A Novel Technique of Computer-Assisted Image Analysis to Quantify Molecular Stress in Cetaceans

Images, Data and Discussion Generously Contributed by Anne C. Allen and Sarka O. Southern, NMFS, Southwest Fisheries Science Center, La Jolla, CA

* This application note does not constitute federal endorsement of the commercial product Image-Pro™ Plus

Introduction

To gain a better understanding of how the cetacean molecular stress response reacts to such stressors as disease, marine pollution, and fishery-related stress, a preliminary study was undertaken. A group led by Dr. Sarka O. Southern at the Southwest Fisheries Science Center in La Jolla, CA, developed and evaluated a method to quantify levels of stress in several species of marine mammals. This method is based upon identifying the presence and abundance of proteins commonly generated with stress, through immunohistochemical staining of thin skin sections. Dr. Southern and her group determined the presence and relative amounts of these proteins, and then used Image-Pro Plus to verify and correlate the results with traditional methods.

A side benefit exists to this type of research. Similar stress proteins are found in a number of mammalian species including humans. Quantifying the expression of these proteins then has a direct medical benefit to us, and allows us to further investigate the effects of stress on the human body.

Abstract

As part of an effort to develop a test for chronic stress in cetaceans, we needed to quantify the expression levels of stress-response proteins (SRP) in immunohistochemically stained skin. We have developed a custom method of computer-assisted image analysis (CIA) using the commercially available software Image-Pro Plus. The immunoreactive signal, corresponding to the SRP expression level, was calculated based on the product of the average staining intensity and the percentage of positively stained epidermal cells. This new method allows for exact measurement of signal directly in the area-of-interest. We demonstrate the application of CIA for quantification of immunohistochemical staining through expression analysis of metallothionein in 22 specimens of beluga, bottlenose dolphins, and spotted dolphins. Image analysis results were compared with the more standard, qualitative method of human-scored visual assessment using light microscopy. Both visual and CIA methods can accurately separate known healthy and diseased cetaceans. However, CIA is able to distinguish staining differences on a much finer scale than a subjective observer can do visually. We conclude that our method of CIA is both accurate and repeatable and provides an efficient means for rapidly quantifying the expression levels of stress-response proteins.

Methods

Metallothionein was visualized in thin skin sections from 22 specimens using immunohistochemical (IHC) staining.

Using identical microscope and camera settings, three digital images per sample were taken to accurately reflect the overall staining. In a separate session, three additional images per sample were taken to assess reproducibility.

All images were analyzed using the commercially available software program Image-Pro Plus version 4.1 for Windows 95/98/NT (Media Cybernetics, Silver Spring, MD).

Hardware system used: Gateway E-4200 computer and VX900 monitor, DVC high resolution digital camera 1310C, Olympus System light microscope model BX50.

Skin samples were taken from three different species of marine mammals- beluga, bottlenose dolphins, and spotted dolphins..Taking the samples involved removing a small plug of skin and in no way harmed the animals.

MT was detected in a 5-micron section of beluga skin using immunohistochemical staining with rabbit antiserum to horse MT. As a result of the IHC staining, MT was visualized as a red pigment. MT expression was localized to the immature keratinocytes in the epidermal ridges (panel A). Using the free-form drawing tool in Image-Pro Plus, we selected the epidermis as the area-of-interest. All calculations were performed only within this selected area (panel B). A color file was created that exactly selected the hue, saturation, and intensity that indicated the protein expression. This color file defined the range of the signal and was applied to all samples. When superimposed on the image, the color file was visualized as an artificial blue color that showed the localization of the MT signal (panel C). (Original magnifications x400).

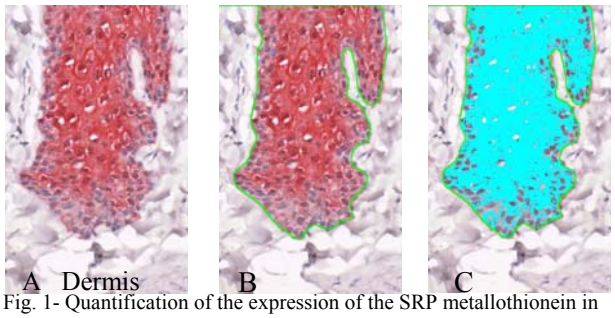


Fig. 1- Quantification of the expression of the SRP metallothionein in beluga skin using computer-assisted image analysis. (A)MT (stained red) in the epidermis. (B) Enclosing MT within a Free-form AOI. (C) Thresholding the range of signal.

Image Analysis

MT was visualized in 22 different skin specimens, 3 of which are shown in Figure 2 (panels **A** and **B**, **C** and **D**, **E** and **F**). MT levels were evaluated using two methods: subjective visual analysis (panels **A**, **C**, and **E**) and computerized image analysis (panels **B**, **D**, and **F**). Visual analysis involved three repeat evaluations of the specimens using light microscopy at 40x, 100x, and 400x magnifications. CIA was performed on three images per specimen at 400x magnification, and the average ELS score was obtained. Only one image per specimen is shown here; however, the expression level score shown is the average ELS (panel **B**, **D**, and **F**). The ELS found using the computer-assisted method was concordant with the visual assessment of staining (Kendall Coefficient of Concordance, W= 0.975, $0.001<p<0.01$). (Original magnifications x400).

