Assessment of cell viability in intact glandular tissue in *Chironomus ramosus* using dye-exclusion and colorimetric assays

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Abstract

Conventionally, dye-exclusion test for determining cell viability has been restricted only for cells in suspension in tissue culture. In this paper, salivary gland of *Chironomus* has been proposed as a simple tissue model system where dye-exclusion test can be reliably employed for the intact gland. We have compared suitability of commonly used vital dyes and nigrosin was found suitable for the salivary gland cells. Biochemical tests using tetrazolium salts are also commonly used for determining quantitative indices of cell viability in metabolically active cells. Ours is the first attempt to extend the same technique for the whole tissue. We standardized the conditions and prepared a protocol for MTT-based colorimetric assay suitable for the salivary gland of *Chironomus*. A strong correlation ($r^2 = 0.9893$) was obtained where increasing O.D. correlated linearly with the number of live glands. We concluded that nigrosin dye-exclusion and MTT metabolic inclusion assays are suitable methods for the viability test of metabolically active intact salivary gland of *Chironomus* which can serve as a potential model for the assessment of cytotoxicity in future.

Introduction

Over the years, a number of techniques have been reported for the direct and indirect determination of cell viability. Among these, dye exclusion assays, release of lactate dehydrogenase from the cytoplasmic compartments, amino acid incorporation into acid precipitable proteins, cleavage of tetrazolium salts by active mitochondria, uptake of ^3H -thymidine by the living cells in culture are the widely practised methods for the assessment of viability, detection of redox activity and proliferative index of cells (Loo and Rillema 1998; Freshney 2000). Nevertheless, dye-exclusion techniques are

mostly popular due to its simplicity, rapidity and reliability.

Trypan blue, neutral red and nigrosin are common indicators of viability. These vital dyes mark cells that have lost membrane integrity. Therefore, dead cells take up the dye, whereas, viable cells exclude the dye and appear refractile and colourless. Application of fluorescent dye has also been reported for the determination of cell viability (Jones and Senft 1985; Bank 1987). These methods proved to be reproducible and sensitive when carried out, to determine cell viability either in suspension culture or for cells in monolayer culture. The major problems encountered in intact

tissue slices happened to be the proper choice of incubation conditions and the lack of viability of constituent cells during the test period. Additionally, poor penetration of vital dyes and accessibility to inner cell layers created another major drawback in determining cell viability in tissue slices. Unfortunately, in the past, simple tissue models have never been explored. Only in few cases, dye exclusion techniques were successful in selected tissue sections (Means et al. 1995; Yamane et al. 1998).

Since the introduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by Mosmann (1983) for the quantitative estimation of viable cells, many other tetrazolium salts (e.g. XTT, MTS, WST) have also been used for colorimetric assays (Riss and Moravec 1992; Tan and Berridge 2000). These assays are based on the capability of reductases and electron carriers of actively growing viable cells to transform the tetrazolium salt into coloured formazan derivatives. Amongst various tetrazolium salts reported so far, MTT continued to be the popular choice for quantitative determination of cell viability due to its sensitivity and reliability (Stevens and Olsen 1993; De Coste et al. 1999; Traore and Meyer 2001). Although most cellular reduction of MTT has initially been attributed to mitochondria, considerable evidence in the literature now indicates that cellular MTT reduction also occurs in the cytosol and by membrane bound NAD(P)H dehydrogenases (Bernas and Dubrucki 2002). Over the years, MTT-based assays have been mostly used for various cell types in culture. However, applicability of MTT-based assays in tissue slices has been reported only in limited studies (Olinga et al. 1997; Drixler et al. 2002). It is commonly experienced that conventional assays can be reliably carried out for cells in monolayer and/or suspension culture. Unfortunately, conventional dye-exclusion-based cell-viability assays are not always successful when generally applied to tissues.

In this paper, we have presented two techniques for determining the viability and metabolic active status of cells in the intact salivary gland of *Chironomus* insect using both dye exclusion and colorimetric methods. The assay described here provides both qualitative and quantitative data using a simple model of intact glandular tissue.

Materials and methods

Chironomus and salivary glands

The fourth instar larvae of Chironomid midge belonging to species *Chironomus ramosus* (Figure 1A) were reared following the methods described by Nath and Godbole (1998). The larval salivary glands are paired and are situated on either side of the oesophagus in the second and third thoracic segments. These glands are suspended in the haemolymph. A secretory duct, one from each gland, originates from the lumen of each gland (Figure 1B). The larval salivary glands are very active physiologically and produce a proteinaceous secretion, used by the aquatic larvae to spin housing tubes from the detritus (Grossbach 1977). Cells of the salivary gland of *C. ramosus* are located in a single peripheral layer surrounding the central lumen. There are about 22 cells in a single larval salivary gland of *C. ramosus* larva.

