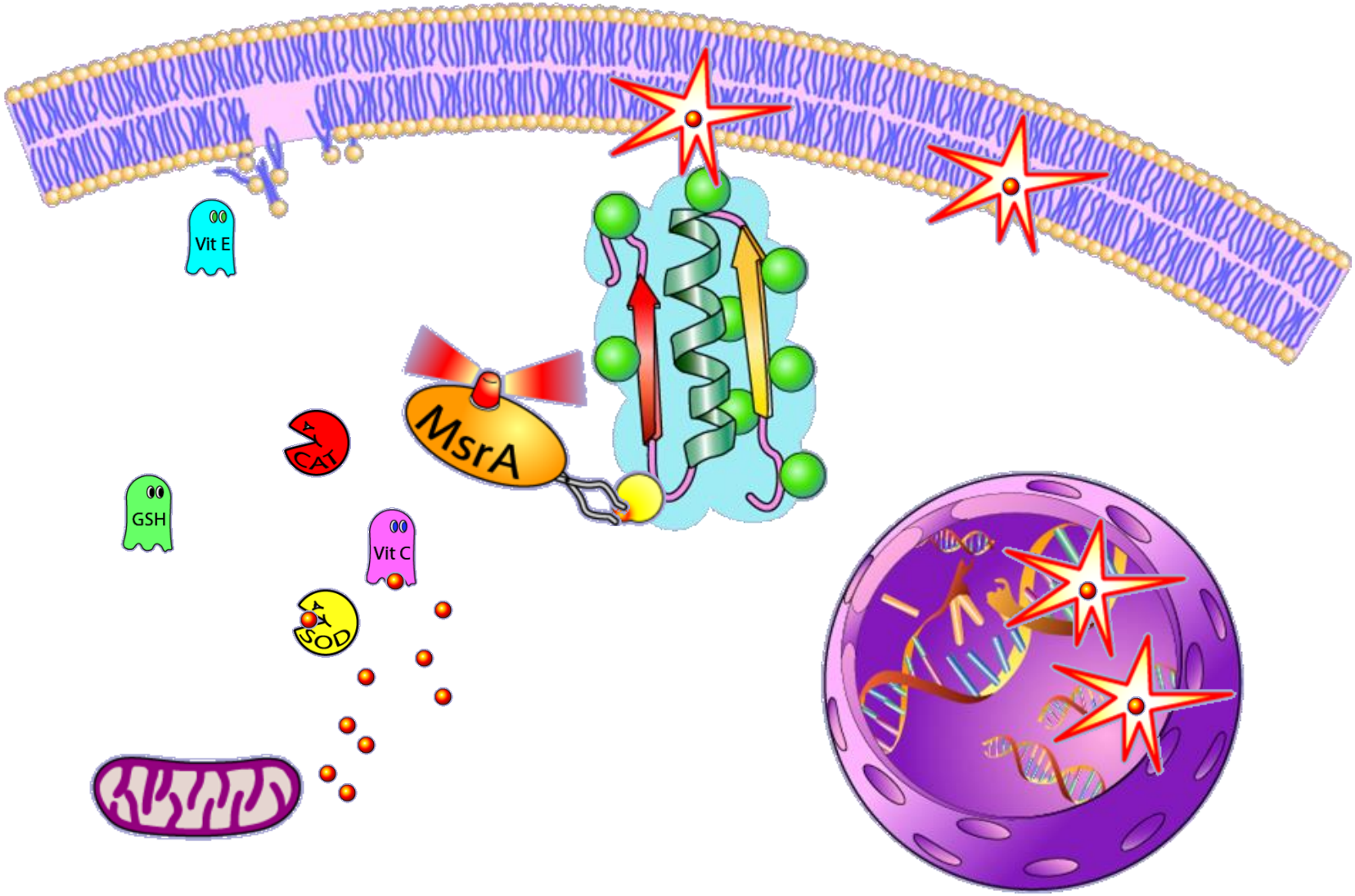
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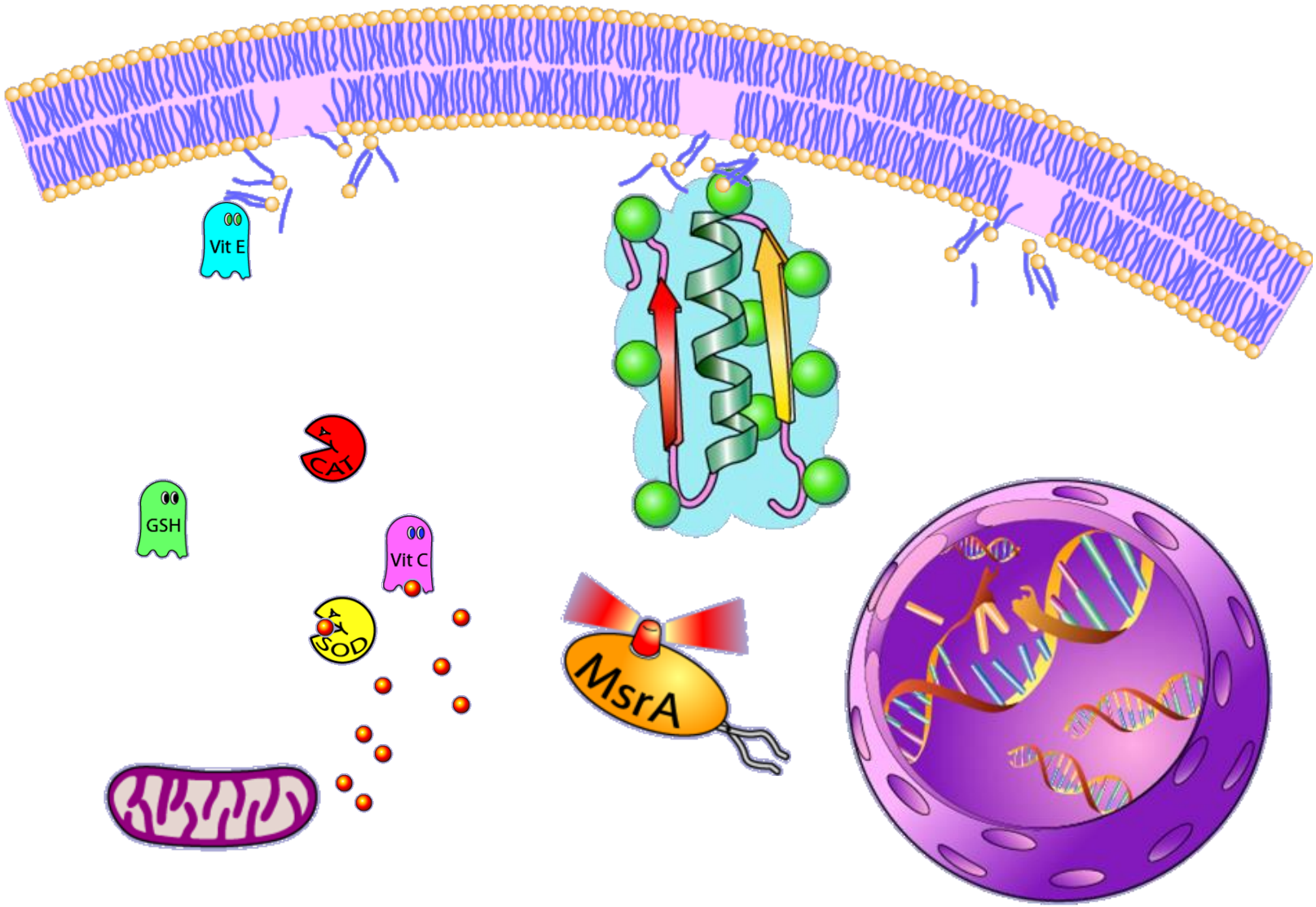
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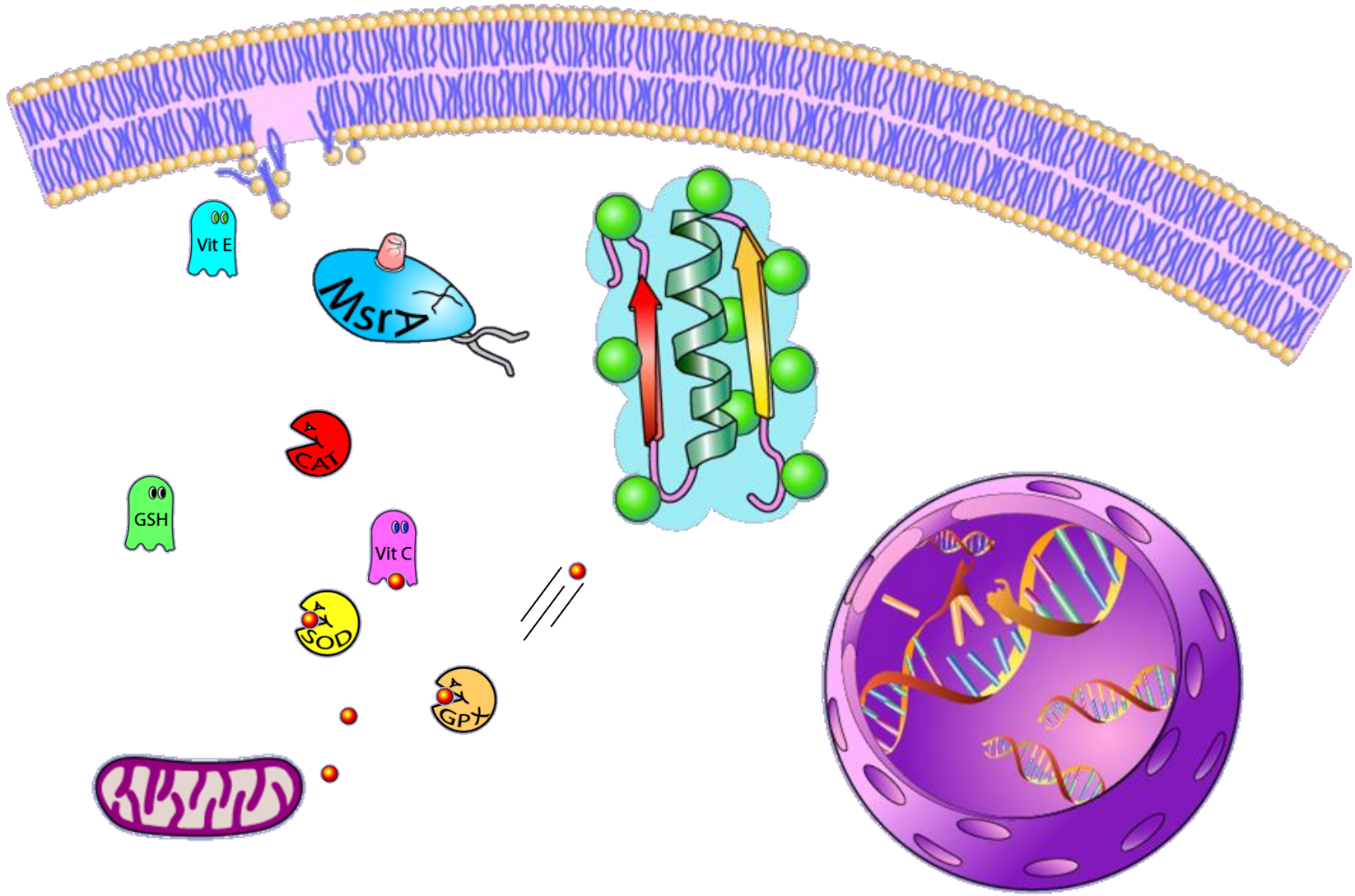
