ORIGINAL ARTICLE

A SEMI-AUTOMATED MORPHOMETRIC ASSESSMENT OF NUCLEI IN PAP SMEARS USING IMAGEJ

R. Vijayashree[1], K. Ramesh Rao[2]

ABSTRACT: BACKGROUND AND PURPOSE: Carcinoma of cervix is the fourth commonest malignancy in women. Its incidence is progressively falling due to the routine use of Pap smears to detect precancerous lesions. However, routine Pap smear examination is time consuming and, as it is based on descriptive morphological assessment, false positive or negative reports are likely to occur. Using morphometric techniques, several attempts have been made to improve the accuracy of reports. In the present study, we have used Image morphometric software and some of its plugins to create a macro to analyse large number of cells at a time. **MATERIALS AND METHODS:** Using Image and three of its plugins, namely, BEEPS, Kuwahara filter and Mexican Hat filter, we created a macro to morphometrically analyse normal, reactive and neoplastic Pap smears. We also compared the macro measurements with manual measurements. **RESULTS AND CONCLUSIONS:** Results obtained with macro showed strong positive correlation with manual measurement. Although the neoplastic nuclei were on an average larger than reactive/normal nuclei, there was considerable overlap. More than the enlargement, anisonucleosis (variability in the size) appeared to be a better indicator of neoplasia. The macro that we developed works rapidly and gives results comparable to manual measurements provided the smears and the photographs are technically acceptable.
KEYWORDS: Anisonucleosis, HSIL, ImageJ, Kuwahara filter, LSIL, Mexican Hat Filter, Morphometry, Pap Smears, Squamous cell carcinoma
MESHTERMS: Cervical neoplasia, Imagej cytometry, Pap smear; Pap test; Papanicolau Test; Morphometry.

INTRODUCTION: The carcinoma of cervix is the 4th most common cancer in women globally.[1] At one time it was the commonest malignancy in women. In India, carcinoma of cervix remains the most common type of cancer in women, particularly in rural areas. The decline in its incidence particularly in developed countries like United States and United Kingdom, where it does not figure in the top five cancers affecting women, is mainly due to the regular use of "Pap Smear"[2] as a screening procedure to detect precancerous conditions. The "Pap Smears" are usually qualitatively evaluated by using well established descriptive morphological criteria and are categorised according to Bethesda System.[3] These morphological criteria are mainly based on descriptive nuclear changes. As some of the benign and reactive conditions can produce significant nuclear alterations that mimic neoplasia, false positive results may sometimes occur. In order to overcome the subjective nature of qualitative assessment which may lead to misinterpretation, some authors have recommended the use of morphometry.[4] In fact, several commercial devices/protocols are available aimed at improving the quality of preparation of smears and its interpretation (Pap Net.[5] BD Focal Point.[6]) However, these are expensive and are not suitable for small volume work. In a recent article,[7] Image has been used

ORIGINAL ARTICLE

to measure individual cell radius and perimeter. However, it is time consuming as the nuclei are measured one at a time.

In the present study, we have used Imagej[8] and some of its plugins to develop a simple macro to measure the nuclear dimensions of a large number of cells simultaneously. We have analysed the common cervical lesions using this macro. We have also compared the results obtained by using macro with the manual measurements of individual cell nuclei by "Cell Magic Wand Tool",[9] another plugin of Image.

