MarBEF TRAINING COURSE ON BIOASSAY METHODS IN MARINE CHEMICAL ECOLOGY

September 9-14, 2006

Bioassay techniques and toxicological tests on marine invertebrates

Giovanna Romano Ecophysiology Laboratory
Stazione Zoologica “Anton Dohrn” Napoli

Bioassay techniques and toxicological tests on marine invertebrates

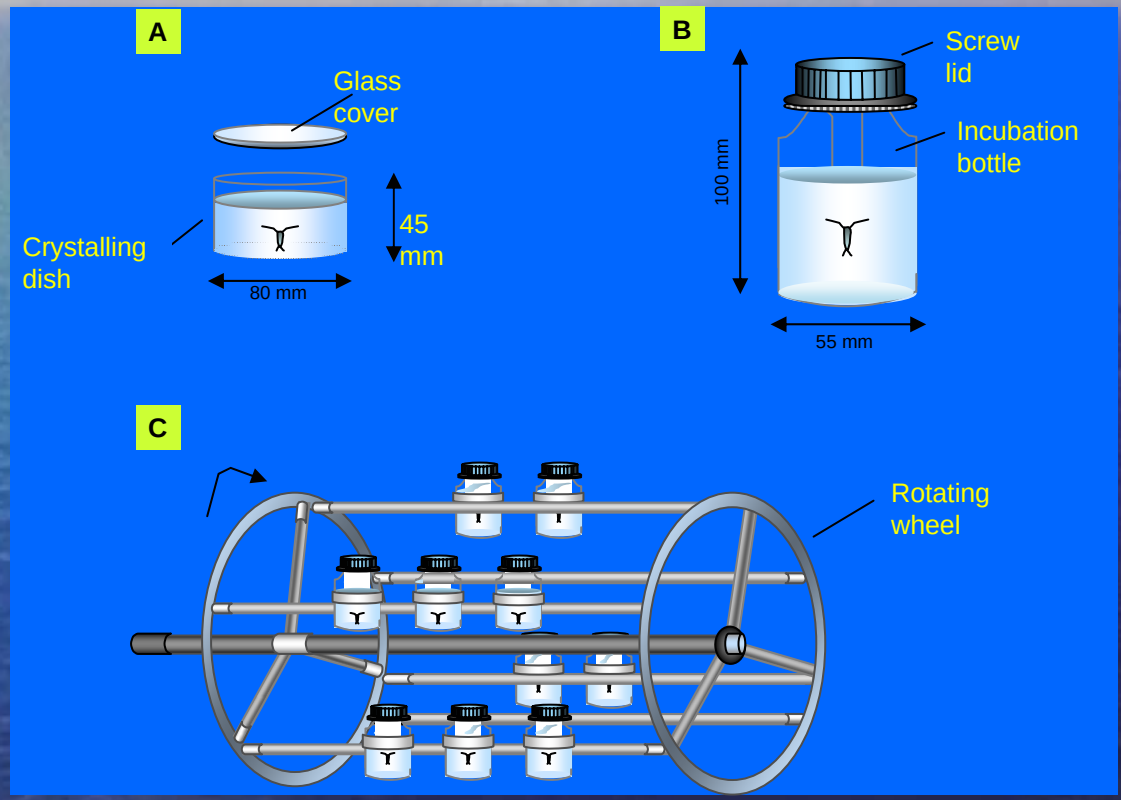
Classical assay methods:

-Copepod feeding, egg production, egg hatching success and larval development

- Antimitotic assay on sea urchin embryos

Fluorescence-based method for determining cell viability and for assaying cytotoxicity

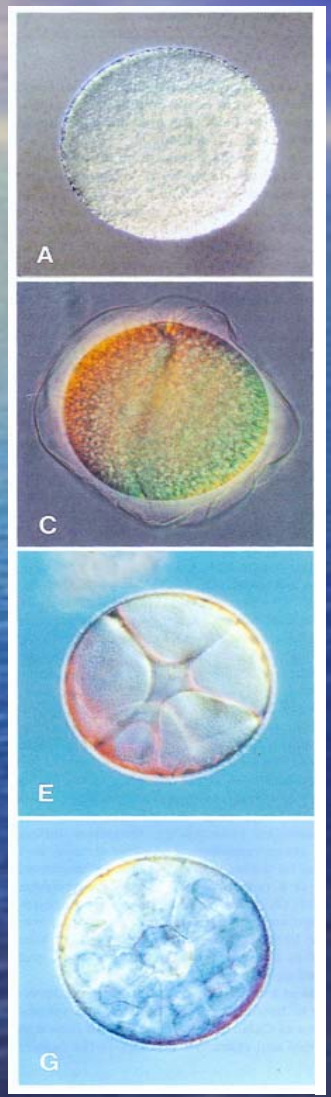
Bioassays to study copepod feeding, egg production, egg hatching success and larval development



In these assays copepods are incubated either in (A) crystallizing dishes or in (B) jars filled with known quantities of food. Experimental jars are mounted on a (C) rotating wheel (0.5 rpm) in a controlled temperature room and on a fixed dark:light cycle.