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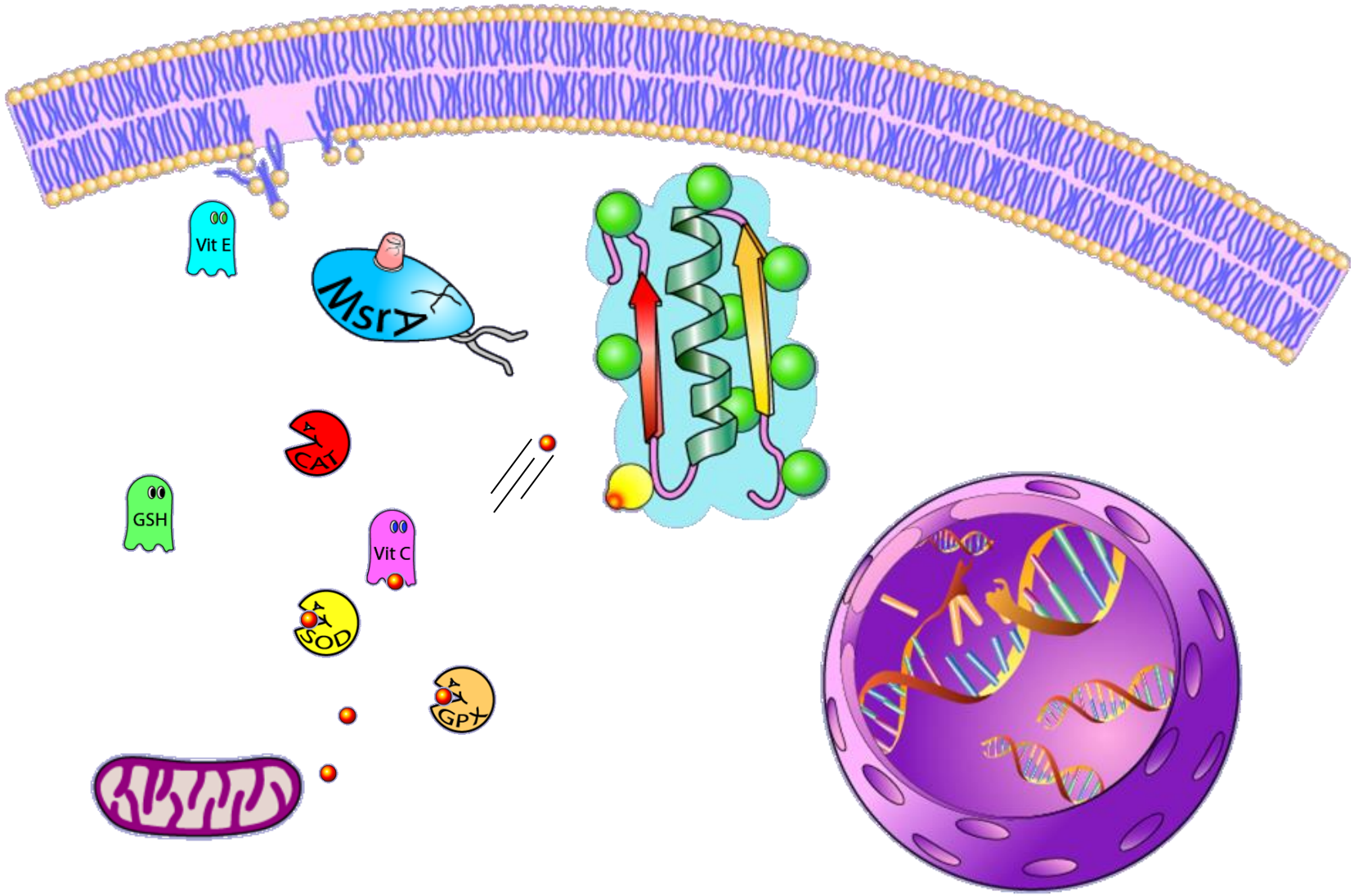
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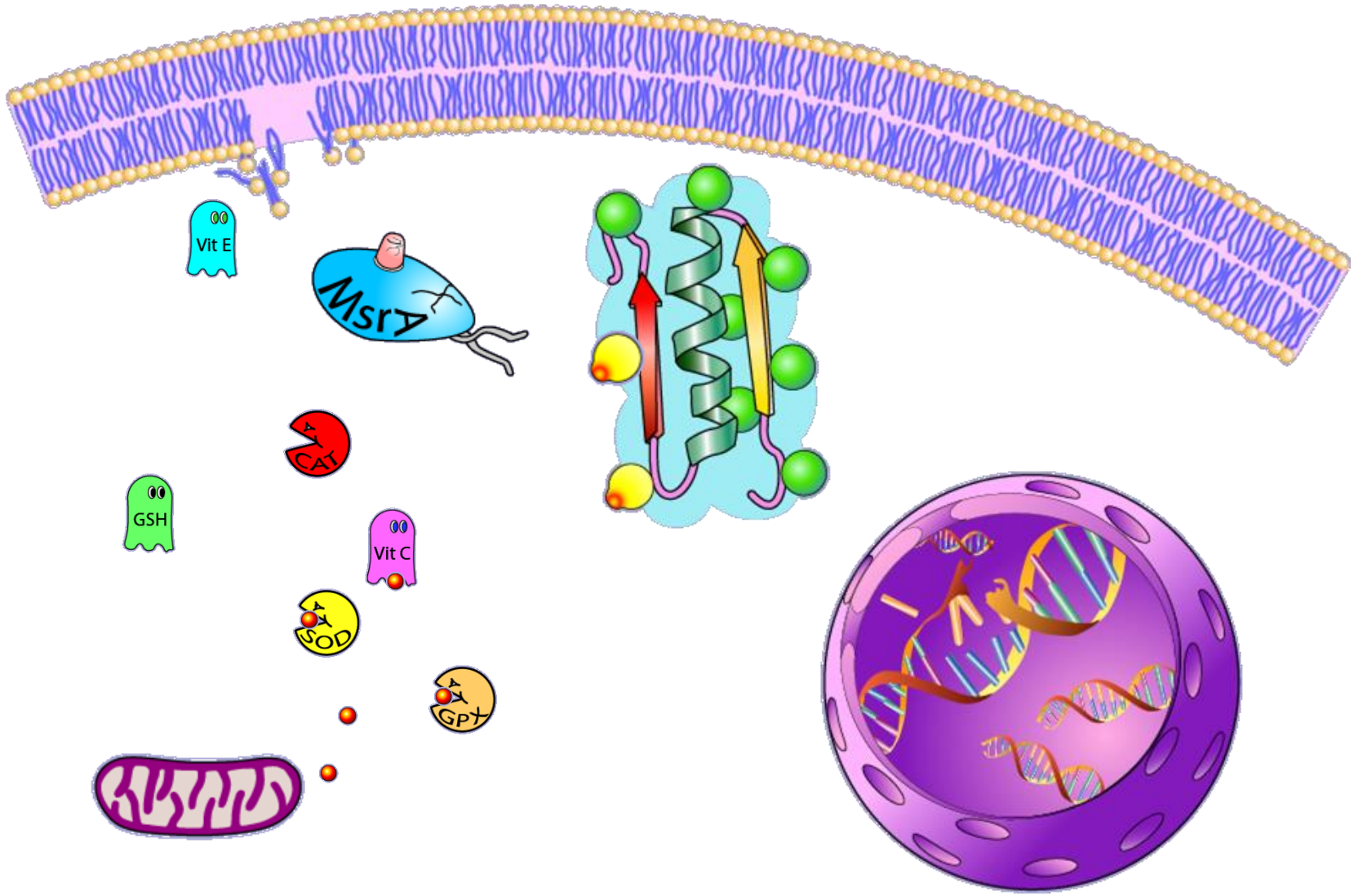
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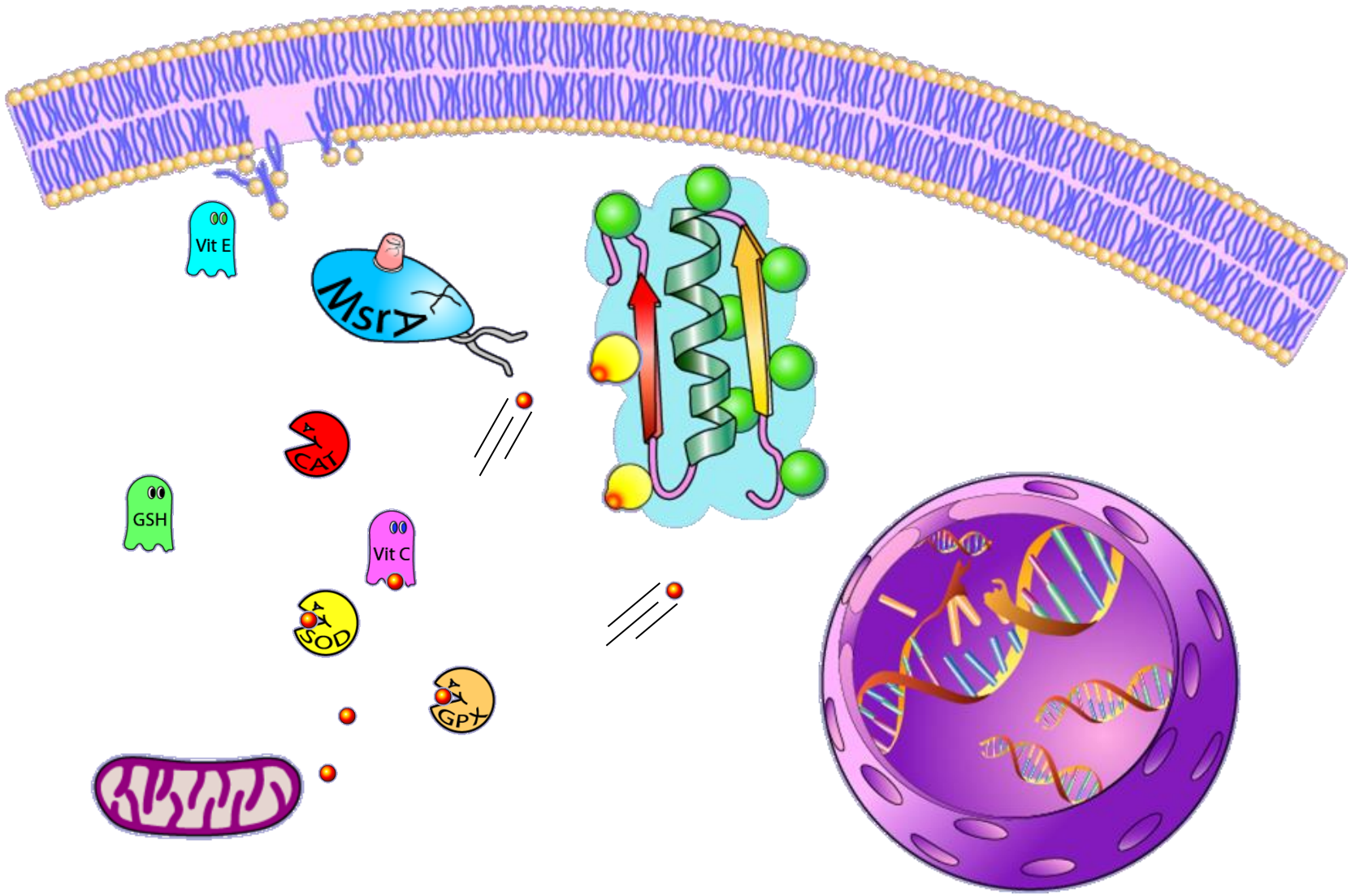