MATERIALS AND METHODS: Nuclear measurements were carried out on normal as well as abnormal Pap Smears. Abnormal smears used in the study include biopsy proven low grade squamous intraepithelial lesion (LSIL), high grade squamous intraepithelial lesion (HSIL) and squamous cell carcinoma (SCC). In addition we also used reactive smears and smears with atypical squamous cells of uncertain significance (ASCUS). Representative areas (those areas where the cells are distributed in a monolayer) were digitally photographed with Olympus SP 350 compact Zoom Camera attached to Olympus CX 41 trinocular microscope through a microscope adopter. The photographs were taken using 40X objective and the images were processed in an editing software to improve the contrast. In all samples, nuclear analysis was done in an area equivalent to 1,92,0000 (1600X1200) pixels. The morphometric analysis was done using ImageJ[8] and three plug-ins: BEEPS (Bi-exponential edge preserving smoother) filter [10], Kuwahara filter[11] and Mexican Hat Filter.[12] BEEPS filter removes unnecessary details without affecting the edges; the Kuwahara filter reduces the noise while preserving the edge; and the Mexican Hat Filter helps to separate signal from the noise by applying Laplacian of Gaussian filter. In addition, inbuilt median filter of Imagej was also used. A macro was developed for automated analysis using the processing algorithm shown in fig. 1.
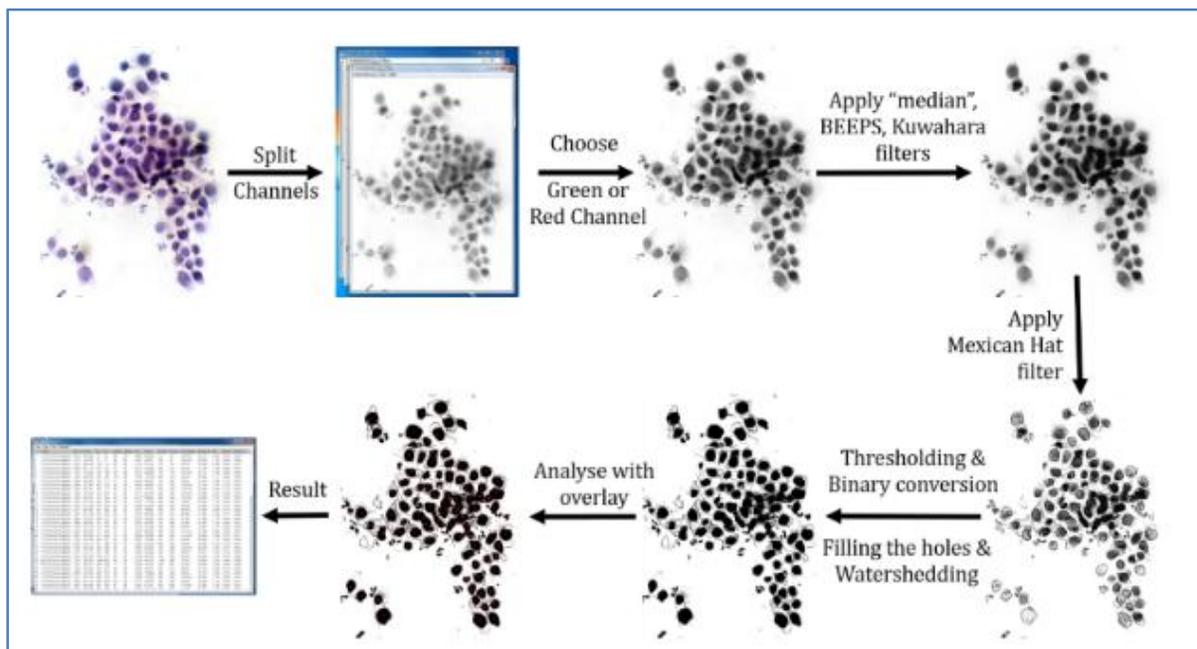

Fig. 1: Sample processing algorithm

ORIGINAL ARTICLE

After channel splitting, green or red channel was chosen because of the greater contrast (red channel is preferable if the smear has too much blood in the background). For Kuwahara Filter, sampling window was kept at default; for median filter, a radius of 5 was chosen and for Mexican Hat Filter, a radius of 3 or 4 was selected. However, other values can be tried. A bit of experimentation is required to arrive at the optimal values. The quality of smears and photographs determine the accuracy of the results obtained. The "Analyze tool" in Image J was configured to measure Area, Perimeter, Width, Height and Feret diameter. In addition, the diameter was calculated manually from the Area. Perimeter is the boundary length of a region of interest (ROI in this case is nucleus). Width and breadth are the dimensions of the smallest rectangle that can enclose the ROI. Feret diameter is also called caliper diameter and is the longest distance between two points on the selection boundary. Further, "Analyze tool" settings were set as follows: size = 500 to infinity (to exclude structures less than 500 pixels; circularity = 0.50-1 (to ignore elliptical and linear structure); show = overlay outlines or outlines.

HSIL and invasive squamous cell carcinoma and their analysed images are shown in figure 2.

