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Method Article

# Cultured fish epithelial cells are a source of alarm substance

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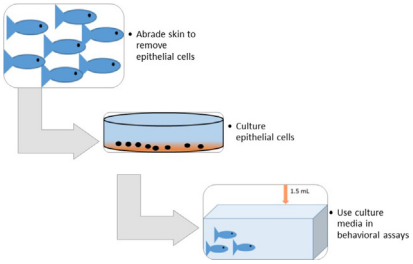
GRAPHICAL ABSTRACT




ABSTRACT

In various species of fishes, the importance of visual cues in the determination of environmental threat and subsequent predator avoidance is clear. Chemical cues also play an essential role facilitating predator avoidance. Among fish in the superorder Ostariophysi, club cells in the epidermis produce an alarm substance. Damage to the skin during a predation event releases an alarm substance (AS), which diffuses through the water column and binds to olfactory receptors of conspecifics. Fish then engage in a number of anti-predator behaviors that may include darting, schooling, or hiding.

Behavioral responses to AS and physiological mechanisms that underlie those responses is an active area of study. However, because the precise chemical composition of the alarm substance is unknown, AS is not commercially available. Thus, when fish are challenged alarm substance in various experiments and assays it is obtained from skin extracts or via perfusion of shallow cuts in the epidermis. Both procedures are effective but require the animal to be sacrificed.

In this manuscript, we report:

- A non-invasive primary cell culture protocol to obtain alarm substance and does not require the model organism to be killed.

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• The demonstration of anti-predatory behaviors in fish exposed to alarm substance collected by this method.



ARTICLE INFO
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## Method details

### *Animals and housing*

Sexually immature (40–70 mm in length) male and female creek chub (*Semotilus atromaculatus*) were used for these studies as they have been observed to express more club cells than adult. Given their abundance and accessibility, the animals were wild caught. Fish were housed in glass aquaria at 20C and maintained on a 12:12 light cycle. Food pellets were provided daily. Filters and aeration maintained water quality and fish were visually inspected daily for normal behavior and obvious signs of infection.

### *Cell collection*

All reagents should be used at room temperature. Remove fish from the home tank and place in 1.5 L of water containing 400 μL of an oil of cloves solution (1:10 clove oil in 95% ethanol (should yield a concentration of 100 mg/L) (store in a dark bottle as solution is light sensitive). The solution should induce a light plane of anesthesia in fish in approximately 5 min. When the fish is unable to right itself in the water and/or does not try to avoid handling, collection can begin. Place the fish on its ventral side on a wet paper towel and gently abrade the epidermis with a sterile scalpel blade. Focus cell collection on the anterior end of the animal and avoid removal of scales. Place epidermal cells in a 15 mL polypropylene conical vial containing 10 mL room temperature phosphate buffered saline (PBS) (no calcium or magnesium) (Life Sciences) supplemented with 1.5% penicillin/streptavidin solution (10,000 IU/mL, GIbco) and 1.5% Fungizone ® (antimycotic, Gibco), 1.5% kanamycin (Gibco), and 1.5% tetracycline (Gibco) (this solution will be referred to as supplemented PBS). Sample collection should take less than 60 s. Immediately afterwards, place the fish back into its home tank and observe for normal behaviors. Recovery from anesthesia should occur within minutes. Pool cells from six fish.

### *Primary cell culture*

All reagents should be used at room temperature (Fig. 1). Vortex the conical vial containing cells for approximately 15 s at the highest speed (speed 10 using a VWR Mini Vortexer VM3000) to assist is disrupting the mucus that was collected with the cells. Centrifuge the cells 5000 rpm for 5 min. Remove the supernatant and discard. Add 10.0 mL fresh supplemented PBS. Repeat the vortex/wash 2X. After the final wash, remove as much PBS as possible without disrupting the pellet of cells in the bottom of the tube.

Resuspend the final pellet in 6.0 mL Leibovitz's L-15 culture medium supplemented with 20% fetal bovine serum, 1.5% penicillin/streptavidin and 1.% Fungizone, 1.5% kanamycin and 1.5% tetracycline (this solution will be referred to as supplemented Leibovitz's). Leibovitz's medium was selected for cell culture because it is $CO_2$ independent; thus, cells require no $O_2$ or $CO_2$ supplementation. This media also maintains physiological pH through salts, high concentration of basic amino acids and galactose. and vortex. If scales are evident in the tube, allow the tube to stand vertically for 15 s—the scales will settle to the bottom. Avoiding the scales if present, remove the media/cell solution from the conical vial with a sterile Pasteur pipet and transfer to a sterile T25 flask with a vented cap (Corning).