Control embryos

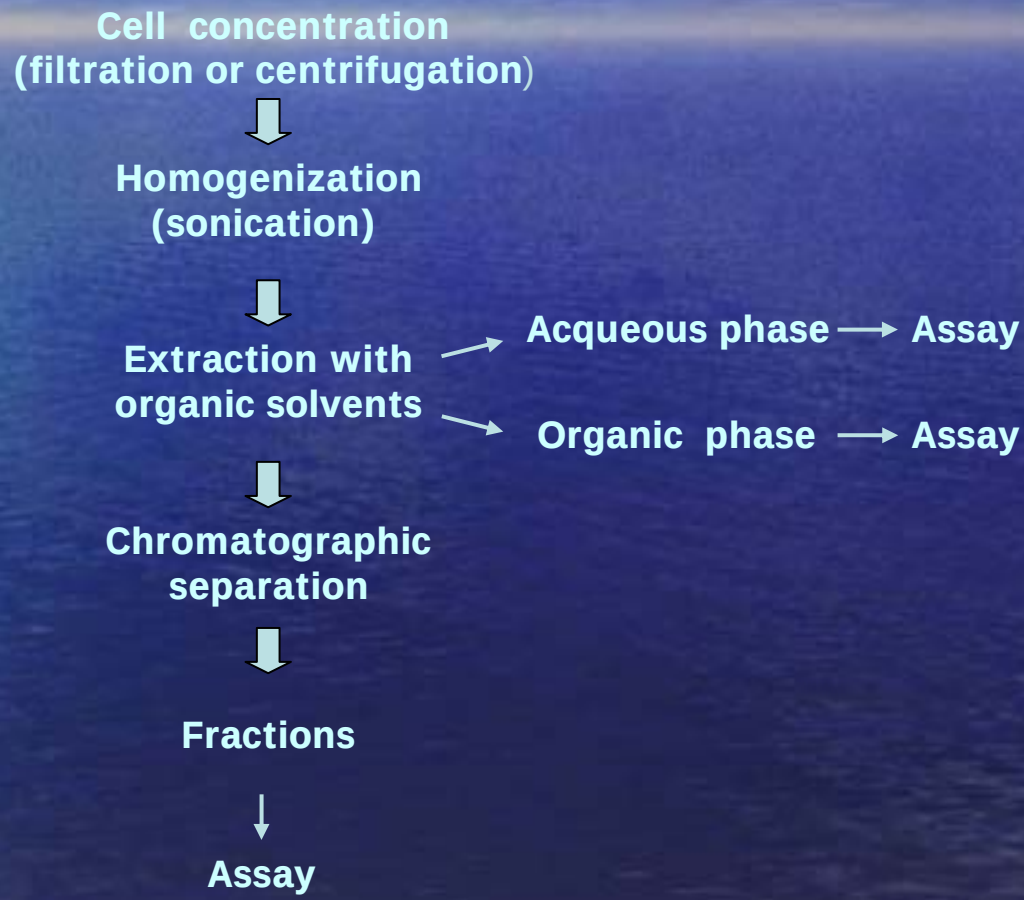
Embryos Treated with diatom extract



Calanus helgolandicus early embryos

Poulet et al. MEPS 1995

Isolation of
Biologically active molecules from marine microalgae



Antimitotic assay on Sea Urchin embryos

-Sample to test diluted in sea water at increasing concentrations

-Eggs and sperm collection

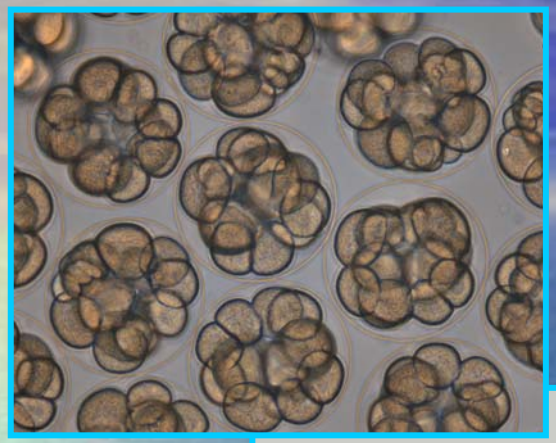
-Fertilization

-Incubation in test mixture

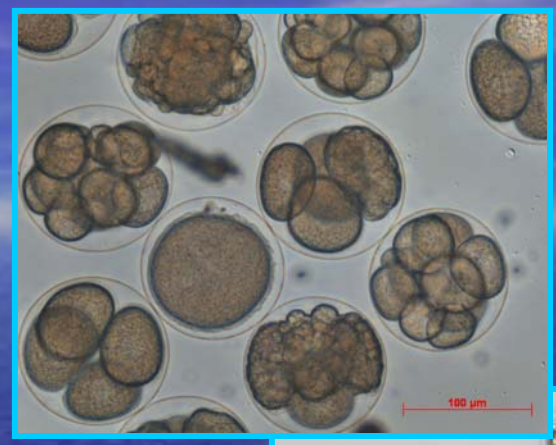
-Observation each 30 min with an inverted microscope