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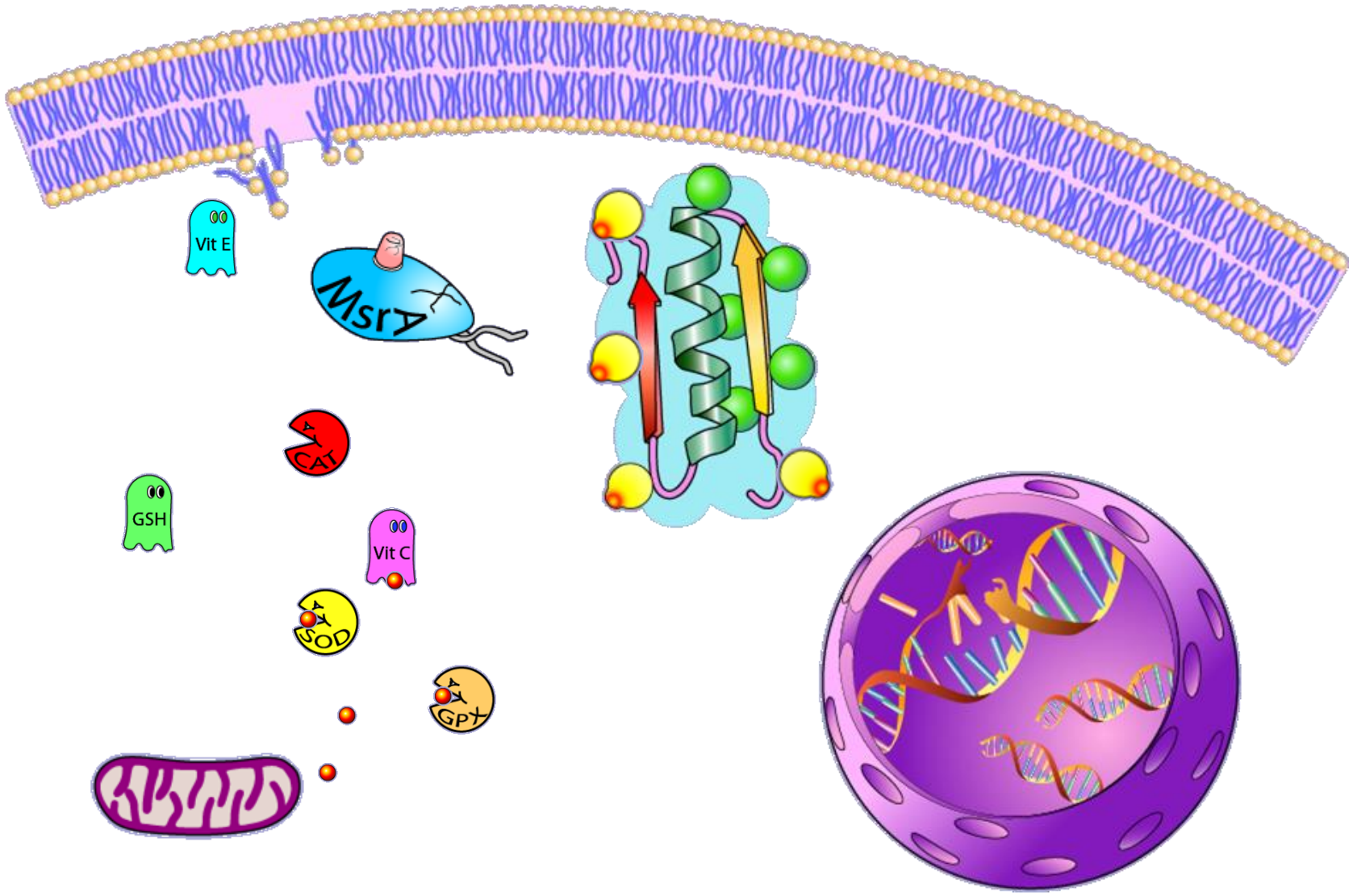
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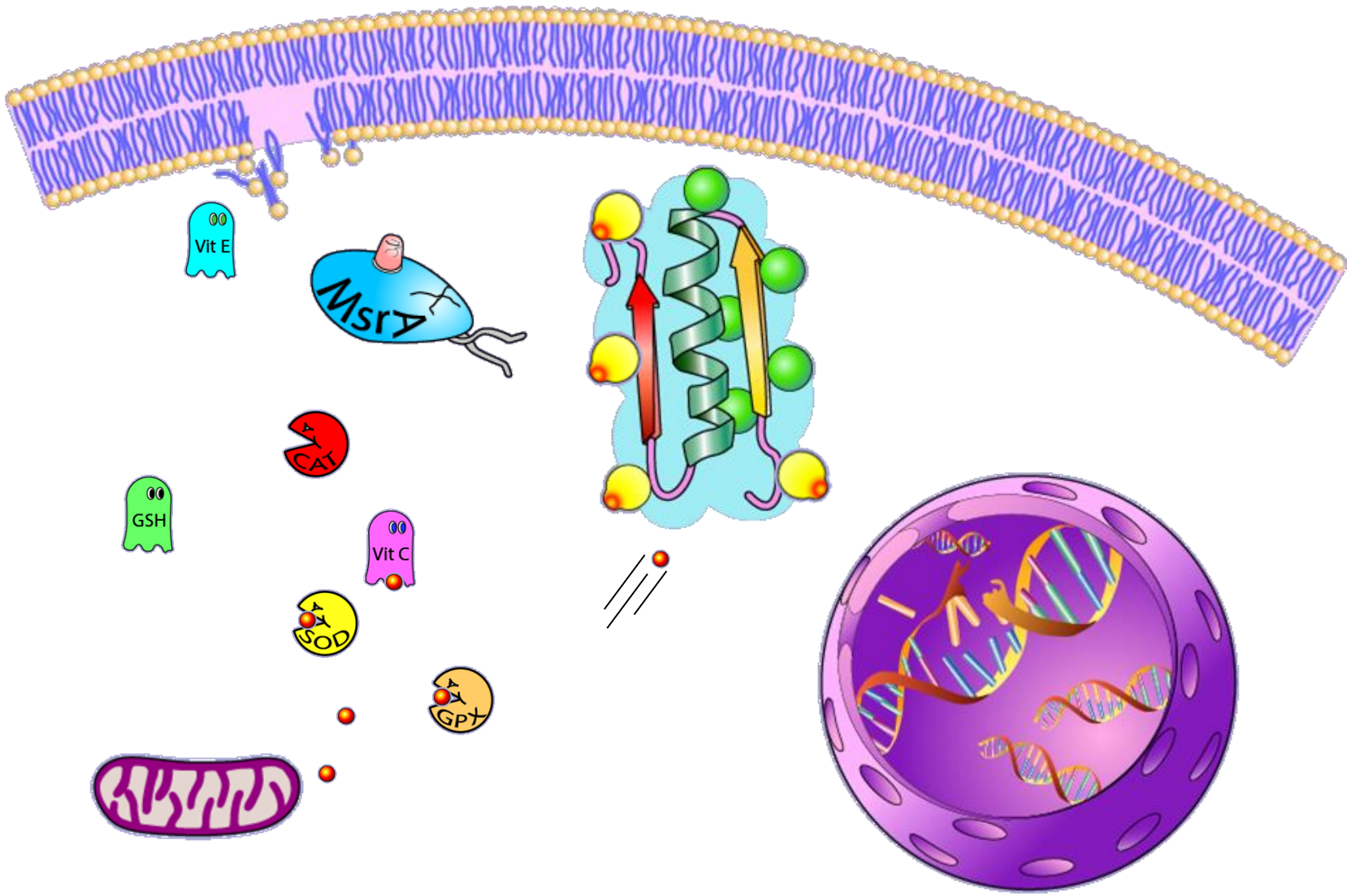
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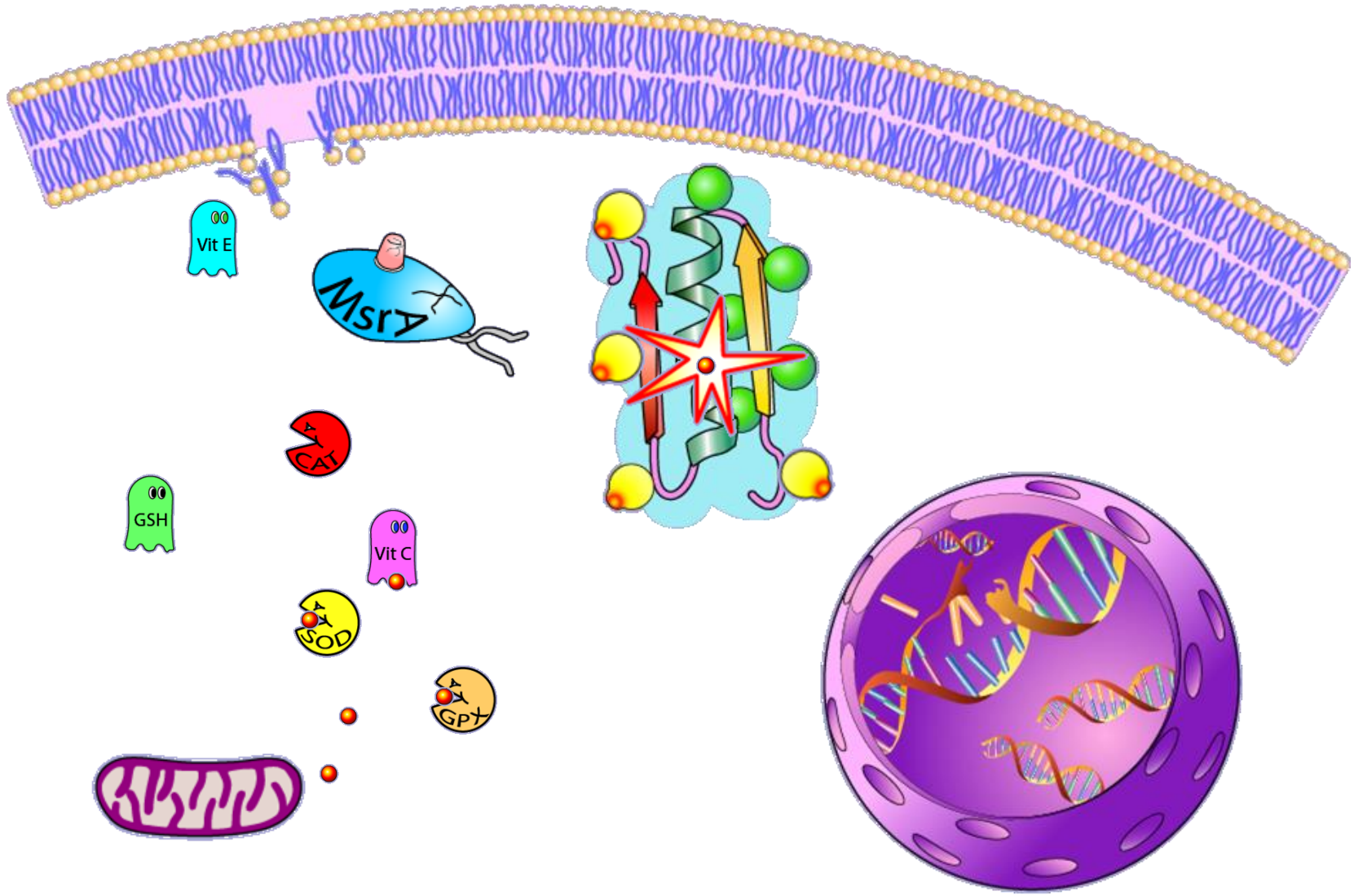