Figure 2: A1, A2 & A3 show (left to right)Pap smear of HSIL, its thresholded image and analysed image having nuclei larger than 1500 pixels; B1, B2 & B2 show Pap smear of squamous cell carcinoma with its thresholded and analysed images.
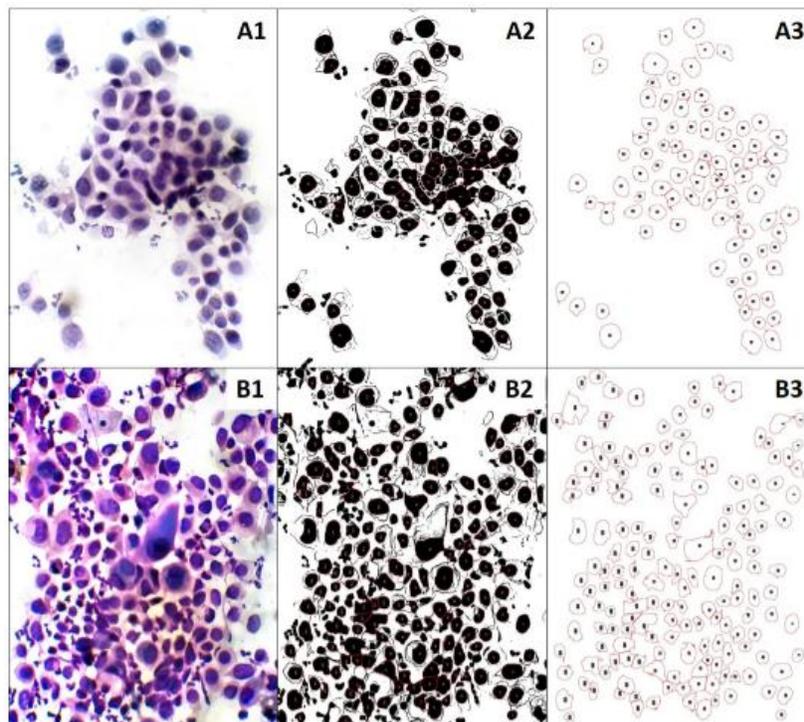

Fig. 2

As a quality control, a limited number of nuclei (200) were measured directly on the unaltered digital photograph using "Cell Magic Wand Tool".[9] With this tool, one can get with a simple click on the nucleus/cell, the delineation of its perimeter (Figure 2). However, each cell needs to be

ORIGINAL ARTICLE

measured separately. The values obtained by this method were correlated with those values obtained on the same nuclei by running the macro and the correlation coefficient was determined.
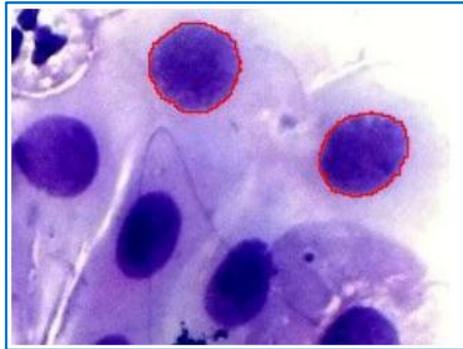
Fig. 3: Perimeter of the nucleus delineated by Cell Magic Wand

Statistical analysis was done and Pearson's correlation coefficient, mean t test and F test for standard deviation were determined wherever required.

	Area-M	Perim-M	Width-M	Height-M	Feret-M
Area-A	r=0. 991 p<0. 001				
Perim-A		r=0. 948 p<0. 001			
Width-A			r=0. 983 p<0. 001		
Height-A				r=0. 961 p<0. 001	
Feret-A					r=0. 960 p<0. 001
Table 1: Correlation matrix between the parameters measured by automated (A) and manual (M) methods					

RESULTS:
Legend: M = Manual measurement; A= automated measurement using macro; r=Pearson's Coefficient; p=p value;

The results for five parameters (area, perimeter, breadth, width and Feret) of 200 nuclei obtained with macro were correlated with manual assessment of the same 200 nuclei using Pearson's correlation coefficient. The correlation matrix is given in table 1.

As is obvious, there is an excellent correlation among the tested parameters of the two methods with p value being less than 0.001 in all cases. The greatest correlation was (0.991) found between the area parameter measured by the two methods. These results are highly significant. It

ORIGINAL ARTICLE

suggests that the results obtained by two methods are close enough so that these two methods could to be used interchangeably.