Control embryos

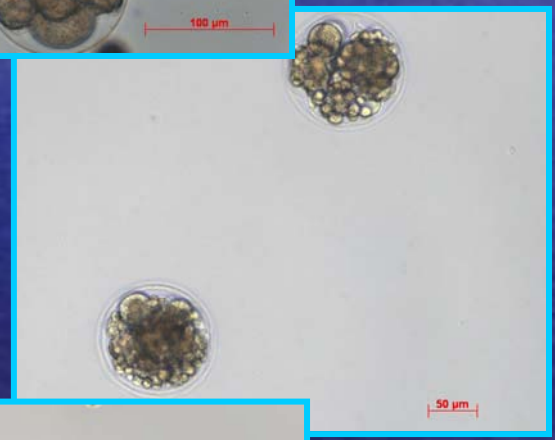
Aldehydes-treated Embryos



2 hours



24 hours



72 hours

Fluorescence-based method for determining cell viability and for assaying cytotoxicity

Cell-permeant nuclear stains

- DAPI
- Hoechst
- SYTO 9

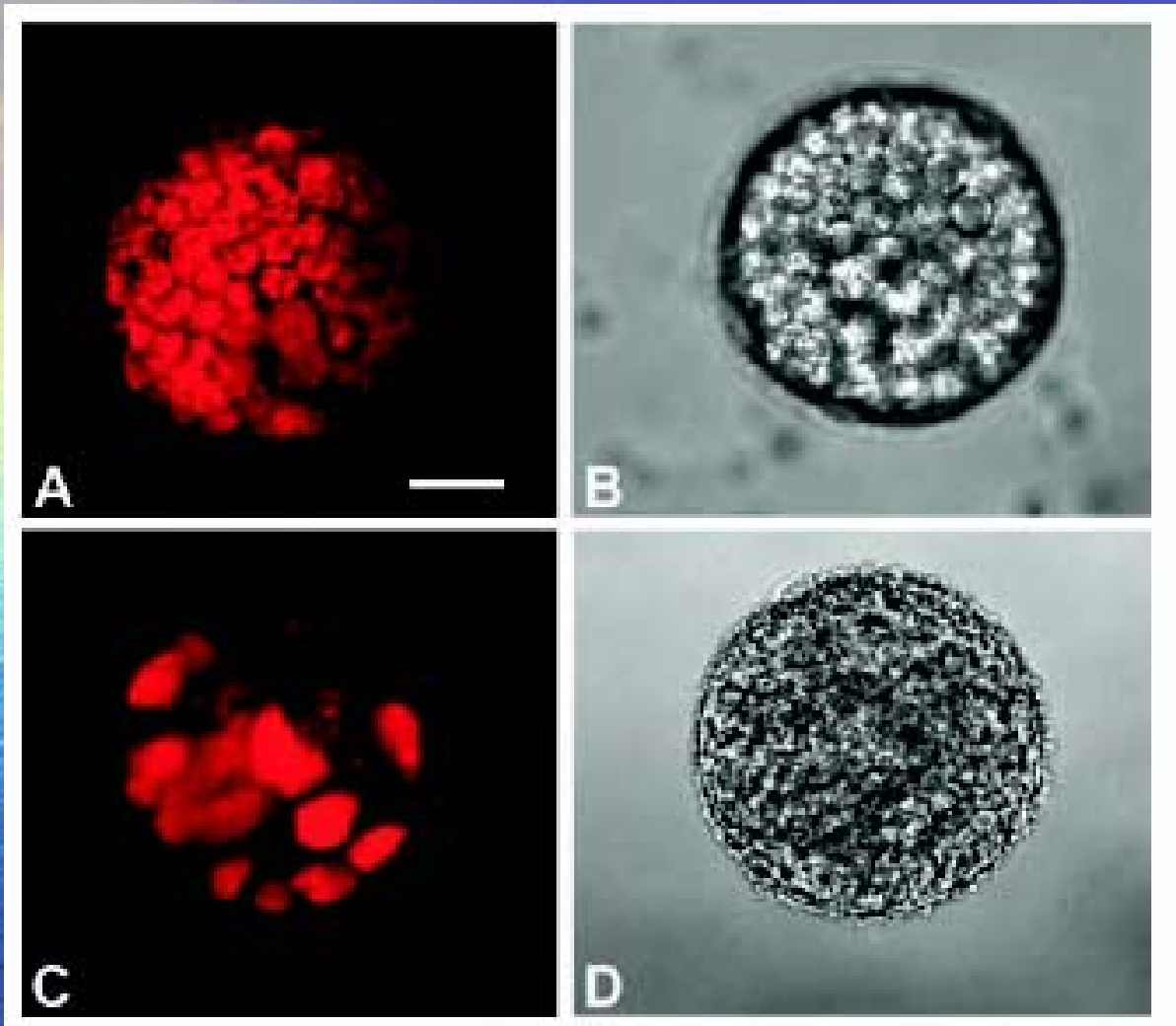
→ Stain live cells or tissues that have been minimally processed. Have little effect on cell function, allowing live cells to be traced as they move during development or as progress trough apoptotic events.