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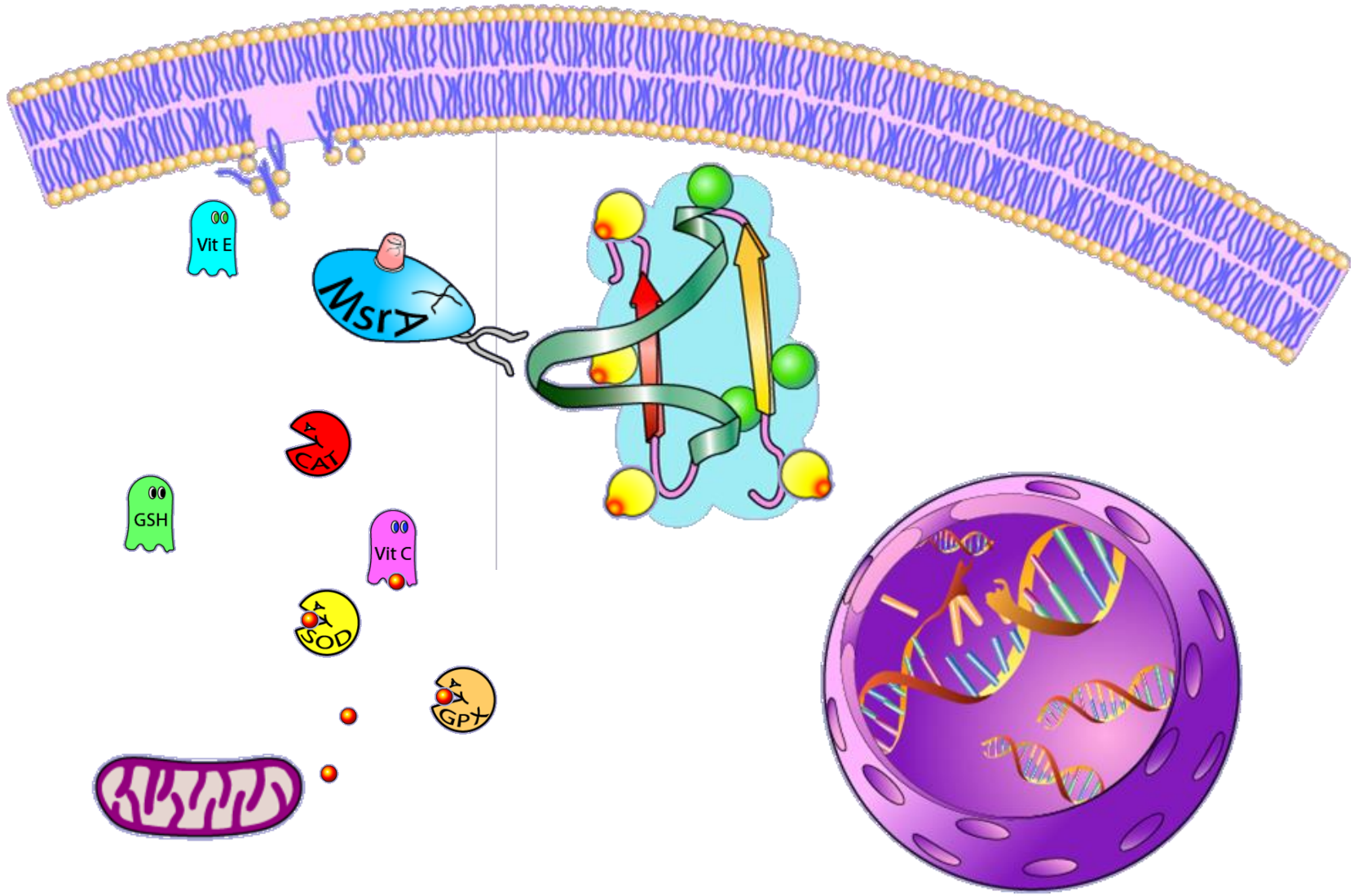
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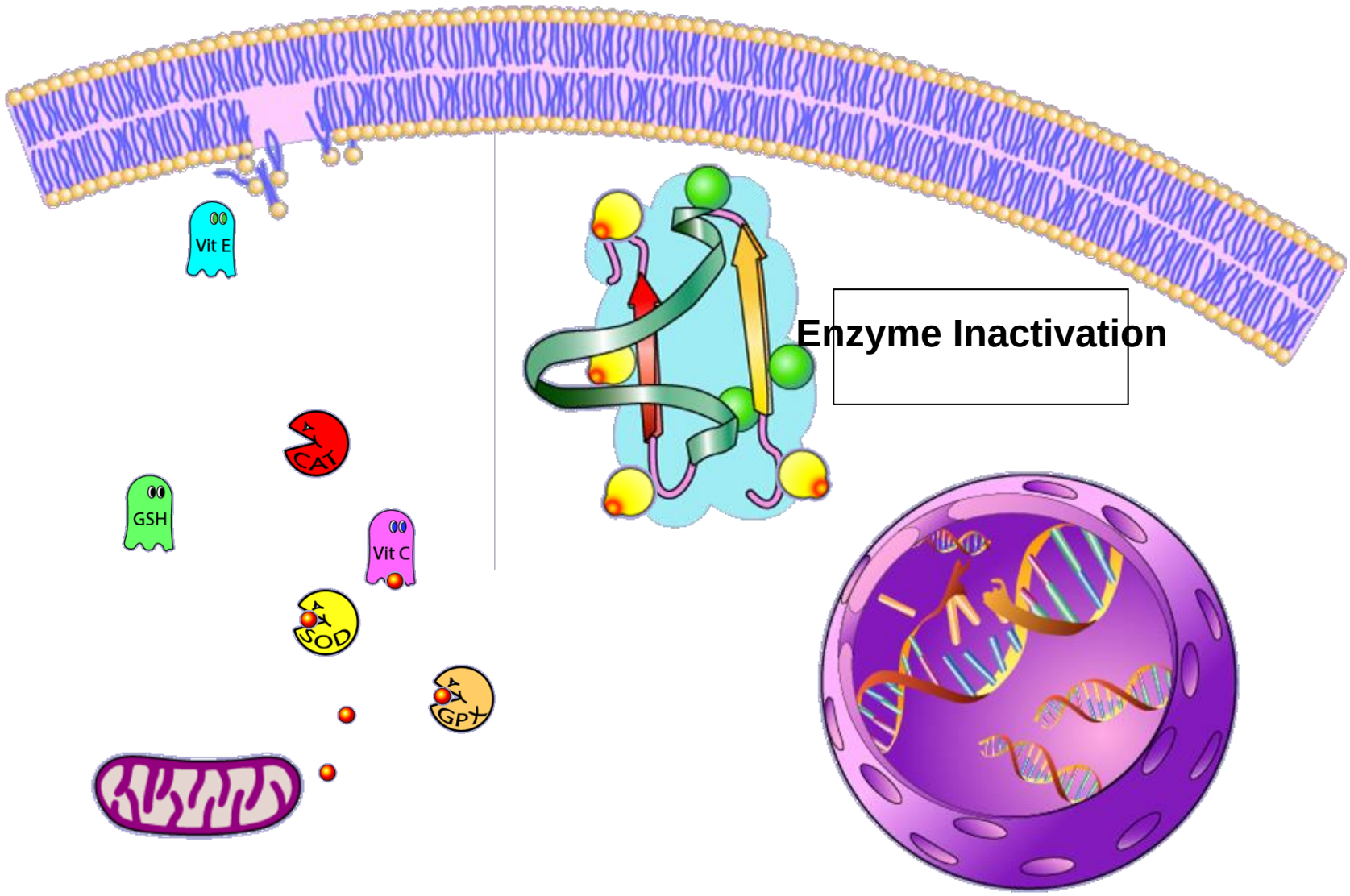
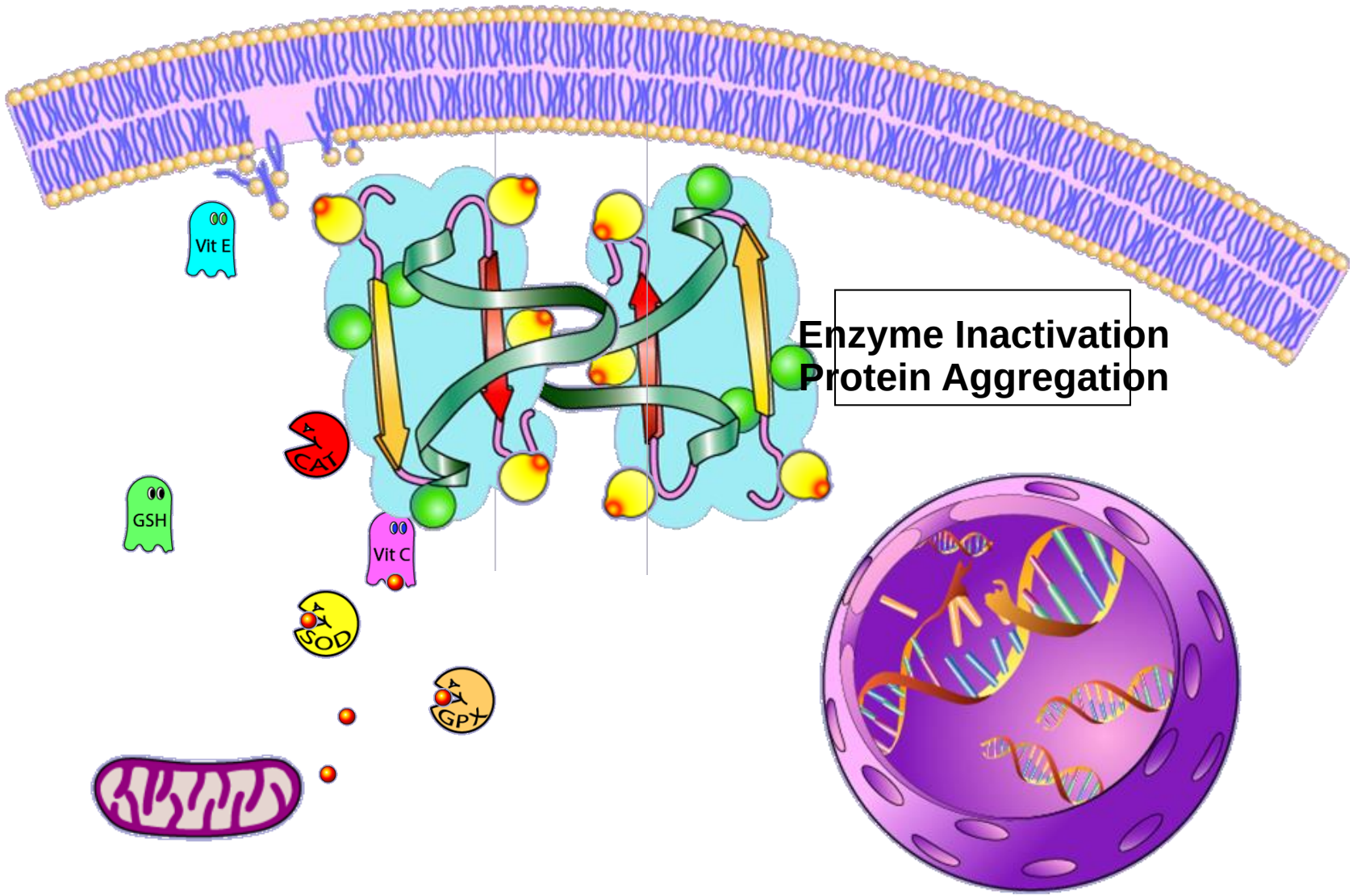
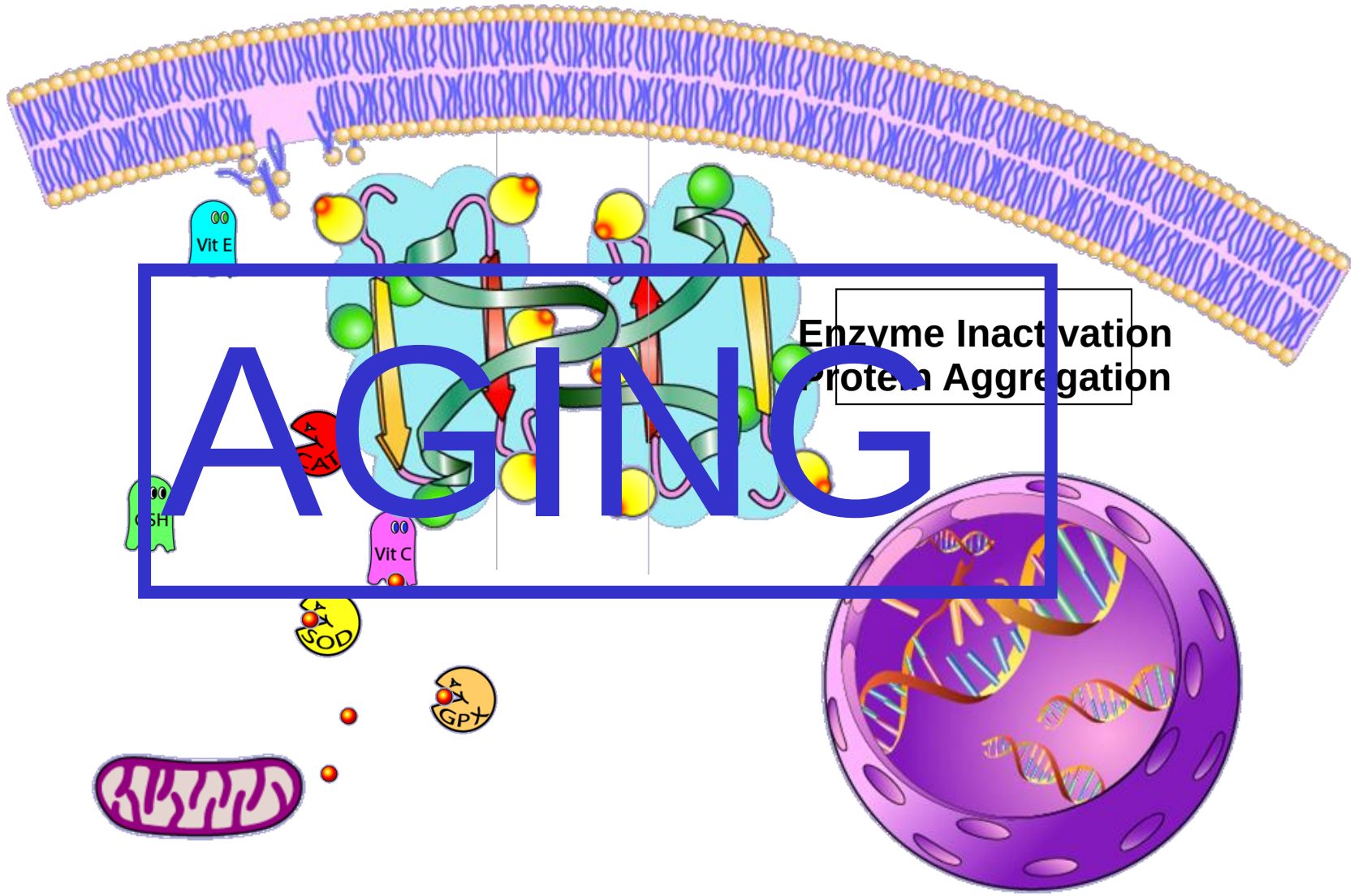