Cell/Lesion		Area	Perimeter	Diameter	Feret	width	Height
Superficial cells	Mean	710.353	105.897	29.999	35.439	30.18	32.47
	SD	100.247	8.547	2.0969	2.747	3.15	3.223
Intermediate cells	Mean	1089.17	138.66	37.17	47.476	38.83	41.42
	SD	132.563	11.641	2.12	3.593	7.04	4.621
Parabasal cells	Mean	2211.56	186.162	53.0196	72.5	53.44	56.44
	SD	167.121	11.439	2.021	5.8	4.93	3.609
Reactive smear	Mean	974.317	129.3085	35.208	45.505	37.20	38.13
	SD	329.565	28.423	20.4628	9.8025	9.15	8.741
ASCUS	Mean	1866.57	167.395	48.733	59.123	50.14	48.45
	SD	1169.33	49.674	38.57	16.696	16.21	15.77
LSIL	Mean	1784.73	167.6205	47.65	58.341	50.47	47.85
	SD	1187.11	56.6735	38.868	18.097	17.76	16.70
HSIL	Mean	2486.16	194.471	56.249	67.705	59.53	55.48
	SD	1229.84	49.42567	39.549	16.499	15.73	15.55
SCC	Mean	2340.19	191.1512	54.573	66.353	57.15	53.80
	SD	1515.57	62.448	43.91	20.303	19.57	18.08
Table 2: Measurements of nuclear parameters in normal & abnormal smears							

Legend: ASCUS = atypical squamous cells of uncertain significance; LSIL= low grade squamous intra-epithelial lesion; HSIL=high grade squamous intraepithelial lesion; SCC = squamous cell carcinoma.

The nuclear assessments of normal and abnormal smears were done using the newly written macro. The mean values along with the standard deviations obtained for these are given in table 2. As expected, there is progressive decrease in the dimensions of the nuclei as the cell matures from parabasal to superficial cell. The nucleus of parabasal cell measured on an average about 3 times larger (2211.56 pixels) area-wise than superficial cell (710.353 pixels; p=<0.0001). Similarly, the perimeter of parabasal cell (186.162 pixels) was 1. 8 times as long as the perimeter of superficial cell (105. 897). However, these cells showed little variation in size as reflected by low standard deviations. In an inflammatory smear rich in intermediate cells, the latter were found to be similar in size to the Intermediate cell in a normal smear (1089 vs. 974 pixel) but the standard deviation was more than double (329 vs. 132 pixels) indicating a greater variation in cell size (anisonucleosis). The normal parabasal cell nuclei, on an average, were larger (2211 pixels) than nuclei of ASCUS (1866 pixels) and LSIL (1784 pixels) but they were more uniform with lesser variation (SD: 382 vs. 1169/1187 pixels). Both HSIL and squamous cell carcinoma had cells, whose nuclei were not only larger but also exhibited marked variation in size. This was reflected in high standard deviation (SD = 1227/1515). The greater degrees of abnormality appeared to be associated with greater anisonucleosis. More than the increase in overall dimensions, high SD (reflecting anisocytosis) appears to be an important characteristic of abnormal cells.

ORIGINAL ARTICLE

DISCUSSION: Pap smear continues to be one of the most important screening procedures for carcinoma cervix, at least, in the developing world [13]. In developed world, large scale screening is done using one of the commercially available automated protocols viz, Pap Net,[5] BD Focal Point.[6] However, these are expensive and are not cost effective for low volume work. In India and other developing countries, the pap smears are still evaluated qualitatively using descriptive nuclear morphological features. This approach carries certain inherent drawbacks. It is subjective and time consuming. Time constraints and fatigue may adversely affect the quality of report.[14] Besides, several benign conditions may induce nuclear changes that mimic neoplastic conditions. So, several attempts have been made to make these assessments more objective by using PC based morphometry.[15],[16],[17],[18]

In the present study we have utilised ImageJ and three of its plugins to develop a simple macro to analyse nuclear parameters in normal and abnormal pap smears. In order to achieve this, we had to enhance the point of interest while removing the noise and suppressing the distracting background details. This was realised as follows: BEEPS plugin filter was used to remove irrelevant details without affecting the edges.[9] Kuwahara filter plug-in[10] was used to remove the noise while preserving the edge. Mexican hat plug-in [11] was particularly useful in enhancing spherical points or objects in a noisy image. Final effect of these filters is to enhance points of interest (in the present study, the nuclei) while smoothening out the background.

