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## An improved acridine orange staining of DNA/RNA<sup>☆</sup>

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### ABSTRACT

New tools are desirable to examine the metabolic state of individual cells within tissues. We proposed a fluorescence-based procedure consisting of acridine orange staining and fast green counterstaining (AO-FG) to improve the selectivity of the former for nucleic acids (acridine orange stains both DNA and RNA with different fluorescence colors), with no interference from proteins. We compared this test with the biochemical quantification of the relative amounts of RNA and DNA in selected rat ventral prostate samples and PC3 cells. The epithelium of the prostate gland is highly active metabolically for the production of secretions. Differences in AO-RNA staining were revealed and correlated with the metabolic state of the epithelium. Specificity was confirmed by RNase A. To assess how AO-FG staining correlates with the metabolic state of the cell, we cultured PC3 cells in different concentrations of glucose and measured the ratios between the amounts of RNA and DNA. In parallel, similar cultures were subjected to AO-FG, and the staining pattern correlated closely ( $r^2 = 0.886$ ) with the obtained biochemical results. The results confirmed that the combined use of AO and FG is useful for detecting DNA and RNA simultaneously, as well as for assessing quantitatively the transcriptional activity of individual cells and their changes in response to experimental manipulation.

### 1. Introduction

Nucleic acids are polyanionic and their negatively charged nucleotides allow the binding of cationic dyes, such as acridine orange (3,6-dimethylaminoacridine). Acridine orange (AO) was first synthesized in 1889, but its ability to bind nucleic acids was only reported in 1940, with greater improvement by 1950's (von Bertalanffy and Bickis, 1956). Since then, a number of biological applications have been assigned to this fluorophore (Robbins and Marcus, 1963).

AO interacts with double-stranded DNA by intercalation, while the single-stranded RNA interacts by ionic interactions and dye stacking, resulting in different fluorescence with maximum emission at  $\lambda = 530$  nm and 640 nm for DNA and RNA, respectively (McMaster and Carmichael, 1977). The basophilic AO binds to other anions, such as carboxyl groups, from biological molecules other than nucleic acids. In fact, although it has long been used for staining DNA and RNA, the use of AO in tissue sections is hampered by the non-specific staining of other anionic groups, in particular those in the extracellular matrix.

The objective of this study was to describe a protocol for the simultaneous DNA and RNA staining by acridine orange plus fast green (AO-FG), improving the selectivity for nucleic acids and allowing

observation of the metabolic status of individual cells, either within tissues or in cell cultures. Given our interest in studying the prostate gland, we employed rat ventral prostate tissues under different physiological situations and the PC3 human prostate cancer cell line to demonstrate the ability of the procedure to reveal modifications in the metabolic state of the epithelial cells.

### 2. Materials and methods

#### 2.1. Animals, treatments, and tissue processing

Fifteen male Wistar rats were used throughout this study. The ventral prostates of castrated animals ( $n = 6$ ) were obtained as before (Bruni-Cardoso et al., 2010). Three sham-castrated animals were used as controls. Those from alloxan-induced diabetic animals ( $n = 3$ ) as well as those obtained after the administration of insulin ( $n = 3$ ) were acquired as before (Damas-Souza et al., 2010). The organs were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 24 h. Fixed prostates were then processed for routine paraffin embedding. The animal handling and experimental protocols were approved by the University Ethics Committee for Use of Animals (Protocol nos. 1887-1).

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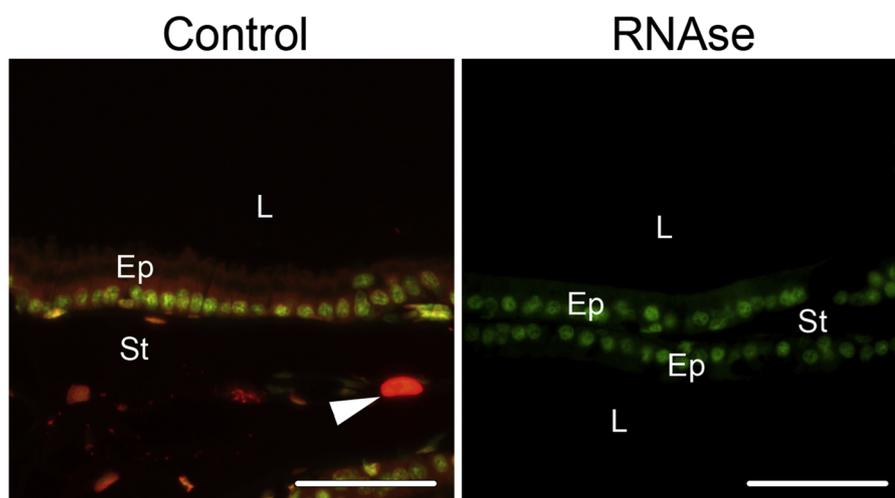
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**Fig. 1.** Simultaneous identification of DNA (green) and RNA (red) in the ventral prostate (VP) using a modified protocol for acridine orange staining. Most of the RNA found in the VP of control rats occurs in the epithelium, with a clear concentration in the supranuclear region. Red fluorescence was abolished by RNase A treatment. Ep = epithelium, St = stroma, L = lumen. Bar = 50  $\mu$ m (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