Cell-impermeant nuclear stains

- SYTOX Green
- Ethidium homodimer-1
- Propidium iodide
- 7-AAD (7-aminoactinomycin D)

→ Employed to detect the dead-cell population. Penetrate only cells with compromised plasma membrane.

Embryos stained with 7-AAD



Buttino et al. Mar. Biol. 2004

7-AAD is a fluorescent DNA probe that is excluded from live cells; dead cells appear with red fluorescent nuclei.

Fluorescence-based method for determining cell viability and for assaying cytotoxicity

Viability/Cytotoxicity Assays Using Esterase Substrates

- Calcein AM
- Dihydrocalcein AM
- Fluorescein Diacetate (FDA)
- Carboxyfluorescein Diacetate (CFDA) and its derivatives

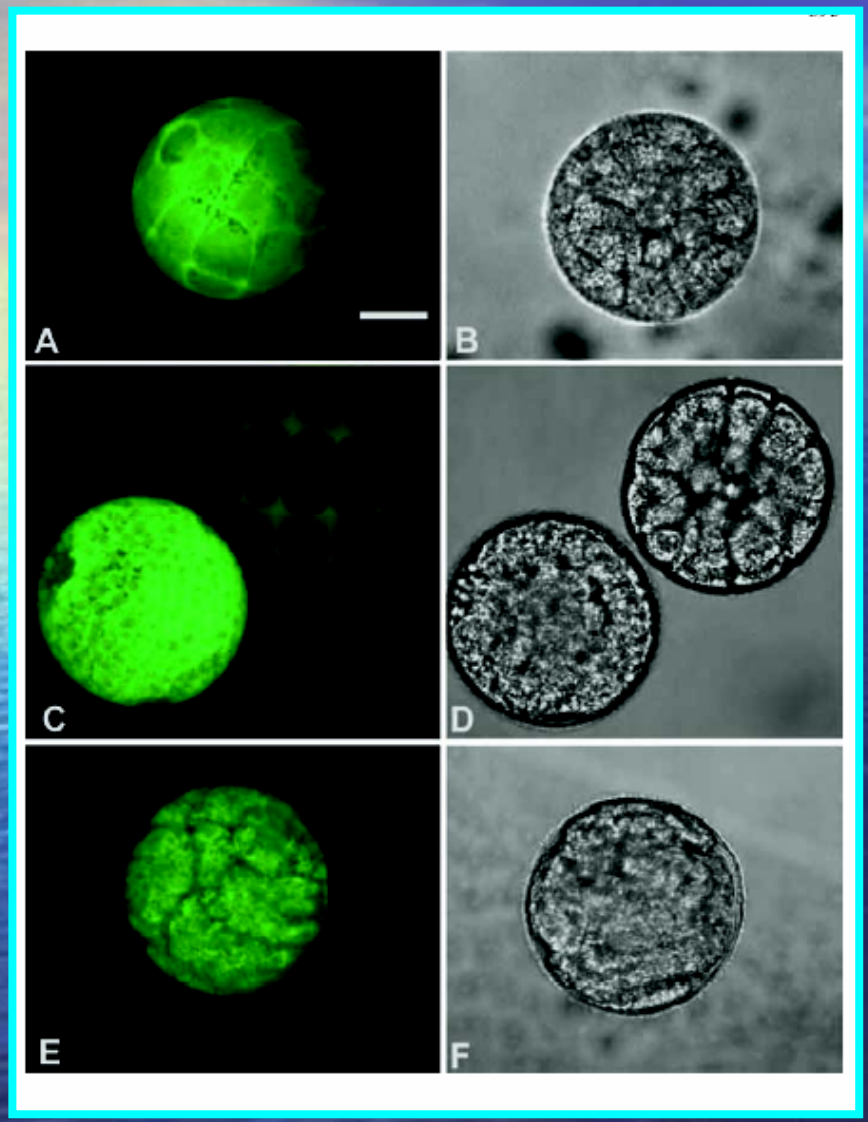
Viability probes that measure both enzymatic activity, which is required to activate their fluorescence, and cell-membrane integrity, which is required for intracellular retention of their fluorescent products.