When we had a working macro, we had to ascertain if the measurements obtained using it were comparable to the values obtained by measuring manually using Cell Magic Wand tool. Area and other measurements obtained with the macro showed such an excellent correlation with manual assessments of same nuclei (r=0.991; p=<0.001) that these two methods could be used interchangeably.

The nuclear dimensions of cells in abnormal pap smears were in general significantly much higher than the corresponding nuclear measurements in normal smears (Table 2). But the most important characteristic appeared to be the variability in the nuclear dimensions in abnormal cells. For example, the mean nuclear dimensions of normal parabasal cells appeared to be higher than nuclear dimensions in some abnormal smears like ASCUS and LSIL. But, unlike normal parabasal cells which had fairly uniform nuclei, the nuclei in ASCUS and LSIL exhibited much greater variability in size (anisonucleosis) as reflected by higher standard deviations. This difference in anisonucleosis was statistically significant (F test for SD: p = <0.01). In smears with more advanced abnormalities like HSIL and squamous cell carcinoma, as expected, the nuclei were much larger on an average but there was some overlap in the values with non-neoplastic nuclei. But, they showed marked anisonucleosis. These findings suggest that more than the overall enlargement of the nuclei, anisonucleosis appears to be the characteristic feature of neoplastic PAP smears. This is in agreement observations of Mudaliar and Hutchens [19] who found nuclear pleomorphism (as reflected by high standard deviation) to be the most useful cellular change in distinguishing neoplastic lesions from benign lesions in their morphometric study on neoplastic and non-neoplastic skin lesions. In addition, anisonucleosis is also the parameter that is least affected by methods of assessment.

The image analysis macro that we developed requires properly exposed images of well-made PAP smears without cellular overcrowding or clumping. The images should be adjusted for optimal brightness and contrast. The filter parameters should be determined by experimentation. A single macro may not give satisfactory results in all cases. It may be a good idea to have more than one

ORIGINAL ARTICLE

macro with slightly different algorithms to obtain satisfactory results in all. In our study, one macro (with the configuration outlined above) worked very well in majority of situations. In low contrast or very high contrast images we had to modify the settings of the plugins slightly. When it is properly configured, macro gives satisfactory results in less than a minute.

CONCLUSION: In the present study we have designed macro to carry out morphometric analysis of nuclei in Pap smears. For this, we have utilised ImageJ software and three of its plugins. The macro was used to analyse normal, reactive and neoplastic cells in Pap smear. It appears to be fast (45 seconds), processes all the cells in the field and consistent. Results obtained show excellent correlation with manual measurements. In Pap smears, anisonucleosis is a better marker of neoplasia than changes in the dimensions of the nuclei.

REFERENCES:
1. Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, et al. GLOBOCAN 2012 v1. 0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer; 2013. Available from: http: //globocan. iarc. fr, accessed on 13/12/2013.
2. National Institutes of Health. Cervical Cancer. NIH Consensus Statement. 1996. p. 1-38.
3. Solomon D, Davey D, Kurman R, Moriarty A, O'Connor D, Prey M, et al. The2001 Bethesda System: terminology for reporting results of cervical cytology. JAMA. 2002 287 (16): 2114-9.
4. Po Chi Huang, Yung Kuan Chan, Po Chou Chan, Yung –Fu Chen, Rung ChingChen, Yu–Ruei Huang. Quantitative Assessment of Pap smear Cells by PC Based Cytopathologic Image Analysis System and Support Vector Machine. Lecture Notes in Computer Science 2007; 4901: 192-99.
5. Koss LG, Lin E, Schreiber K, Elgert P, Mango L. Evaluation of the PAPNET cytologic screening system for quality control of cervical smears. American journal of clinical pathology. 1994 101 (2): 220-9.
6. Levi AW, Galullo P, Gordy K, Mikolaiski N, Schofield K, Elsheikh TM, et al. Increasing cytotechnologist workload above 100 slides per day using the BD FocalPoint GS imaging system negatively affects screening performance. American journal of clinical pathology. 2012; 138 (6): 811-5.
7. Divya Rani MN, Aparna N, Harendra Kumar ML, Sheela SR. Evaluation of Pre-Malignant and Malignant Lesions in Cervico Vaginal (Pap) Smears by Nuclear Morphometry. Journal of Clinical and Diagnostic Research. 2014 Nov, Vol-8 (11): FC16-FC19.
8. Rasband W. ImageJ V. 1. 49[o] USA: National Institute of Health; [cited 2015 20/02/2015]. Available from: http: //imagej. nih. gov/ij/.
9. Walker T. Cell Magic Wand [Internet]. 2014 [cited 2015 20/022015]. Click on aCell. Get an ROI around the cell]. Available from:
   http: //www. maxplanckflorida. org/fitzpatricklab/software/cellMagicWand/index. html.
10. Thévenaz P. Bi-Exponential Edge-Preserving Smoother. An ImageJ plugin thatsmoothes an image without altering its edges Biomedical Imaging Group, Swiss Federal Institute of Technology, Lausanne. Available from: http: //bigwww. epfl. ch/thevenaz/beeps/.