## 2.2. Cell culture

PC3 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and grown on glass coverslips in 24-well culture plates (Sarstedt, Newton NC, USA). Cells arrived at passage 30 and were maintained in the laboratory for three years and tested periodically for mycoplasma by PCR. Each well received 1 mL of RPMI 1640 (Sigma Chemical Co., St. Louis MO, USA) with different concentrations of glucose, which were composed by mixing 11.1 mM of glucose-containing medium and glucose-free medium in different proportions (75, 50, 25, and 0% of the former), 10% fetal calf serum (Nutricell, Campinas SP, Brazil), 10 mL of insulin/transferrin/selenium (ITS; Gibco, Auckland, New Zealand), and 1% penicillin/streptomycin (Nutricell, Campinas SP, Brazil). The cells were incubated at 37 °C, under 5% CO<sub>2</sub> and 95% relative humidity. The medium was changed every 24 h. After six days, the cells were washed in warm PBS, fixed in methanol/acetone (1:1) for 15 min, air dried at room temperature for 30 min, and then stained with acridine orange.

## 2.3. DNA and RNA extraction

Total DNA and RNA were extracted with Trizol from PC3 cells grown under different glucose concentrations, using the protocol recommended by Amersham-Pharmacia Life Sciences. The nucleic acids were then quantitated by spectrophotometry, using a Nanovue spectrophotometer (GE Healthcare, Chicago IL, USA).

## 2.4. Acridine orange staining for the simultaneous identification of DNA and RNA

Paraffin sections of the rat ventral prostate (VP) and human PC3 cells were stained as follows. The cells were

- 1 Washed once in 150 mM phosphate buffer pH 6.0 (PB),
- 2 Stained with 0.05% acridine orange (C.I. 46,005; Sigma) in PB, for 3 min
- 3 Washed in 1.65% calcium chloride in distilled water for 3 min
- 4 Washed in running tap water for 10 min

Stained with 0.1% fast green FCF (C.I. 42,053; Vetec Fine Chemicals, Rio de Janeiro RJ, Brazil) in 3% acetic acid (pH 2.7) for 30 s, (<http://cshprotocols.cshlp.org/content/2008/7/pdb.rec11382.full>)

- 5 Washed in 1% acetic acid for 1 min
- 6 Air dried and
- 7 Mounted in mineral oil (Nujol).

The stained material was then observed under the fluorescence microscope. Controls were carried out by treatment with 20 mg /mL RNase A (DNase free, cat. 12091-021; Invitrogen; Carlsbad CA, USA) in saline sodium citrate (SSC) diluted twice (1.5 M NaCl and 150 mM sodium citrate, pH 7.0) for 30 min at room temperature, and rinsed in the same solution before staining as above. Quantitative analyses were performed using the open access software Image J of the images obtained with 410 excitation/ 522 emission (green fluorescence) and 560 excitation 600 emission (red fluorescence) in a Zeiss Axioskop fluorescence microscope.