Viability/Cytotoxicity Assays That Measure Oxidation or Reduction

- Resazurin
- Dodecylresazurin
- "dihydro" derivatives of Fluorescein, rhodamine and various other dyes

Metabolically active cells can oxidize or reduce a variety of probes, providing a measure of cell viability and overall cell health. This measure of viability is distinct from that provided by probes designed to detect esterase activity or cell permeability

Assesment of viability using esterase substrate



When FDA penetrates into viable cells, esterases produce free fluorescent fluorescein and cells appear fluorescent in green, whereas cells with an inactive metabolism are not fluorescent.

Buttino et al. Mar. Biol. 2004

Fluorescence-based method for determining cell viability and for assaying cytotoxicity

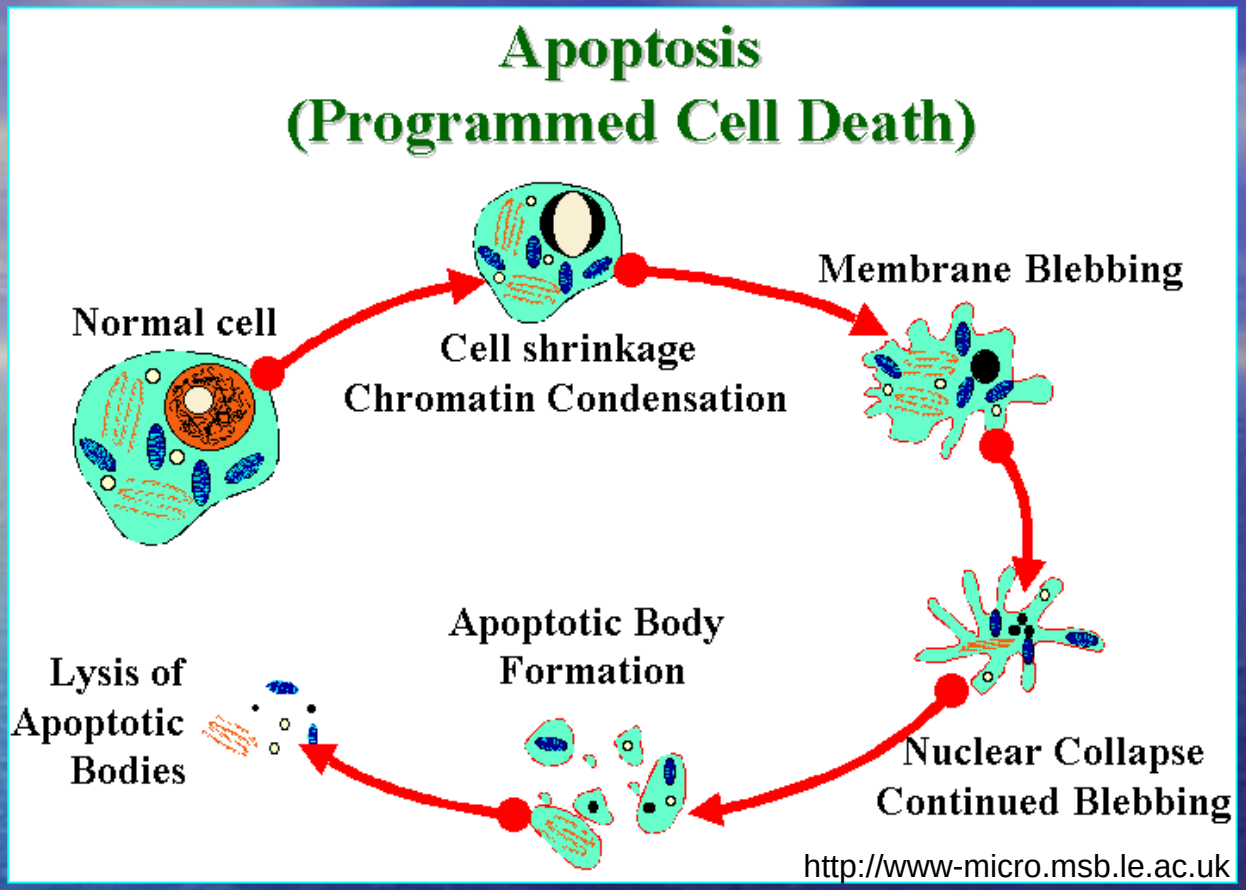
Double stain technique

- **1)**calcein AM - **esterase substrate**
  2)ethidium homodimer-1 - **Cell-impermeant nuclear stains**

- **1)**green-fluorescent SYTO 10 - **cell-permeant nuclear stains**
  2)red-fluorescent DEAD Red (ethidium homodimer-2) - **cell-impermeant nuclear stain**

- **1)**C12-resazurin - **metabolic activity probe**
  2)SYTOX Green - **impermeant nuclear stain**

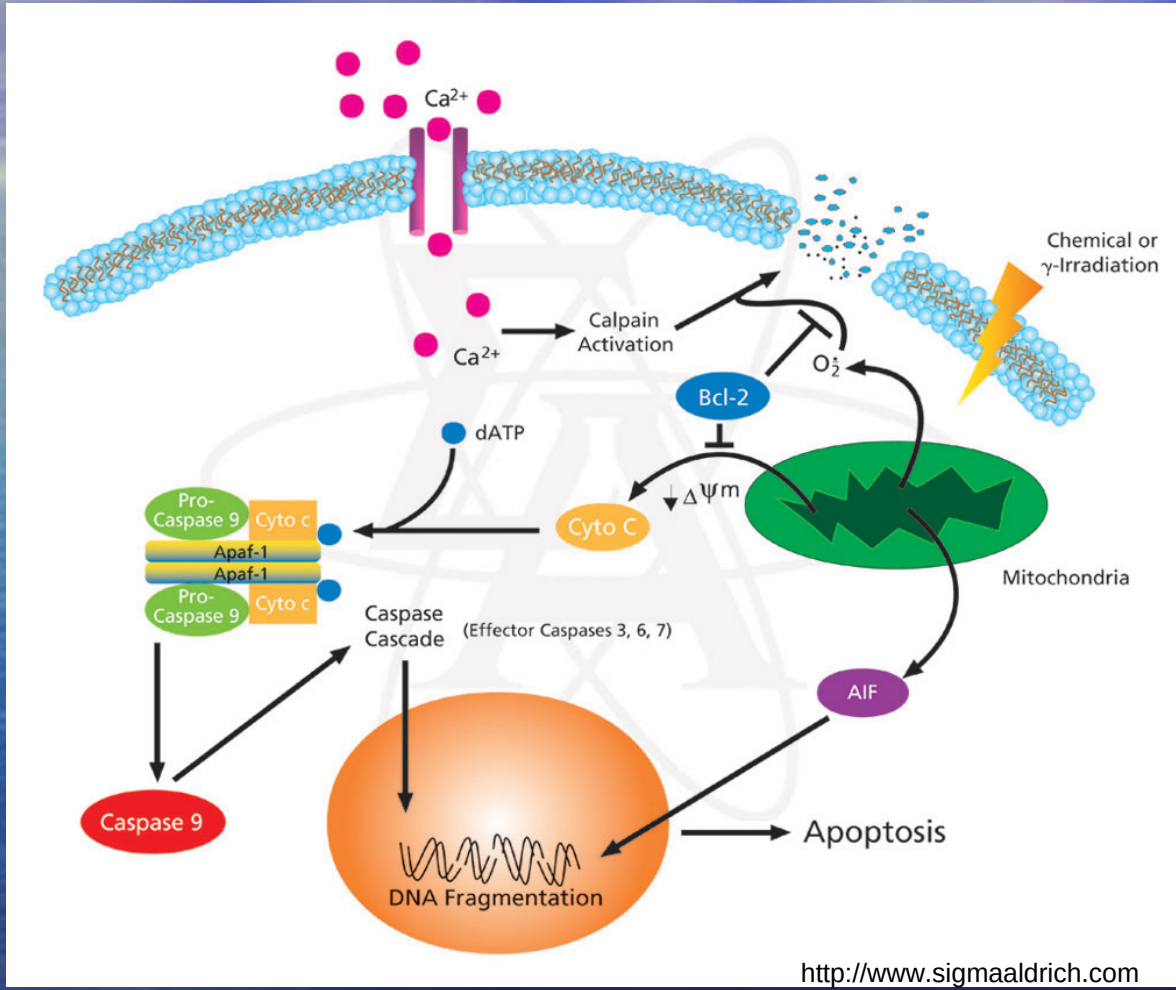
Two-color discrimination of the population of live cells from the dead-cell population by simply adding the reagents, incubating for a brief period and observing the results without any wash steps required



