Chapter 4

Quantification of a Substance Using Color Scores

4.1 Introduction

The goal of development of the staining intensity quantification algorithm was to be able to measure the stained biological substance present in a specimen. The substance under study is usually stained, which takes up various shades of a color and the digital image of the specimen is acquired. The reaction to the stain would be more if the substance is present in a densely packed manner and if it is present in a sparsely distributed manner the reaction would be weaker, taking up paler shades of the color. The color scores are designed to be higher for pixels expressing strong staining representing high concentrations compared to the cases where the stain expression is weak representing low concentrations of the substance. The color score for each pixel represents the concentration of the substance at that point. The sum of color scores of pixels of the image would then represent the overall amount of the substance present in the specimen. A small change in the amount of substance can also be measured, which is not possible to be evaluated manually. Also, manual evaluation of the amount of substance present based on staining intensities is very much prone to inter-observer and intra-observer variations.
However, if absolute quantification is required, a calibration step is required. The color scores for a few images with known amount of substance need to be obtained. This can be used to calibrate the rest of the measurements. Also, color scores would not have a linear variation with the amount of substance present. This will require a curve fitting step to interpolate the color scores. Another issue to be considered is that the calibration curve for each color is different and hence needs to be established at the beginning of the study.

The use of color scores for evaluating the effect of an antiosteoporotic drug is described in the following section. The color images of bone sections are obtained and the bone cells are quantified and compared. The pattern of growth of bone cells indicates the effectiveness of the drug.

4.2 Use of Color Scores to Evaluate Growth of Bone Cells

Osteoporosis is considered an important health problem which is characterized by low bone mass and micro-architectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture. According to a study carried out by Osteoporosis Society of India, the number of patients suffering from osteoporosis in India was approximately 26 million in 2003 and is projected to increase to 36 million by 2013. In a study among Indian women aged 30-60 years from low income groups, the bone mineral density at all the skeletal sites was much lower than values reported from developed countries. Osteoporosis can be treated to reduce the risk of fracture. Treatment can be effective if osteoporosis is diagnosed early. At present, the most effective approach to prevent osteoporosis is the hormone replacement therapy. Estrogen, calcitonin, biphosphonates, ipriflavone, calcium products and anabolic steroids are being used clinically as effective medications (McLean and Nakane, 1974). However, these medications are associated with numerous side-effects (Mori et al., 1988). Hence there
are many attempts still being vigorously pursued to identify new inhibitors of bone resorption that minimizes the necessity for drug therapy. Therefore there is a need to search the natural compounds for the treatment of postmenopausal symptoms with no toxic effects. This study evaluates the effect of petroleum-ether extract of Cissus Quadrangularis Linn. (CQ) which is a plant used in folk medicine, on osteoporotic Wistar rats.

Use of Northern Eclipse image analysis software (Empix Imaging, Inc., Mississauga, Canada) for determining the area and density of the mineralized matrix and bone matrix has been reported in [Harada and Rodan 2003]. The green and red color channels were used to set the thresholds. The thresholds were determined interactively and empirically. Study reported in [Nelson et al. 2009] also makes use of this software. Another group working on stained bone sections reported use of Lucia software (Nikon Instruments, Florence, Italy) where the red color thresholding was done to obtain the percentage area with the red precipitation of the osteoblasts [Davison and Davis 2003]. General purpose image processing software namely Scion software and Image Pro Plus can also be used. However, all these techniques are based on threshold and area measurement, which does not take into account the presence of the substance under study at varying concentrations across the specimen.

4.2.1 Method

Healthy female Wistar rats were divided into four groups of six animals each. Group 1 was sham operated (SH). All the remaining groups were ovariectomized. Group 2 was fed with an equivolume of saline and served as ovariectomized control (OV). Groups 3 and 4 were orally treated with raloxifene (RA) and petroleum-ether extract of Cissus Quadrangularis (CQ) respectively, for 3 months. At the end of the 3 months of treatment, rats in all groups were weighed and sacrificed by cervical dislocation and femur bones were dissected. These procedures were carried out in accordance with the ethical committee guidelines of the institution.
The antiosteoporotic activity was assessed with histochemical localization of Alkaline Phosphatase (ALP) and Tartarate Resistant Acid Phosphatase (TRAP). Digital images of stained bone sections were acquired and the images were analyzed to study the amount of bone cells namely osteoclasts and osteoblasts. A reference color each for ALP and TRAP staining was decided after studying a few of the images randomly. The color score for each image was calculated based on the TissueQuant algorithm. These color scores provide quantification of the bone cell activities in these sections. Figure 4.1 shows the screenshot of use of TissueQuant image analysis software for assessment of ALP staining.

Validation

To confirm the applicability of this technique, a two-step validation was performed. As a first step, randomly selected 20 images of the sections (10 images each representing ALP and TRAP staining) were assessed by four persons within the research group for quantification of ALP and TRAP staining. Out of the ten images, one was used as reference in both cases. The analysis of other images was done in comparison to the reference image. Average values of the four readings were compared with the quantification obtained by TissueQuant. The difference of automated quantification and average manual quantification was within 15% in all cases. The second step was to use sections from the same specimen. Three different sections from the same specimen were evaluated using the tool and the score obtained in each case was compared with the mean. Ten such specimens were used for validation. The percentage error was within 10% in all cases.

4.2.2 Results and Discussion

The images were analyzed and the color scores were obtained for each of the images. The result images in Figures 4.2 and 4.3 represent the color scores which are mapped to gray levels 0 to 255 and displayed as an image. The mean color score of this image
provides quantification of bone cell activity in the section represented by this image.

From the plots of the results shown in Figures 4.4 and 4.5, it can be observed that the osteoblast activity indicated by the ALP marker, is the highest in the case of CQ and the osteoclast activity as indicated by the TRAP marker is minimum. The results were compared with manual evaluation. It was found to correlate well with the manual evaluation, $p < 0.001$. 
Fig. 4.3: Tartarate resistant acid phosphatase (TRAP) stained images (A) of bone sections in Sham control (SH), Ovariectomised (OV), Raloxifene treated (RA) and Cissus Quadrangularis (CQ) extract treated groups, the color score representation (B) of the images in A, and the color coded representation (C) of images in (B).

Fig. 4.4: Plot of color scores of ALP stained section images
4.3 Conclusion

This chapter demonstrates the use of color scores to quantify a substance present which upon staining, expresses in different shades of a specific color. The application considered here is a study of efficacy of a drug for treatment of osteoporosis. The treatment of osteoporosis is expected to bring down the amount of osteoclasts and also increase the amount of osteoblasts present in bones. TissueQuant algorithm was used to evaluate the changes in the amounts of osteoblasts and osteoclasts in bone sections. This study helps establish the role of CQ as a possible solution to osteoporosis.

The next chapter describes the development of computer aided diagnostic systems using color scores.