## 3. Results

### 3.1. Validation of DNA and RNA staining

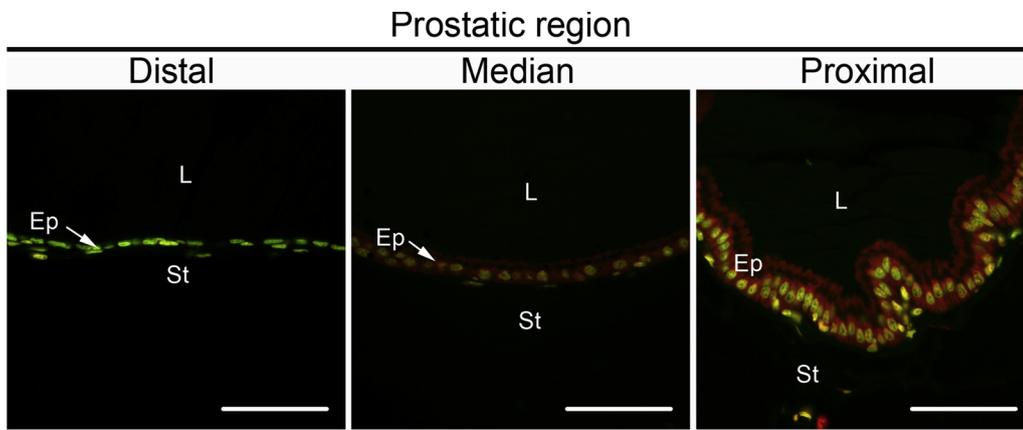
The results show simultaneous identification of DNA (green fluorescence) and RNA (red fluorescence) in the VP using a modified protocol for acridine orange staining. Most of the RNA in the VP of control rats is found in the epithelium, with a clear concentration in the supranuclear region (Fig. 1A). RNase treatment abolished the red fluorescence (Fig. 1B).

### 3.2. Variations in RNA contents along the VP ductal system

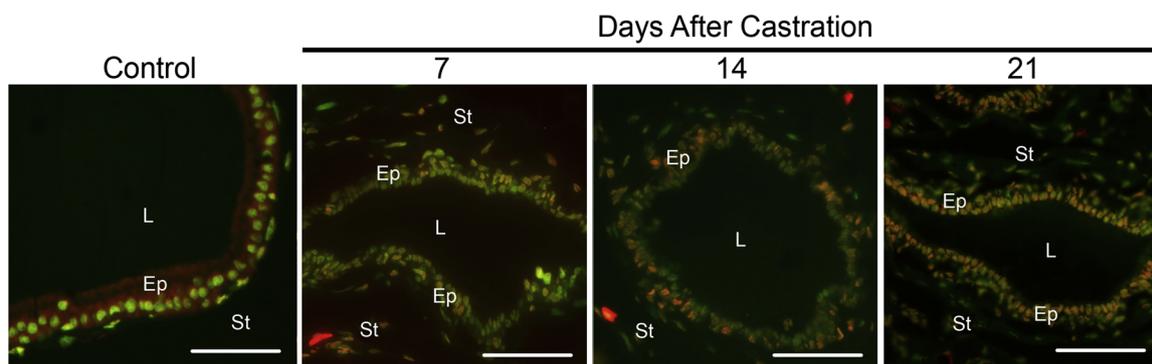
Three main regions of the VP ductal system can be distinguished (Lee et al., 1990; Nemeth and Lee, 1996). The proximal region, consisting cuboid cells, showed little fluorescence (Fig. 2A), reflecting a specialized structural function of the ducts. The distal and intermediate (median) regions showed intense red fluorescence, compatible with their secretory activity (Figs. 2B and C).

Castration is known to reduce the secretory activity of the epithelium. Accordingly, there was a marked reduction in the amount of red fluorescence exhibited by epithelial cells at different periods after castration. Most of the RNA was located in the epithelial cell nuclei, with an observed increase at 21 days after castration. Little effect was observed on the stromal cells (Fig. 3).

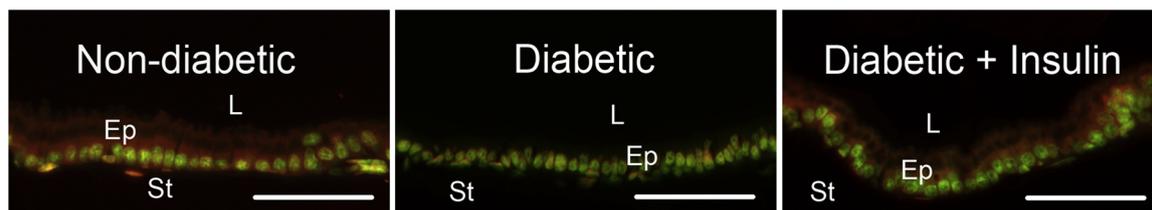
There was a marked reduction in the amount of RNA produced by epithelial cells in diabetic animals, as shown by the reduction in red fluorescence in both the cytoplasm and the cell nucleus (Figs. 4A and B). Insulin administration to diabetic rats resulted in the recovery of the red fluorescence, indicating that the transcriptional activity was recovered, at least partially (Fig. 4C). The staining results were quite uniform among the animals in each experimental group.