Apoptosis is a form of cell death in which a programmed sequence of events leads to the elimination of cells without releasing harmful substances into the surrounding area. Many types of cell damage and biologically active substances can trigger apoptosis, and it also occurs normally during development in multicellular organisms.

Apoptosis plays a crucial role in developing and maintaining health by eliminating old cells, unnecessary cells, and unhealthy cells. It is an active process requiring metabolic activity by the dying cell, often characterised by cleavage of the DNA into fragments that give a so called laddering pattern on gels. Apoptosis is distinguished from necrosis, a form of cell death that results from injury.

Apoptotic machinery



http://www.sigmaaldrich.com

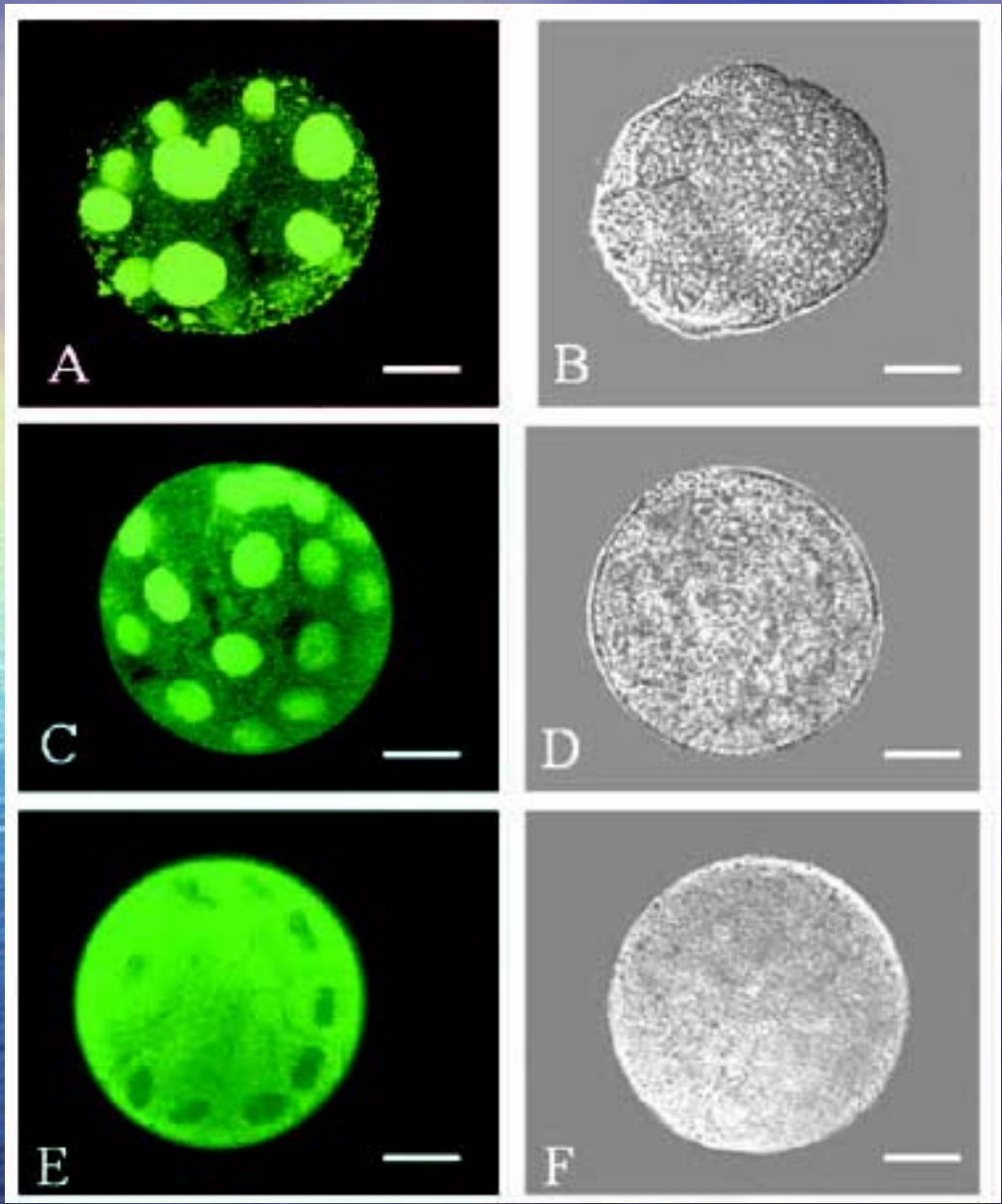
Fluorescence-based method for the detection of apoptosis