Vit E
GSH
CAT
Vit C
SOD
GPX
Enzyme Inactivation


Vit E
CAT
GSH
Vit C
SOD
GPX
Enzyme Inactivation
Protein Aggregation


Vit E
CAT
GSH
Vit C
SOD
GPX
AGING
Enzyme Inactivation
Protein Aggregation

POLITECNICO DI MILANO





AOPs (Advanced Oxidation Processes)

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Dipartimento CMIC “Giulio Natta”

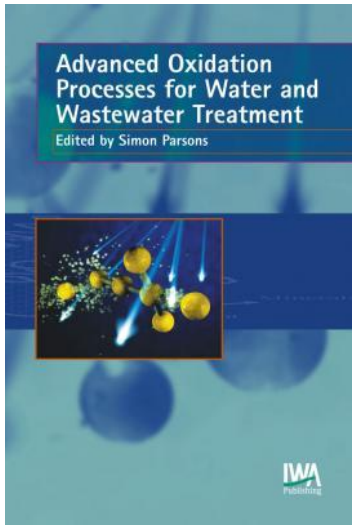
AOPs
(Advanced Oxidation Processes)

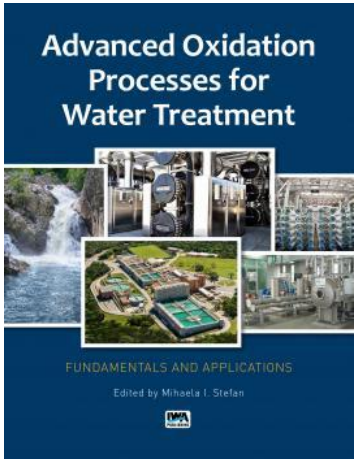
One of the most promising technologies for treatment of low-level refractory organic pollutants in water, sterilization, etc. - intensively investigated for the last decade.

Use of hydroxyl radicals (•OH) as the main oxidant.

Various methods for generation of •OH

- Radiation chemical methods
- Sonochemical methods
- Photochemical methods
- Chemical methods





In Situ Oxidants with More Than Ten Years of History

Permanganate

- Potassium permanganate ($KMnO_4$)
  - Crystalline solid
- Sodium permanganate ($NaMnO_4$)
  - Concentrated liquid

Ozone

- O_3 (gas)

Peroxide (Fenton's Reagent)

- H_2O_2 and ferrous iron react to produce radicals
- More accurately catalyzed peroxide propagation

Peracetic acid

Emerging Oxidants

Persulfate

- Sodium persulfate - most commonly used
- Potassium persulfate - very low solubility
- Persulfate anions ($S_2O_8^{2-}$) dissociate in water
- Oxidative strength greatly increased with addition of heat or a ferrous salt (Iron II)
  - Attributed to production of sulfate free radical ($SO_4^{-\bullet}$)

Other oxidants – solid peroxides

- Magnesium peroxide (MgO_2)
- Calcium peroxide (CaO_2)
- Sodium percarbonate ($Na_2CO_3 \bullet 3H_2O_2$)

Permanganate Chemistry

pH < 3.3

- $MnO_4^- + 8H^+ + 5e^- \rightarrow Mn^{2+} + 4H_2O$ (1)

3.5 < pH < 12

- $MnO_4^- + 2H_2O + 3e^- \rightarrow MnO_2(s) + 4OH^-$ (2)

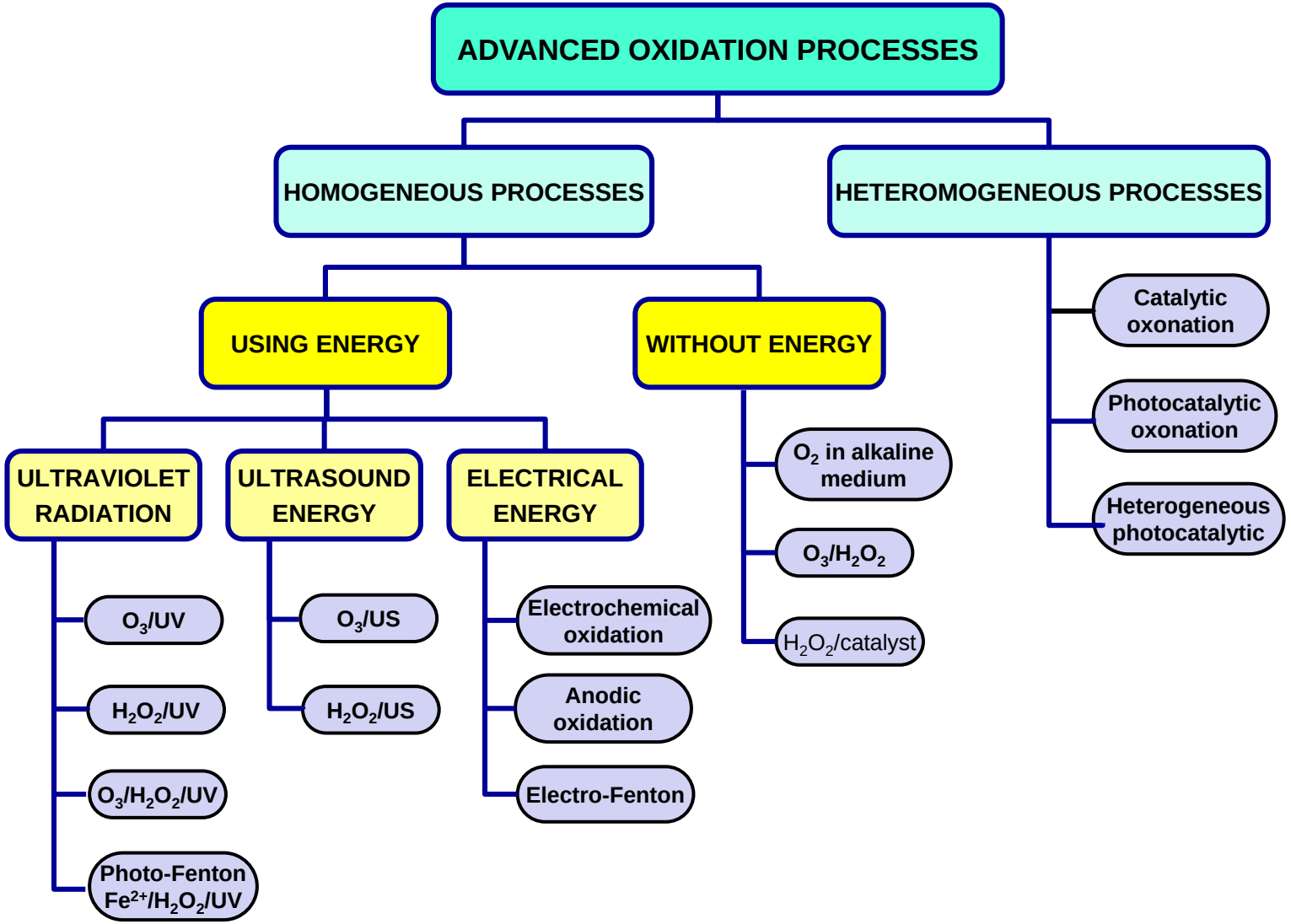
pH > 12

- $MnO_4^- + e^- \rightarrow MnO_4^{2-}$ (3)