**Fig. 2.** Simultaneous identification of DNA (green fluorescence) and RNA (red fluorescence) in the three different VP ductal regions using a modified protocol for acridine orange staining. **A** Proximal region with the red fluorescence in the perinuclear region. **B** Intermediate (median) region with the red fluorescence clearly localized in the supranuclear region. **C** Distal region with similar distribution of the red fluorescence in the supranuclear region. The arrowhead indicates a mast cell. Ep = epithelium, St = stroma, L = lumen. Bar = 50  $\mu$ m (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).



**Fig. 3.** Simultaneous identification of DNA (green fluorescence) and RNA (red fluorescence) in the VP using a modified protocol for acridine orange staining. Most of the RNA found in the VP of control rats occurs in the epithelium, with a clear concentration in the supranuclear region. There is a marked reduction of the red fluorescence exhibited by epithelial cells in castrated rats. There was an apparent increase in the RNA content in the nuclei of epithelial cells 21 days after castration. Little effect was observed in the stromal cells. Ep = epithelium, St = stroma, L = lumen. Bar = 50  $\mu$ m (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).



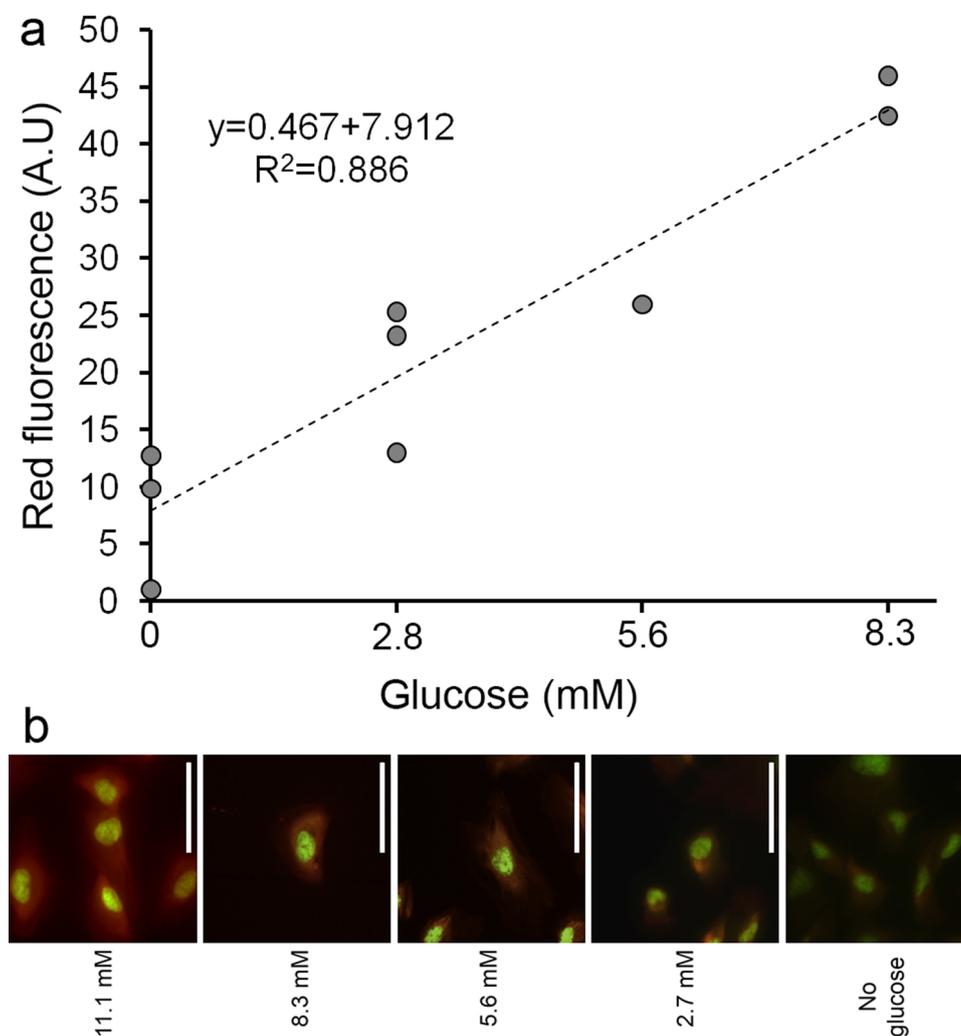
**Fig. 4.** Simultaneous identification of DNA (green fluorescence) and RNA (red fluorescence) in the VP of a diabetic rat using a modified protocol for acridine orange staining. **A** Most of the RNA found in the VP of control rats is located in the epithelium, with a clear concentration in the supranuclear region. **B** There was a marked reduction in the amount of RNA produced by epithelial cells in diabetes, as shown by the reduction in red fluorescence in both the cytoplasm and the cell nucleus. **C** Insulin administration to diabetic rats resulted in the recovery of the red fluorescence, indicating an increased amount of RNA. No evident effect was observed on stromal cells. Ep = epithelium, St = stroma, L = lumen. Bar = 50  $\mu$ m (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