ORIGINAL ARTICLE

11. Rasband W. Kuwahara Filter [updated 2010/03/11; cited 2015 20/02/2015]. The Kuwahara filter is a noise-reduction filter that preserves edges.]. Available from: http: //rsb. info. nih. gov/ij/plugins/kuwahara. html.

12. Prodanov D. Mexican Hat Filter [updated 2012/11/26; cited 2015 20/02/2015]. This plugin applies a Laplacian of Gaussian (Mexican Hat) filter to a 2D image.]. Available from: http: //imagej. nih. gov/ij/plugins/mexican-hat/index. html.

13. Bukhari MH, Saba K, Qamar S, Majeed MM, Niazi S, S. N. Clinicopathological importance of Papanicolau smears for the diagnosis of premalignant andmalignant lesions of the cervix. J Cytol. 2012; 29 ((1)): 20-5.

14. Koss LG. The Papanicolau test for cervical cancer detection. A triumph and atragedy. JAMA. 1989; 261 ((5)): 737-43.

15. Harris MV, Cason Z, Benghuzzi H, Tucci M. Cytomorphological assessment of benign and malignant dense hyperchromatic groups in cervicovaginal smears. Biomed Sci Instrum. 2000; 36: 349-54.

16. Nunobiki O, Sato M, Taniguchi E, Tang W, Nakamura M, Utsunomiya H, et al. Color image analysis of cervical neoplasia using RGB computer color specification. Anal Quant Cytol Histol. 2002; 24, (289-294).

17. Bacus JW. Cervical cell recognition and morphometric grading by image analysis. Journal of cellular biochemistry Supplement. 1995; 23: 33-42.

18. Yung-Fu C, Po-Chi H, Ker-Cheng L, Hsuan-Hung L, Li-En W, Chung-Chuan C, et al. Semi-Automatic Segmentation and Classification of Pap smear Cells. Biomedical and Health Informatics, IEEE Journal of. 2014; 18 (1): 94-108.

19. Mudaliar K, Hutchens K. Morphometric Image Analysis as a Tool in the Diagnosisof Transected SquamousNeoplasms. J Clin Anat Pathol 1: 1-5.

AUTHORS:
1. R. Vijayashree
2. K. Ramesh Rao

PARTICULARS OF CONTRIBUTORS:
1. Associate Professor, Department of Pathology, Chettinad Hospital and Research Institute, Rajiv Gandhi Salai, Kelambakkam, Kanchipuram District, Tamilnadu.
2. Professor, Department of Pathology, Chettinad Hospital and Research Institute, Rajiv Gandhi Salai, Kelambakkam, Kanchipuram District, Tamilnadu.

FINANCIAL OR OTHER COMPETING INTERESTS: None

NAME ADDRESS EMAIL ID OF THE CORRESPONDING AUTHOR:
Dr. K. Ramesh Rao,
Professor, Department of Pathology,
Chettinad Hospital and Research Institute,
Rajiv Gandhi Salai, Kelambakkam-603103,
Kanchipuram District, Tamilnadu.
E-mail: rameshkrao@gmail.com