Detection of Apoptosis

- Nucleic acid stains combination
- **Annexin** V
- **TUNEL** (terminal deoxynucleotidyl transferase dUTP nick end labeling)
- **TUNEL** in combination with Propidium iodide or Hoechst
- Apoptosis Assays Based on Protease Activity: detection of **Caspase** activity
- Apoptosis Assays Using Mitochondrial Stains (**Mitotracker**)

TUNEL Staining Protocol

- Fixation
- Permeabilization
- Incubation in staining mix solution
- Observation with an epifluorescence microscope

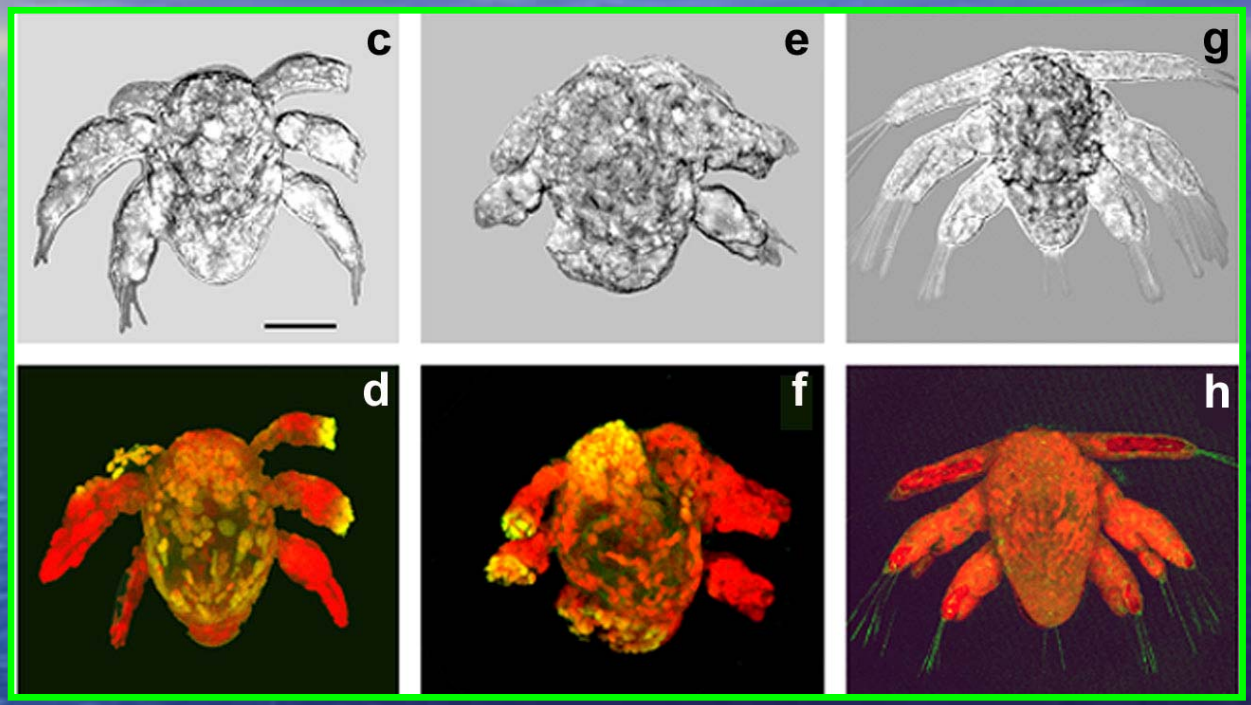


Calanus helgolandicus **embryos stained with TUNEL dye to detect apoptosis (cell death)**

Apoptotic nuclei show a green fluorescence, whereas in control embryos only a backgraund fluorescence is present and nuclei appear dark

Romano et al. J Exp Biol 2003

Embryos stained with TUNEL (yellow) + Propidium iodide (red)



Ianora et al. Nature 2004