Detection of mineral nodules in marine fish mineralogenic cell lines

Vincent Laizé, Marta S. Rafael, Sara Mira, M. Leonor Cancela
Centre of Marine Sciences (CCMAR), University of Algarve, Faro, Portugal

PREAMBULE. Mineralization of the extracellular matrix (ECM) of fish cell lines is previously induced by supplementing culture medium with 4 mM calcium chloride, 10 mM β-glycerophosphate and 50 mg/ml of ascorbic acid (Pombinho et al., 2004). Volumes and quantities given below are for a well of a 24-well plate.

PHOSPHATE-SPECIFIC STAINING (adapted from von Kossa, 1901)

1. Discard medium and wash cell culture 3 times with 1 ml of PBS.
2. Fix cells for 1 h at 4ºC with 0.5 ml of 4% (v/v) formalin (in PBS).
3. Discard fixative and wash cell culture 3 times with 1 ml of MilliQ water.
4. Stain mineral nodules for 15 min at room temperature and under UV light with 0.5 ml of 5% (w/v) silver nitrate solution (in MilliQ water).
5. Discard stain and wash cell culture 3 times with 1 ml of MilliQ water.

   Optional: Fix silver staining for 5 min at room temperature with 0.5 ml of 2.5% (w/v) sodium thiosulfate (in MilliQ water). Discard fixative and wash cells with 1 ml of MilliQ water.
6. Observe black-stained nodules under an inverted microscope equipped with phase contrast, then scan the plate at a resolution compatible with subsequent densitometry analysis (e.g. 600 dpi).

CALCIUM-SPECIFIC STAINING (ALIZARIN RED S / AR-S)

1. Discard medium and wash cell culture 3 times with 1 ml of PBS.
2. Fix cells for 1 h at 4ºC with 0.5 ml of 4% (v/v) formalin (in PBS).
3. Discard fixative and wash cell culture 3 times with 1 ml of MilliQ water.
4. Stain mineral nodules for 15 min at room temperature with 0.5 ml of 40 nM AR-S solution (in MilliQ water).
5. Discard stain and wash cell culture 4 times with 1 ml of MilliQ water.
6. Observe coloration under an inverted microscope and document with micrographs and/or a plate scan.
7. Discard water and distain for 15 min at room temperature with 250 µl of 10% (w/v) cetylpyridinium chloride (CPC) solution.
8. Collect extracts and measure absorbance at 550 nm (dilute if necessary). Determine stain concentration using AR-S standard curve (in CPC).

Apparatus: An inverted microscope equipped with phase contrast, a UV light transilluminator, a spectrophotometer.

Solutions: phosphate-buffered saline solution (PBS: 137 mM NaCl, 2.7 mM KCl, 15.8 mM Na_2HPO_4, 1.23 mM KH_2PO_4; adjust pH to 7.4); Formalin solution (4% in PBS; dilution from 36% commercial solution); AR-S solution (40 nM alizarin red S in MilliQ water; adjust pH to 4.2 with 1% NH_4OH). CPC solution (10% in 10 mM sodium phosphate pH 7.0).

Plasticware: 24-well cell culture dishes and serologic pipettes.

All chemicals were purchased from Sigma-Aldrich, unless otherwise stated.

Additional information:

Pombinho AR, Laizé V, Molha DM, Marques SMP, Cancela ML *(2004) Development of two bone-derived cell lines from the marine teleost Sparus aurata; evidence for extracellular matrix mineralization and cell-type-specific expression of matrix Gla protein and osteocalcin. Cell Tissue Research 315: 393-406*

von Kossa J *(1901) Uber die im organismus kunstlich erzeugbaren verkalkungen. Beitr Path Anat 29:163-202*

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