Under acidic conditions

- $3MnO_2 + 2MnO_4^- + 4H^+ \rightarrow 5MnO_2(s) + 2H_2O$ (4)
- $MnO_2(s) + 4H^+ + 2e^- \rightarrow Mn^{2+} + 2H_2O$ (5)

AOP Classification



Classification of AOPs

Classification			Process
Reaction Phase	External Energy	Generation of Hydroxyl radicals	
Homogeneous	Light	Photochemical Process / Chemical Process	UV^{*}/O_3
			UV/H_2O_2
			$UV/H_2O_2/O_3$
			$UV/F^{2+}(Fe^{3+})/H_2O_2$
	Light /Ultrasound	Photochemical/ Sonochemical process	UV/US^{**}
	Ultrasound	Sonochemical process	US/H_2O_2
			US/O_3
	High-energy electrons	Ionising radiation process	Electron Beam
	None	Chemical process	H_2O_2/O_3
	None	Chemical process	O_3/H_2O_2/high pH
	None	Chemical process	F^{2+}/H_2O_2(Fenton)
	None	Electrochemical/ Chemical process	Electro-Fenton
Heterogeneous	Light	Photochemical process	$UV/TiO_2/O_2$
			$UV/TiO_2/H_2O_2$
	None	Chemical process	Iron Oxide/H_2O_2

*** : Ultraviolet, ** : Ultrasound**

AOP (advanced oxidation processes): generating $^{\bullet}OH$

→ Fenton's reagent: H_2O_2 / Fe^{2+}

→ Fenton-like reagent: H_2O_2 / Fe^{3+}

→ Combination of O_3 , H_2O_2 , and UV light

→ Combination of H_2O_2 (or O_3) and metal ions (e.g. iron salts)

→ Combination of UV light and semiconductors (e.g. TiO_2)

→ electron beam

Commercialized industrial wastewater treatment technologies

Fenton, UV/O_3

Peroxide (Fenton's) Chemistry

Fenton's Reaction (pH 2.5/3.5; 300 ppm peroxide)

- $H_2O_2 + Fe^{2+}$ (acid) $\rightarrow$ $OH^{\bullet} + OH^- + Fe^{3+}$ (1)
- **Organic Contaminant $\rightarrow$ Alcohols, Acids, CO_2, H_2O**

Chain Initiation Reactions (>1 % peroxide)

- $OH^{\bullet} + H_2O_2 \rightarrow HO_2^{\bullet} + H_2O$ (2)
- $H_2O_2 + Fe^{3+} \rightarrow Fe^{2+} + HO_2^{\bullet} + H^+$ (3)

Catalyzed Peroxide Propagation

Chain Propagation Reactions (excess peroxide):

- $HO_2^{\bullet} + Fe^{2+} \rightarrow HO_2^{-} + Fe^{3+}$ (4)
- $OH^{\bullet} + H_2O_2 \rightarrow HO_2^{\bullet} + H_2O$ (5)
- $HO_2^{\bullet} \rightarrow O_2^{\bullet -} + H^{+}$ (6)
- $OH^{\bullet} + R \rightarrow R^{\bullet} + OH^{-}$ (7)
- $R^{\bullet} + H_2O_2 \rightarrow ROH + OH^{\bullet}$ (8)

Chain Termination Reactions (excess iron):

- $HO_2^{\bullet} + Fe^{2+} \rightarrow O_2 + H^{+} + Fe^{3+}$ (9)
- $O_2^{\bullet -} + Fe^{3+} \rightarrow Fe^{2+} + O_2$ (10)
- $Fe^{3+} + n\ OH^{-} \rightarrow$ Am. iron oxides (precipitate) (11)

Persulfate Chemistry

Chain Initiation Reactions (Me is a metal ion; R is an organic compound):

- $S_2O_8^{2-} \rightarrow 2\ SO_4^{-\bullet}$ (1)

- $S_2O_8^{2-} + RH \rightarrow SO_4^{-\bullet} + R^{\bullet} + HSO_4^-$ (2)

Catalyzed Persulfate:

- $Me^{n+} + S_2O_8^{2-} \rightarrow SO_4^{-\bullet} + Me^{(n+1)+} + SO_4^{2-}$ (3)

  (M = Ag^+, Cu^{2+}, Fe^{2+}, etc.)

Persulfate Chemistry

Chain Propagation Reactions:

- $Me^{(n+1)+} + RH \rightarrow R^{\bullet} + Me^{n+} + H^{+}$ (4)
- $SO_4^{-\bullet} + RH \rightarrow R^{\bullet} + HSO_4^{-}$ (5)
- $SO_4^{-\bullet} + H_2O \rightarrow OH^{\bullet} + HSO_4^{-}$ (6)
- $OH^{\bullet} + RH \rightarrow R^{\bullet} + H_2O$ (7)
- $R^{\bullet} + S_2O_8^{2+} + H^{+} \rightarrow SO_4^{-\bullet} + HSO_4^{-} + R$ (8)

Chain Termination Reactions (excess metal/catalyst):

- $SO_4^{-\bullet} + Me^{n+} \rightarrow Me^{(n+1)+} + SO_4^{2-}$ (9)
- $OH^{\bullet} + Me^{n+} \rightarrow Me^{(n+1)+} + OH^{-}$ (10)
- $R^{\bullet} + Me^{(n+1)+} \rightarrow Me^{n+} + R^{+}$ (11)
- $2R^{\bullet} \rightarrow$ Chain termination (12)

OZONATION

- Ozone is a powerful oxidant agent for water and wastewater.
- Once dissolved in water, ozone reacts with a great number of organic compounds in two different ways:
  - By direct oxidation as molecular ozone or
  - By indirect reaction through formation of secondary oxidants like hydroxyl radical.
- The conventional fine bubble contactor is the most widely ozone generator used because of the high ozone transfer efficiency (90%) and high performance.
- Results presented by a few researchers revealed that ozone decolorize all dyes, except non soluble disperse and vat dyes which react slowly and take longer time.
- Color removal using ozonation from textile wastewater is depended on dye concentration.

Ozone Chemistry

Chain Initiation Reactions:

- $O_3 + OH^- \rightarrow O_2^{\bullet -} + HO_2^{\cdot}$ (1)

Chain Propagation Reactions:

- $HO_2^{\bullet} \rightleftarrows O_2^{\bullet -} + H^+$ (2)
- $HO_2^{\cdot} + Fe^{2+} \rightarrow Fe^{3+} + HO_2^-$ (3)
- $O_3 + HO_2^- \rightarrow OH^{\bullet} + O_2^{\bullet -} + O_2$ (4)

O_3/UV

- According to several authors, conventional ozonation of organic compounds does not completely oxidize organics to CO_2 and H_2O in many cases.
- Remaining intermediate products in some solution after oxidation may be as toxic as or even more toxic than initial compound and UV radiation could complete the oxidation reaction by supplement the reaction with it.
- O_3/UV is the most effective method for decolorizing of dyes comparing with UV oxidation by UV or ozonation alone. (Hung-Yee and Ching-Rong (1995))
- O_3/UV treatment is recorded to be more effective compared to ozone alone, in terms of COD removal.

H_2O_2/UV

- Oxidization of the textile wastewater with H_2O_2 alone has been found ineffective at both acid and alkali values (Olcay et al., 1996),
- Under UV radiation, H_2O_2 are photolyzed to form two hydroxyl radicals ($2OH^{\bullet}$) that react with organic contaminants (Crittenden et al., 1999).
- Non efficient color removal at alkaline pH
- Therefore the instantaneous concentration in HO* is lower than expected.
- Furthermore, the H_2O_2/UV process is more sensitive to the scavenging effect of carbonate at higher pH values.

O_3/H_2O_2

- The addition of both hydrogen peroxide and ozone to wastewater accelerates the decomposition of ozone and enhancing production of the hydroxyl radical.
- At higher pH, even very small concentration of H_2O_2 will be dissociated into HO_2^- ions that can initiate the ozone decomposition more effectively than OH^- ion.
- H_2O_2/O_3 treatment of synthetic dye house highly depended on the pH of the effluent.
- It is documented that it was 74% ozone absorption at pH 11.5 (and 10 mM H_2O_2) whereas at the same concentration of H_2O_2 and pH 2.5, ozone absorption was only 1
- Complete decolourization of C.I. Reactive Blue 220 and C.I. Reactive Yellow 15 using H_2O_2/O_3 process is achieved in 90 min (Tanja et al., 2003).

$O_3/H_2O_2/UV$

- The addition of H_2O_2 to the O_3/UV process accelerates the decomposition of ozone, which results in an increased rate of OH• generation.
- Among all AOPs, for dye house wastewater and acetate, polyester fiber dyeing process effluent; combination of H_2O_2/O_3/UV appeared to be the most efficient in terms of decolouration.
- COD removal efficiency of raw textile wastewater increased from 18% to 27% by using sequential ozonation and H_2O_2/UV.
- In the case of bio-treated textile effluent, a preliminary ozonation step increased COD removal of the H_2O_2/UV-C treatment system from 15% to 62%.
- 99% COD removal from polyester fiber dyeing process effluent in batch mode operation(O_3/H_2O_2/UV in 90 min) was achieved.
- In raw textile effluent, TOC removal rate was accelerated from 14% (H_2O_2/UV-C) and 17% (O_3) to 50%.

Mechanism of AOP Reactions: Photocatalysis and Sonolysis

Reactions involved in Photo catalysis:

$TiO_2 + h\nu \rightarrow TiO_2 (e^- + h^+)$

$TiO_2 (h^+) + H_2O \rightarrow TiO_2 + H^+ + HO^-$

$TiO_2 (h^+) + OH^- \rightarrow TiO_2 + HO^\bullet$

$TiO_2 (e^-) + O_2 \rightarrow TiO_2 + {}^\bullet O_2$

$H_2O_2 + h\nu \rightarrow 2\ HO^\bullet$

Reaction involved in Sonolysis:

$H_2O +))))\rightarrow H^\bullet + HO\bullet$

$H_2O +))))) \rightarrow ½\ H_2 + ½\ H_2O_2$

$H_2O_2 + H^\bullet \rightarrow H_2O + HO^\bullet$

Processing Methods for Elimination of Potential Pathogens such as Bacteria/Virus/Prions

Approaches Towards Virus and Microorganism Safety

1. **Selection of starting materials/raw materials**

2. **Testing of raw materials, blood/cell lines/fermenters**

3. **Methods for virus and microorganism inactivation/removal**

(testing of final product is usually not efficient)

None of these approaches alone can guarantee viral safety. The appropriate combination of complementary measures is important.