Cell viability tests with vital dyes using intact glands

Trypan blue, neutral red and nigrosin were initially tested and compared for their effectiveness as vital exclusion stains for the salivary gland cells. However, among the three stains that were used, nigrosin was found to be the most suitable for salivary gland cells. Nigrosin also provided better contrast in comparison to other stains. Initially, the salivary gland cells looked bluish and then gradually turned blue-black in colour. Unlike trypan blue or neutral red, nigrosin did not diffuse out into the lumen of the gland and had least background. Previously, Kaltenbach et al. (1958) had also shown the advantages of nigrosin over other dyes. Moreover, nigrosin was found to be non-toxic.

For the viability test, salivary glands were excised in insect ringer solution (0.67% NaCl solution in distilled water). One of the salivary glands, was given *in vitro* lethal treatment by subjecting it to heat shock at 41 °C for 60 min (Nath and Lakhota 1989). In another experimental set, glands were exposed to 41 °C only for 30 min (sublethal incubation period) to study mixed population of both live and dead cells. For control, the other sister salivary gland was incubated at room temperature (25 °C) in ringer solution in a

microcavity slide. Both control and heat-killed salivary glands were stained in 1% nigrosin solution for 2 min. Excess stain was removed using a blotting paper and the salivary glands were washed with ringer solution three times. The glandular cells were observed under bright-field objectives (Olympus, BX40 Microscope, Japan) and photographed. In order to achieve a better contrast during photomicrography, cells were stained in nigrosin for an extended period of 5 min.

MTT-based colorimetric assay for cell viability using intact glands

The original methodology for MTT assay employed by Tim Mosmann (1983) and later on by others were modified for the present study using

whole glandular tissue. Larval salivary glands in varying numbers (10, 20, 30, 40 and 50) were dissected out in the insect ringer solution. The glands were collected in different Eppendorf tubes, containing 50 μ l of 1.2 mM MTT (Amersham cat. # 19265) in the insect ringer solution. MTT was removed after 1 h of incubation and the glands were further washed with the ringer solution. The blue formazan generated after the MTT cleavage by the viable cells was extracted with 25 μ l of 50% (v/v) Tween 80 (Sigma cat # P5188) for 24 h. Finally, the formazan product was diluted with 1 ml of ringer solution and the absorbance was measured at 505 nm in a spectrophotometer (Kontron instruments, Uvicon 922). A graph of extinction against varying number of fully viable glands was plotted as standard curve for the next experiment.

In the second experiment, sensitivity of the MTT-based colorimetric assay was tested for the detection of metabolically active viable and dead salivary glands. For this, 10 salivary glands were used for both treated (with heat treatment) and control (without heat treatment) experiments. As for the lethal treatment, 10 glands were incubated in the ringer solution and subjected to heat shock at 41 °C as described for the experiments with vital dyes. Subsequently, the modified protocol for MTT assay was carried out and optical densities of control (live) and treated (dead) glands were measured at 505 nm. All data were expressed as mean absorbance of six replicate samples $\pm$ standard deviation of the mean.

Results and discussion

In vitro dye exclusion techniques are commonly used for differentiating live and dead cells in suspension. The ratio of live to total cells are then used to quantitate the percent cell viability. Alternatively, various tetrazolium salts are used for colorimetric assay to quantitate the viability of metabolically active cells. However, as mentioned earlier, both dye exclusion and tetrazolium salt-based colorimetric assays have so far been employed for cells either in suspension or in monolayer culture. No one has attempted to use ‘whole’ tissue for such viability test. In this paper, we have presented the salivary gland of *Chironomus* as a tissue model system for ‘whole-gland’ cell viability assay.

Figure 1. Schematic sketches showing (A) dorsal view of a fourth instar *Chironomus ramosus* larva measuring 12.5 ($\pm$ 1.3) mm in length and (B) an enlarged diagram of the dissected out salivary glands after pulling off the larval head with a sharp needle [a: head; b: salivary duct; c: salivary gland; d: lumen of the gland].

For many years now, the salivary gland of *Chironomus* has been reported as an excellent model system for cell and developmental biology, cytogenetic and other studies (Sorsa 1988; Armitage et al. 1994). It is one of the simplest types of acinar gland. The *Chironomus* salivary gland is

also considered unique for its limited number of large cells, arranged in single layer, surrounding a central lumen (Grossbach 1977).

