Biorее		
Protocol for lanthanoid staining of biological samples for further examination by scanning electron microscope (ESEM|EP)		
Instructions for preparation of samples for examination by scanning electron microscope (ESEM|EP) with visualization of internal cell structure		**Doc.№** BRLN002 v1.0
Developed: Subbot A.M.	**Tested:** Fedorov A.A.	**Date:** 14.12.2015

Introduction: The technology of elective accumulation of elements of lanthanum group in individual structures of a cell and therefore increasing their contrast for back-scattered electrons detection allows for SEM to be used for visualization of cell ultrastructure. Moderate concentrations do not cause immediate cell death but instead gradually "shut-off" the energy-dependant processes which makes the method "supravital". We noticed that the localization of contrast areas is consistent in cells of all types. The accumulation of lanthanides in cell structures is based on several mechanisms:

- lanthanides bind to RNA (that makes the nucleoli bright)
- lanthanides replace calcium in cadherin-like proteins (that marks cell junction areas)
- paired transfer of two calcium ions replaced by trivalent lanthanoid ion paired with alkali metal (that better corresponds to the location of calcium pumps)
- phosphate residues that appear during the energy-dependant processes, i.e. microtubules development, together with lanthanoid cipitate into insoluble forms (thus we see the traces of cytoskeleton formation)

Subjects: Staining is practical in living cells since for fixed (dead) cells the information content would be incomparably lower. The protocol was tested on staining structural elements of thin tissue samples (scrapes of 1 mm or lower thickness) and cells – cultivated or primarily acquired – on carbon and polymer scaffolds, excluding those based on phosphates.

Kit functioning principle: The first rinsing removes the components of growth mediums and the liquids of tissue ground substances sorbed on the surface of the sample. The following soaking in the rare earth element solution leads to elective accumulation of the REE on tropic cell structures. The second rinsing removes the surplus of the staining agent.

Reagents:

- rinsing solution – normal saline (isotonic solution of Sodium Chloride);
- contrast agent solution, NdCl3+NaCl;
- final rinsing solution – distilled water.

Process of staining cells binded to substrates and tissue samples:

1) The sample is rinsed in NaCl isotonic solution because otherwise part of REE, being tropic to phosphates, could form insoluble compounds with components of growth mediums and liquids of tissue ground substances sorbed on the sample surface which would prevent further observations.

2) The sample is put into aqueous isotonic solution of contrasting agent and left there for the specified duration.

3) The sample is rinsed in distilled water (1-10 seconds) to remove the surplus of contrasting agent, moist surplus can be removed using “air brush”.

4) Prepared sample is put onto the microscope stage.

The best results can be achieved if during the process of staining the object of examination is kept in the conditions close to physiological which can be done by thermostatting the sample and putting it into an orbital shaker with low frequency.

Table of approximate time of staining solution exposure for some objects.

Object of examination	***Exposure time, min***
Cells binded to substrates	
Individual cells on plastic or glass with adhesive coating	15
Cellular monolayer on plastic or glass with adhesive coating	30
Tissue-engineered structure, 3D scaffold	30
Native tissue	45
Suspension cultures	
Cells suspension	15
Cells deposited onto membrane	15
Bacteria, protozoa, yeasts etc.	15

Process of cell suspension staining:

1) The sample is centrifuged, then supernatant is removed and rinsing solution is added. Shaked up with a vortex. Centrifuged, then supernatant is removed leaving 1 ml. Shaked up with a vortex to remove conglomerates.

2) Aqueous isotonic solution of contrasting agent is added to pellet cells and the solution is left for the specified duration.

3) Cells are deposited onto the membrane of syringe filter.

4) Syringe filter cartridge is rinsed with distilled water to remove the surplus of contrasting agent and the membrane with deposited cells is extracted from the cartridge.

5) Prepared object is put onto the microscope stage.