Methods for Virus and Microorganism Inactivation/Removal

Classical Methods:

1. SD-Treatment
2. Pasteurization
3. Dry heat
4. Nano filtration
5. Low pH treatment
6. Purification (Precipitation, Chromatography)

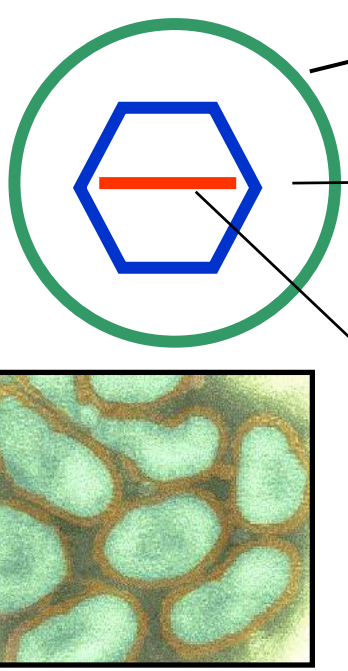
New developments:

7. High pressure
8. UV irradiation
9. Gamma irradiation
10. Nucleic acid modifying chemicals
11. Other chemicals

Virus Structure

enveloped Viruses

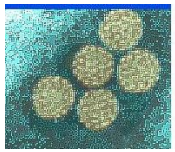
(HIV, HBV, HCV, BVDV)



- labile
- large (60 -220 nm)
- inactivation possible
- filtration possible

non-enveloped viruses

(HAV, MMV, Parvovirus B19)



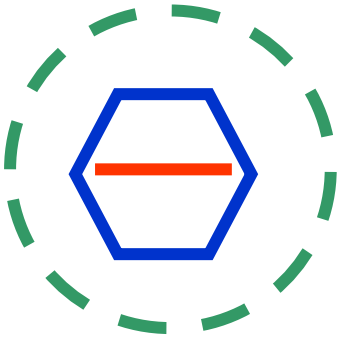
- stable
- small (20 -80 nm)
- difficult to inactivate
- filtration difficult

1. Solvent Detergent Treatment („SD-washing“)

1% Triton X 100 + 0.3% TNBP (>4 h)

1% Tween 80 + 0.3% TNBP (>6h, 24°C)

0.2% Na-deoxycholate + 0.3% TNBP (>6h, 30°C)



Destruction of envelope

- efficient inactivation of all enveloped viruses (HIV, HBV, HCV)
- no inactivation of non-enveloped viruses

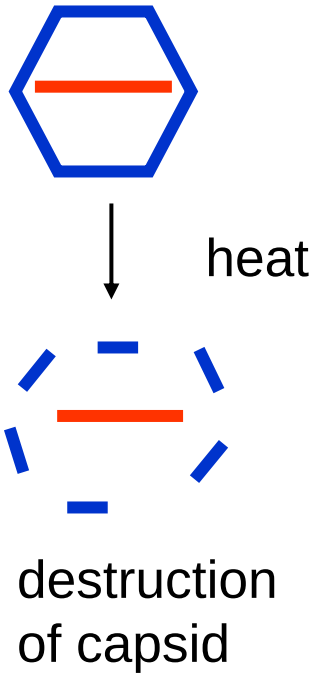
Critical parameters:

- exact concentration of SD-reagent (mixing, tank validation)
- virus aggregates (pre-filtration is required)
- lipid content
- temperature

2. Pasteurization (60°C, 10 hours)

Good inactivation of enveloped and non-enveloped viruses

- HIV, HBV, HCV efficiently inactivated
- HAV, B19: ca. 4 log reduction
- some non-enveloped viruses are heat resistant:
  (animal parvoviruses: MMV, PPV, CPV)



Critical parameters:

- exact temperature, homogeneity of temperature
- exact concentration of stabilizers (e.g. sugar)
- virus aggregates (pre-filtration)

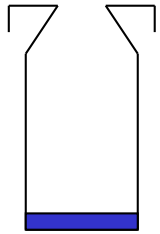
3. Dry heat

- Inactivation of a broad range of viruses

  (HIV, HBV, HCV, HAV); parvoviruses partially inactivated 80°C for 72 hours or 100°C, 30 minutes

- Good inactivation of enveloped viruses and some non-enveloped viruses (HIV, HBV, HCV und HAV parvoviruses are not completely inactivated

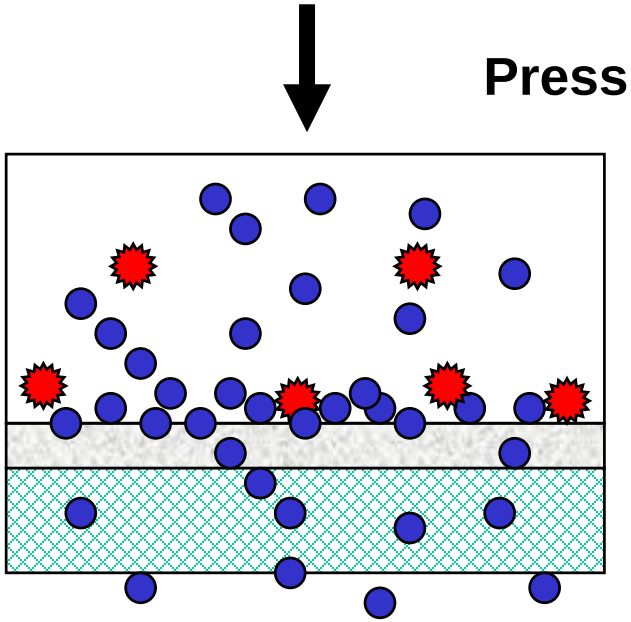
- Drying leads to stabilization of viruses
  residual moisture is very important (0.5 to 2%)

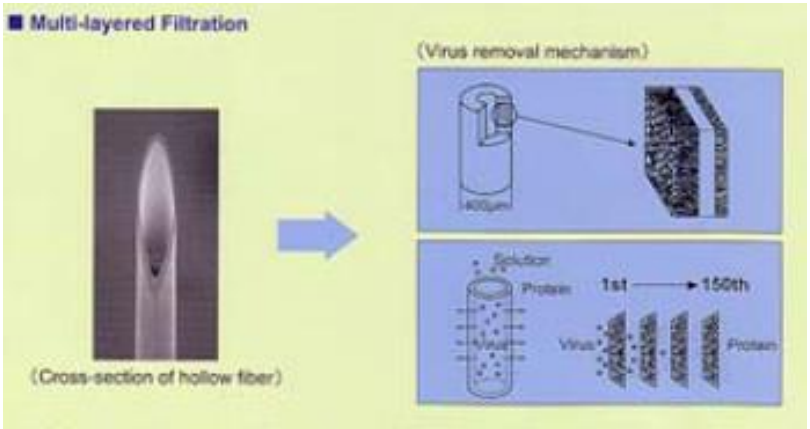


Critical parameters:

- exact temperature
- exact control of residual moisture

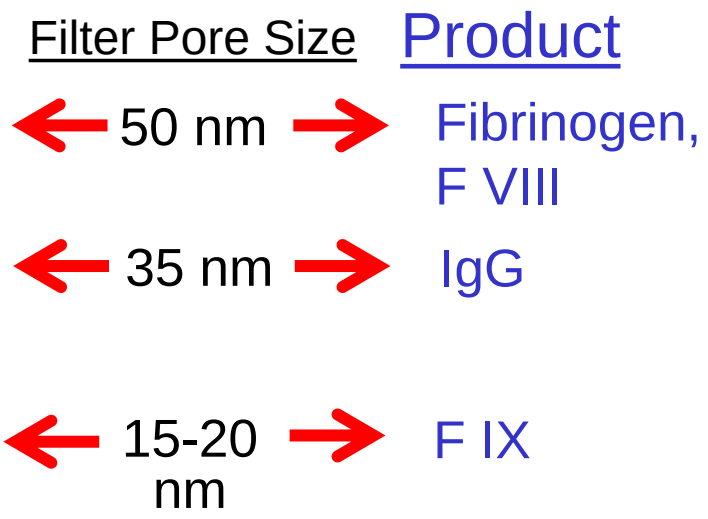
4. Nano filtration





Problem: Some viruses may be smaller than the drug substance

Virus	Size (nm)
HIV retroviruses	80-130
HCV, BVDV	40-50
HBV, SV40	40-45
HAV, EMCV	28-30
Parvovirus B19, MMV, PPV	18-26



Critical parameters:

- pressure, flow rates, time
- filter loading (volume/filter area), do not overload!

5. Virus inactivation at low pH (pH <3.8) (e.g. mAb-Production, Protein A Eluate)

- **X-MuLV: Yes**
- **PRV: Sometimes**
- **BVDV: No/very slow**
- **Non enveloped viruses: (mostly) no**

Critical parameters:

- exact pH
- time
- temperature

6. Chromatography

Effectiveness towards reduction of viruses depends on specific conditions. No general prediction possible

Many critical parameters: column size, flow rates, column loading, pH, ionic strength, age/re-use

7. Precipitation (salt or alcohol) + centrifugation or depth filtration

Sometimes removal of a broad range of viruses
However, removal may be selective for specific viruses
Reduction capacity very variable depending from specific conditions
(>5 log to <1 log)

Many critical parameters: many, concentration of precipitating agent (salt, EtOH), temperature, pH, ionic strength, physical parameters for separation of precipitate (conditions of centrifugation or depth filtration)

New Developments:
1. Pressure or Pressure Cycling

High Pressure:

- 400 MPa, -5°C, 7.4 min

[Bradley et al., *Transfusion*, 40 193-200, 2000]

Pressure-Cycling:

- 540 MPa,
- product specific number of cycles

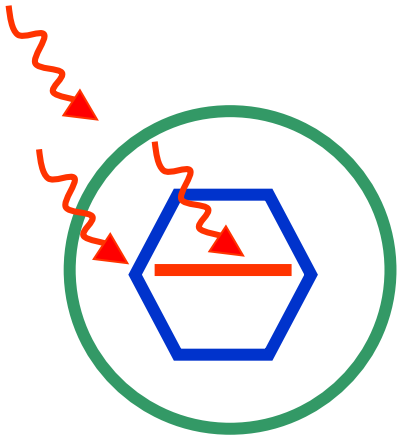
[M. Manak, *IBC conference BSP 2002*, Tysons Corner, VA]

Problems:

- Damage/loss of drug substance (complex proteins)
- (F VIII: 30% loss)
- (Large scale application?)