We have compared the efficiency of commonly used vital dyes for salivary gland cells. Nigrosin dye exclusion was easier for microscopic

Figure 2. Photomicrograph showing (a) control salivary gland with all viable cells; (b) nigrosin-stained heat-killed salivary glands and (c) mixed population of unstained viable (indicated by arrows) and non-viable nigrosin stained cells (Bar indicates 100 μm).

Figure 3. Linearity of the MTT assay ($r^2 = 0.989$). Relationship between number of glands and absorbance (O.D.) at 505 nm of MTT-formazan generated by living salivary gland cells.

examination as compared to the other two vital dyes, viz. trypan blue and neutral red. For cell lethality, we used heat treatment, as it was also employed in earlier studies to test the efficacy of cell viability assays (Krause et al. 1984; Doukes and Flotte 1996). When dye exclusion assays were carried out with salivary glands, all three vital dyes could distinguish live and dead cells (data not presented). However, nigrosin provided better contrast with least artefacts. Nigrosin was found more stable compared to trypan blue and neutral red for the viability assay of salivary gland cells. The apparent nontoxic nature of nigrosin permits the user a reasonable amount of time to count the cells in multiple control samples. Figure 2a shows nigrosin dye excluded control salivary gland with all the viable cells and Figure 2b shows nigrosin-stained heat-killed salivary gland with all dead cells. Under sublethal conditions the cells were found to be randomly affected and it was possible to distinguish both live and dead cells using nigrosin (Figure 2c). There are only 22 cells arranged in a single layer, which are countable under a light microscope. Therefore, one can easily quantify and estimate % viable cells.

One of the limitations of nigrosin dye exclusion assay is its inability to differentiate between metabolically active and inactive cells. In order to circumvent this problem we have developed a colorimetric assay for determining viability of

metabolically active cells in the salivary glands. We used MTT, a tetrazolium salt that is reduced by mitochondrial and non-mitochondrial reductases and electron carriers. Therefore, the reactions occur in living cells only. The original method employed by Tim Mosmann (1983) and subsequently by others (Tada et al. 1986; Olinga et al. 1997) did not yield satisfactory results for intact glandular tissue like the salivary gland of *Chironomus*. We have standardized a protocol suitable for the *Chironomus* salivary gland mainly by varying incubation medium and incubation time. In the original protocol of MTT assay described by Mosmann (1983), HCl-isopropanol was used to dissolve the crystals of formazan for 4 h in the mouse lymphoma cells. In another murine NKC3 cell line Tada et al. (1986) used sodium-dodecyl-sulphate for the overnight extraction of

Figure 4. MTT assay of heat-killed and viable glands.

MTT cleavage product. Olinga et al. (1997) choose DMSO for the liver tissue slices in order to dissolve formazan products. In the present study, we used Tween-80 for an effective extraction of formazan products following the incubation of salivary glands with MTT. The extraction period was longer (24 h) compared to Mosman's original protocol. The MTT assay standardized for the *Chironomus* salivary gland tissue was found to be reproducible and sensitive. A calibration curve was plotted and A_{505} was found to be directly proportional to the number of viable glands over a range from 10 to 50 glands (Figure 3). The linear regression value ($r^2 = 0.989$) indicated very strong correlation coefficient and thereby, reliability of the assay system. MTT assay also proved to be sensitive for the detection of heat-killed glands and live glands (Figure 4).

Data obtained by MTT assay was comparable to nigrosin exclusion results. These combined techniques not only provided both population and individual cell data but also confirmed the applicability of these techniques to non-dividing cell population in an intact glandular tissue. Moreover, till today, dye-exclusion test to determine cell viability has been restricted to suspension cells in tissue culture. Our's is a novel technique for 'whole-tissue viability test' using a simple model system like the *Chironomus* salivary gland. The method described in this paper can potentially be applied in bioassays for the detection of different types of toxin using the salivary gland of *Chironomus*. Nigrosin dye exclusion and MTT assays have been used to study laser-tissue interaction and to quantitate Nd:YAG laser-induced cell death (BBN: unpublished data). Other cytotoxic agents are currently being evaluated. The assays described here is not only easier to perform, it is relatively inexpensive too.

Assessment of tissue viability is a challenging field and recent advancement in drug discoveries demand rapid assessment of tissue viability *in situ* using a simple model system. Previously, the salivary glands of *Chironomus* have been used in many genotoxicological, developmental and cell biological studies. Puffing activity of the salivary gland polytene nuclei served as excellent endpoints in many cytogenetic studies dealing with the effect of metabolites, heavy metals, pharmacological agents and other stressors (Sorsa 1988). Our study with the *Chironomus*

salivary gland opens up additional scope for the *in vitro* assessment of cytotoxic effect using whole-gland dye exclusion and MTT assays.

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