Quantification of the Stress Response Protein Expression Level Score (ELS)-

ELS = Mean Optical Density of Red x Percent Area Positively Stained x 100

- For Figure 1, ELS = 0.230 x 0.725 x 100 = 16.675
- Observed Range of ELS: 0-35

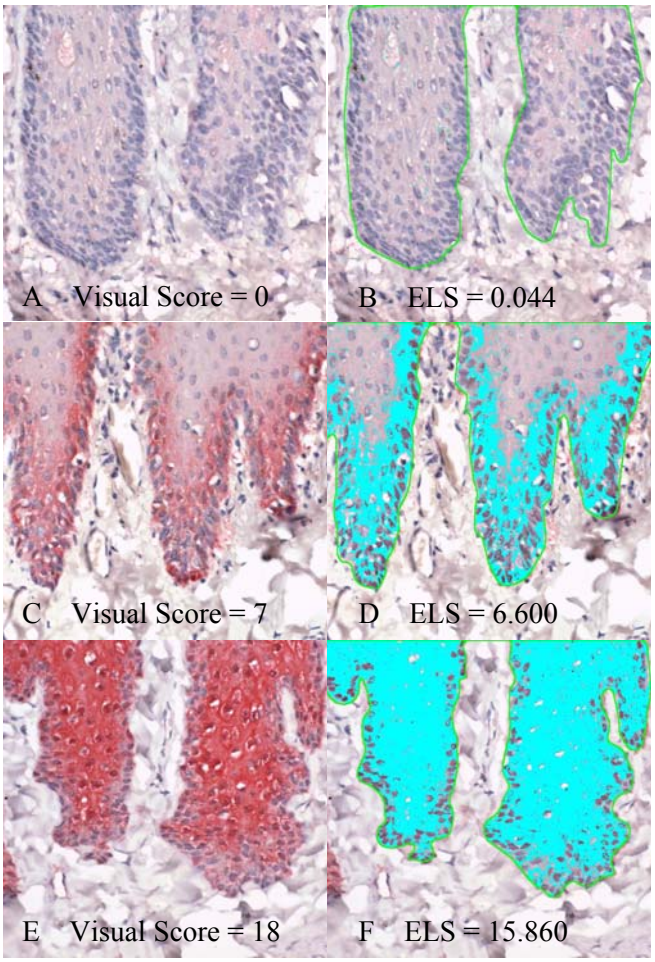


Figure 2. Application of CIA method to cetacean skin sections, comparing with visual analysis. Areas of interest were enclosed using the AOI tool and then thresholded using the Measure|Count/Size…|Manual Intensity Range selection tool.

Reproducibility

For all 22 specimens, 3 additional images were taken in a separate session. CIA was performed in the same manner on these images, and again the average ELS value was calculated. The two CIA sessions were shown to give nearly identical ELS values for every specimen (Kendall Coefficient of Concordance, $W=0.993$, $0.001<p<0.01$).

All 22 specimens were analyzed via subjective visual analysis and computer image analysis. Visual analysis involved three repeat evaluations of the specimens using light microscopy at 40x, 100x, and 400x magnifications. CIA was performed on three images per specimen at 400x magnification, and the average ELS value of all three images was obtained. The eleven field specimens had a range of ELS values

from 0-18. Four of the field specimens fell within the range of the known normals, four were within the range of the clinically diseased samples, and three had intermediate ELS values.

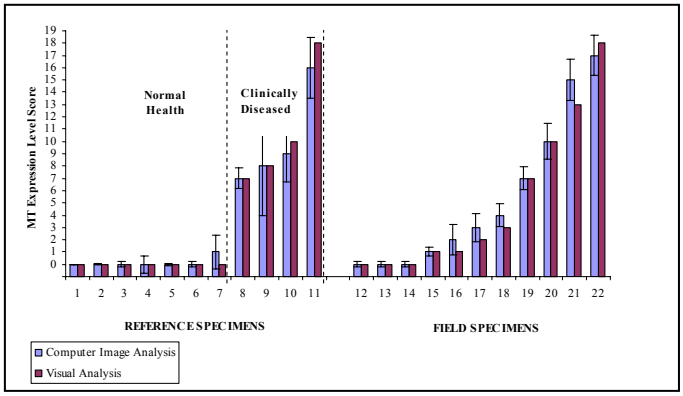


Figure 3. Graph of MT expression level scores for both CIA and visual analyses of 11 reference specimens and 11 field specimens.

The expression of MT was visualized as a red pigment in the immature keratinocytes in the epidermis (panel **A**). In panel **B**, the ELS was calculated from the signal in both the epidermis and the dermis. In panel **C**, the ELS was determined only from the MT signal within the outlined epidermal area of interest. Outlining also allows artifacts of staining, which would alter the true expression level score, to be removed from the analysis. Such artifacts include folds in the skin, areas in which the skin has lifted up (**a** and **b**, panel **C**), tears, and edge effects. (Original magnifications: x400).