Irradiation (General Aspects)

Direct Hits



Indirect Effects

Matrix, (sensitizer)

Reactive Oxygen
Free Radicals

Direct Hits

Drug Substance
Protein

Nucleic Acid
Strand break
Adducts (intra/inter chain)
Nucleotide Oxidation

Proteins:
Chain Breaks
Side Chain Reaction
Denaturation, Cross linking

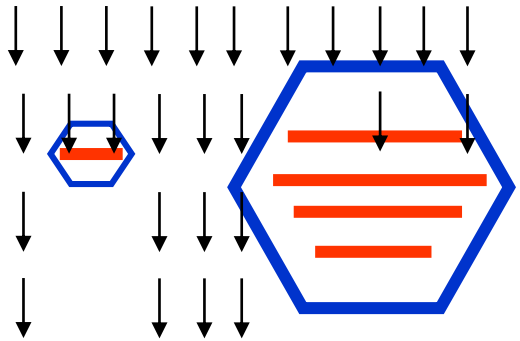
Lipids
Peroxidation

Proteins:
Chain Breaks
Side Chain Reaction
Denaturation,
Cross linking

New Developments:
2. UV-C Irradiation (1)

UVC adsorption mainly by nucleic acids → Viral DNA/RNA damage (photo adducts, T-Dimers)

Virus	Genome	Size (kb)	Flux (J/cm)*
SV40	dsDNA	5.0	0.007
PPV	ssDNA	5.0	0.009
HAV	ssRNA	7.5	0.018
Sindbis	ssRNA	12.0	0.040
Reo	dsRNA	23.5	0.074
Adeno	dsDNA	36.0	0.216



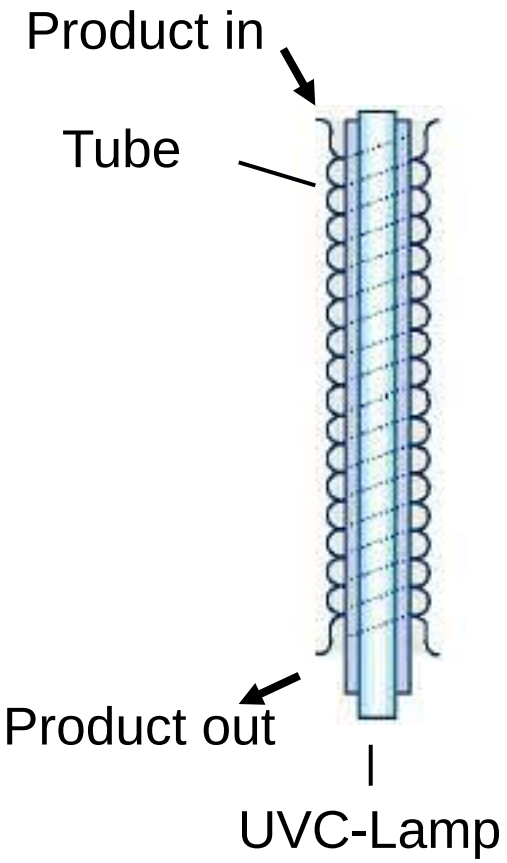
*: Dose required or a log reduction factor of 4.

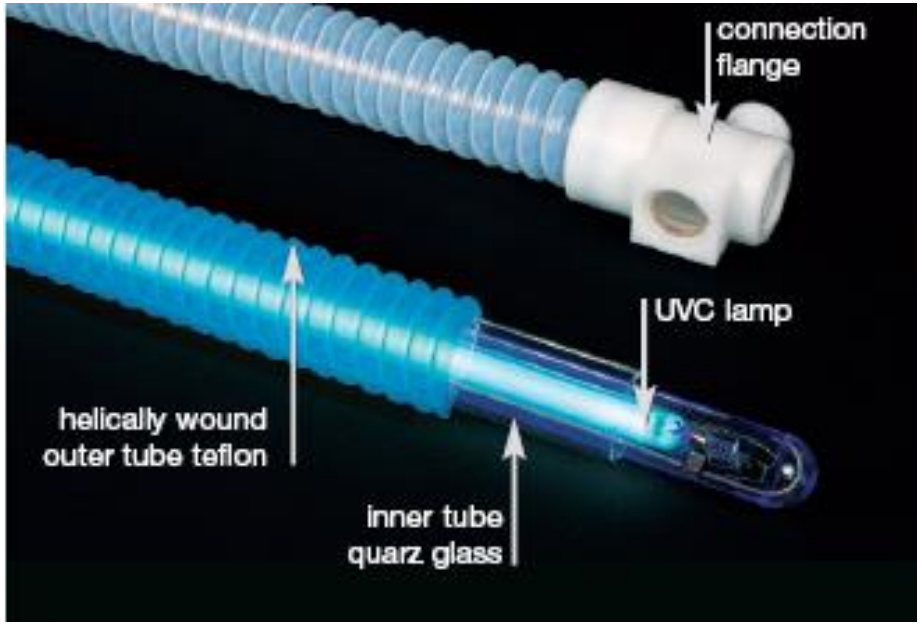
[Wang et al., *Vox Sang* 86, 230-238, 2004]

Good inactivation of small non enveloped viruses (SV40, HAV, PPV)

Problem: Side effects on proteins possible

UV-C Irradiation (2)





Critical Parameters

UV does not penetrate thick liquid layers

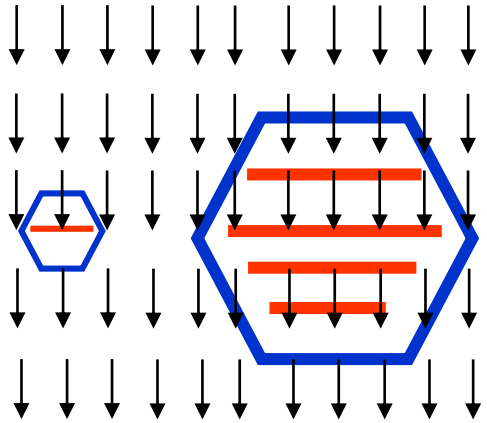
Exact dose (UVC-intensity, time, flow rates)

Composition of product intermediate (turbidity)

New Developments:
3. Gamma- Irradiation

- Direct DNA/RNA strand breaks
- Good inactivation of microbes (fungi, bacteria)
- Good penetration of materials (sterilization of final container
- No toxic chemicals,

Fungi Bacteria:	<10 kGy
Parasites	<10 kGy
Large (enveloped) viruses	15-20 kGy
Small (non-enveloped) viruses	40-45 kGy
Prions	45-60 kGy



Problem:

- High doses 40-45 kGy required for inactivation of small viruses (industry device standard: 25 kGy)
- Secondary reactions (radicals) on drug protein. This may be minimized by stabilizations/quenchers

Critical Parameters:
Dose (chamber validation), oxygen-content, stabilizers, (Temp.)

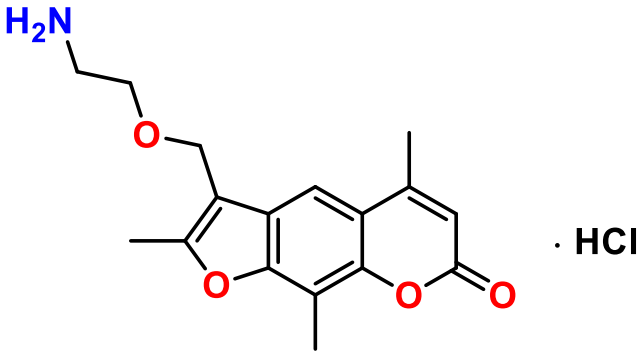
New Developments:
4. Nucleic acid Modifying Substances

- Psoralen derivatives + UVA-light
- Riboflavin + light
- Inactine® (ethylenimin derivative)
- Methylene-Blue

Problem: The nucleic acid modifying substance must penetrate the virus shell in order to get contact to the viral DNA/RNA

- Good inactivation of enveloped viruses
- Difficult to Inactivate non-enveloped viruses
- **Toxic substances must be removed completely afterwards**

Example of Application: Pathogen inactivation of blood components (platelets) with S59, psoralen derivate)



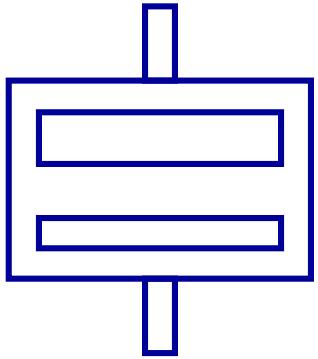
S 59

The S 59 is removed by adsorption (in bag)

5. Other chemicals

Iodine

Iodine-Filter:



PVPP-Iod

PVPP

Good inactivation of Viruses
Problem: Reaction with (therapeutic) proteins.

[Feifel, K et al., (1998) CHI Blood safety Europe]

Iod-acetaldehyd, Chlor-acetaldehyd

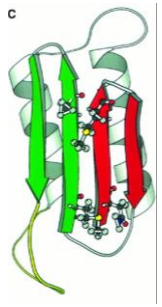
Reaction with nucleotides
Reaction with proteins (SH-groups)
Easy to remove

[Bergval., (1998) ISC Conference BSP 2002, Tysons Corner, VA]]

Reduction of Prions
1. Inactivation

Very resistant small protein.

Harsh methods required (not applicable to therapeutic proteins)



Requirements for specific substances (Guideline EMEA/410/01/rev02)

Tallow derivatives:

a) Trans-esterification at >200°C, 20min

b) Saponification with 12M NaOH (at 95°C for 3h or 140°C >8min)

c) Distillation at 200°C

Gelatin: alkaline lime (20d, pH >12.5) + heat (138-140°C for 4s)

Amino acids: strong hydrolysis pH 1 to 2 followed by pH>11 followed by heat (140°C, 30min, 3 bar).

Charcoal: production at >800°C

Compliance may be certified by EDQM „TSE-CEP“

Reduction of Prions
2. Purification

Example: Chromatographic purification of plasma proteins

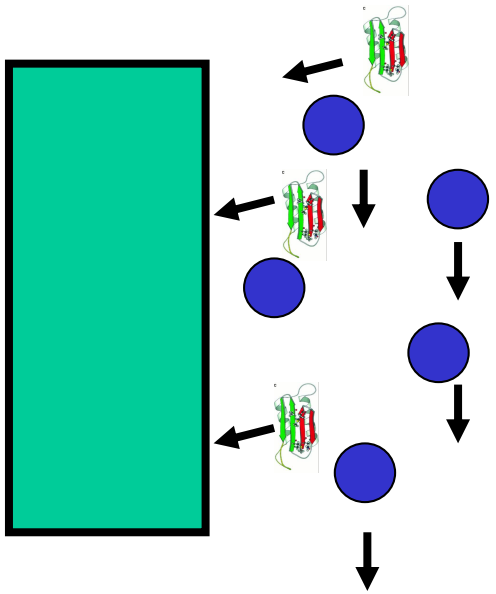
Rf (log10) at **Ion Exchange Chromatography**		
Agent	324K	Mouse adapted BSE 301V
Assay	Western Blot Rf (log_{10})	Mouse Bioassay Rf (log_{10})
Fibrinogen	>3.5	2.9
Factor VIII	3.1	2.7

[P. Foster 2003, Edinburgh]

Good purification may provide significant reduction of prions

Problems: reliability of validation experiments (spike preparation, assay)?,

Reduction of Prions
3. Special Affinity Media



a) Peptide ligand from combinatorial library

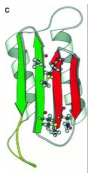
b) Other affinity media

Application: Prion reduction from blood components/plasma?

Problems: reliability of validation experiments (spike preparation)?, binding (loss) or competition of target proteins

[Sowemimo-Coker, Vox Sang (2006) 90, 265-275)]

Reduction of Prions
4. Nano filtration



Monomer: 5-10 nm, 21.5 kDa (not infectious?)
Oligomers: 13-27 nm, 50-600 kDa (most specific infectivity)
Size of fibrils: >100 nm, >1000 kDa (less specific infectivity)

Medium pore size filters (35-80 nm)
Complete removal of infectivity from brain homogenate
No removal of dispersed preparations from brain (sarkosyl, ultrasonic)

Small pore size filters (15-20 nm)
Complete removal of infectivity from brain homogenate
Complete removal of dispersed preparations from brain (sarkosyl, ultrasonic)
No removal of strongly dispersed prion preparations

What is the prion aggregation status in the production intermediate??

References

- B Halliwell and J.C.M. Gutteridge: Free Radicals in Biology and Medicine. 3rd edition OUP, Oxford UK, 1999, ISBN – 0 –19-850044
- Alderton, W.K., Cooper, C.E. and Knowles, R.G. (2001) Nitric oxide synthases: structure, function and inhibition. *Biochem. J.* **357**, 593-615.