### 3.3. *In vitro* modulation of the transcriptional activity and RNA content in PC3 cells by glucose

PC3 cells were used to check the effect of glucose concentration on transcriptional activity and RNA content, and the ability of the present AO-FG staining to reveal these changes. PC3 cells cultured in the presence of increasing concentrations of glucose exhibited a linear increase in the production of RNA, as measured by the ratio of RNA to DNA (Fig. 5A). Examination of PC3 cells grown under the same conditions revealed a clear correlation between red fluorescence staining and the observed increase in the RNA/DNA ratio (Fig. 5B).

## 4. Discussion

In this study, we used the ventral prostate gland as a model system to demonstrate the applicability of a combination of AO and FG staining for the simultaneous staining of DNA and RNA, and variations of RNA content in different physiological conditions. The direct staining of VP sections with AO resulted in diffuse staining of the tissue, with marked labeling of secretory products and the extracellular matrix. These are interfering factors in applying the classical procedure to tissue sections, as compared to isolated cells or cell nuclei (Darzynkiewicz, 1990). The proposed FG counterstaining was based on its specificity for total



**Fig. 5.** A RNA/DNA ratio in PC3 cells. The amount of RNA, as measured by the red fluorescence intensity (integrated density after transforming the fluorescence into an 8 bit image), increases as a function of glucose concentration in the culture medium. B Simultaneous identification of DNA (green fluorescence) and RNA (red fluorescence) in PC3 cells using a modified protocol for acridine orange staining. The red fluorescence is proportional to the concentration of glucose on the individual cell level. Bar = 50  $\mu$ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

protein imino by guanidine groups, and its use resulted in quenching of the AO fluorescence, both in the secretory products in the glandular lumen and in the extracellular matrix. Furthermore, we observed that the different ductal regions exhibited different levels of red fluorescence, which is compatible with the increased secretory activity in the intermediate and distal ductal regions, compared to the proximal region proposed by others (Lee et al., 1990; Nemeth and Lee, 1996).

Still exploring the VP, we also demonstrated that castration promotes a reduction of red fluorescence (RNA), which is compatible with the reduced transcriptional and secretory activity. Knowing that the prostate function is also directly modulated by somatotrophic hormones such as insulin (Damas-Souza et al., 2010; Webber, 1981), we examined the RNA content as indicated by the red fluorescence in the VP of diabetic animals. We found a decreased RNA content in the epithelial cells, which was reversed by insulin administration, thus confirming the role of insulin in contributing to the maintenance of the functional state of the gland.

We were also able to manipulate the metabolic status of the PC3 prostate cancer cells by exposure to different concentrations of glucose. As expected, there was a linear ( $R^2 = 0.886$ ) correlation between the glucose concentration in the culture medium and the amount of RNA extracted from the PC3 cells. Similarly, cultured cells showed increased red fluorescence after AO-FG staining, demonstrating a correlation

between the metabolic state determined by biochemical measurement and the intensity of the AO red fluorescence.

These results support the proposition that the combined use of AO and FG is useful for detecting DNA and RNA simultaneously, as well as for assessing the transcriptional activity of individual cells and its variation in response to experimental factors. As mentioned above, the function of FG is to block the staining of proteins, particularly in the extracellular matrix, which compromises the quality of the results and interferes with the quantitative assessment. It is also important to recognize mast cells in tissue sections, as they appeared strongly fluorescent after staining using the present protocol.

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