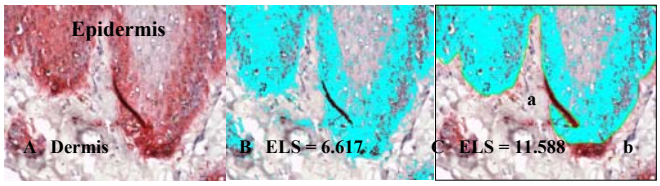
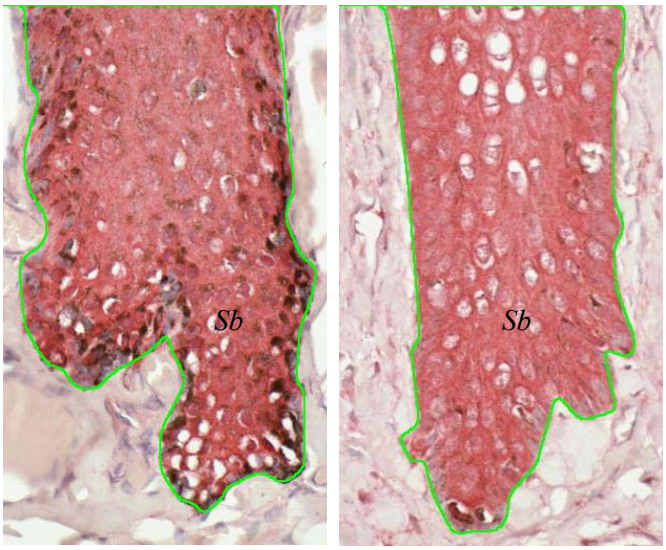
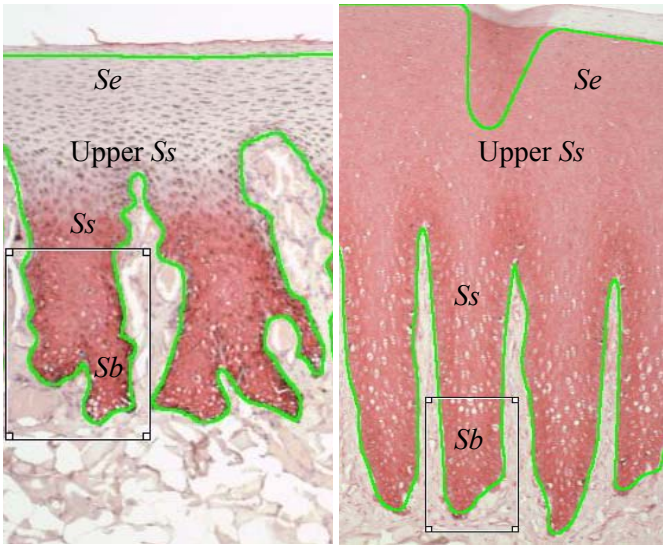


Figure 4. Quantification of metallothionein expression in *Stenella attenuata* skin using Image-Pro Plus, comparing non-outlined and outlined images.

Many stress-response proteins can be expressed in all layers of the epidermis, from the *Stratum basale (Sb)*, through the *Stratum spinosum (Ss),* to the *Stratum externum (Se)* (panel **C**). When two samples at 400x magnification were compared, similar ELS values were obtained for expression in the *Stratum basale* (panels **A** and **B**). The same samples analyzed at 100x show very different patterns of cellular accumulation of antigen, reflected by their much more representative ELS values (panels **C** and **D**). When staining is homogeneous and the cellular area of staining is the same on the sections being compared, a higher magnification can be used. When there is variability in the staining pattern, however, a lower magnification can discriminate staining differences better. (Samples stained with a combination of 40 SRP's).



A. ELS = 21.043 B. ELS = 17.260



C. ELS = 7.420 D. ELS = 15.540

Figure 5. Comparison of CIA for two *Stenella attenuata* samples at different magnifications.

Results and Discussion

Computer-assisted image analysis can be used to quantify immunohistochemical signal in cetacean skin.

CIA results corresponded significantly with the subjective visual assessment ($0.001<p<0.01$), (**Figure 2**).

Both the subjective visual assessment and the CIA methods accurately separated known healthy and diseased animals (**Figure 3**).

ELS values obtained from the CIA method in two separate sessions were shown to be significantly concordant ($0.001<p<0.01$).

This CIA method allows calculations to be performed within a selected area-of-interest, avoiding artifacts of staining which can confound results (**Figure 4**).

CIA can be used at different magnifications, which is important for non-homogeneous stainings (**Figure 5**).

References

An Improved Technique of Computer-Assisted Image Analysis to Quantify Molecular Stress in Cetaceans. Allen, A.C., Kellar, N.M., Southern, S.O., Dizon, A.E. 14th Biennial Conference on the Biology of Marine Mammals, Vancouver, Canada. Nov. 28 – Dec. 3, 2001.

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