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QUANTITATION OF SCANNING ELECTRON MICROSCOPIC URINARY CYTOLOGY

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Abstract

Using scanning electron microscopy (SEM), differences in cell surface morphology are identifiable between normal urothelium and malignant urothelia including the presence of pleomorphic microvilli (PMV). PMV have been reported in carcinomas of the urinary bladder, and they appear early in the pathogenesis of these tumors in animal models. Preliminary studies in our laboratory and others' demonstrated similar changes in tissue and cytologic specimens from patients with bladder cancer. We observed and evaluated normal and neoplastic cells of the human bladder by SEM in a preliminary assessment of surface topography with regard to tumor growth, grade, and stage. However, while these SEM observations indicated that differences between normal and abnormal urothelia could be readily recognized, the distinction between the various types of atypical cells was not as clearly defined. Data collected so far indicates that changes in the surface topography of the exfoliated cells may possibly vary both in relation to tumor grade and stage. Based on these qualitative observations, we expanded our approach by using a computerized image analysis system directly interfaced with the SEM. The measurements which can be made include cell surface area, diameter, length, width, perimeter, orientation and number of PMV per unit area. Statistical analysis is also performed. The boundaries between cells are not recognizable by the system, making the single cells present in cytologic specimens ideal for evaluation. Uniform short microvilli are readily distinguished from PMV. Preliminary evaluation of 23 patients has distinguished specimens from malignant cases compared to patients with benign lesions.

KEY WORDS: Urinary cytology, Bladder cancer, Quantitative scanning electron microscopic cytology, Pleomorphic microvilli (PMV), Uniform microvilli (UMV), Automatic image analysis.

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Introduction

Cytologic evaluation of urine and bladder washing specimens has proven useful in the detection and follow-up of patients with bladder cancer, particularly those with higher grade lesions (8). However, it has not been useful in the detection of the more common low grade papillary tumors which often recur in patients after initial removal. Numerous methods have been evaluated for their potential for detecting these low grade lesions, including flow cytometry (8), karyotype analysis (10), A, B, H-isoantigen and other blood group antigen markers (9), various histochemical markers (2), carcinoembryonic antigen (5), and ultrastructural analysis (6). By scanning electron microscopy (SEM), numerous surface changes have been observed on cells in voided urine or bladder washings of patients with bladder cancer (6). The most striking feature has been the appearance of pleomorphic microvilli (PMV). In studies of bladder carcinogenesis, PMV were initially observed as early features in the process of bladder carcinogenesis in experimental animals. However, PMV have also been detected on the cells of the bladder in benign, regenerative hyperplasia in experimental models (3,4).

Studies utilizing SEM observations of tissue and cytologic specimens from humans have also been extensively evaluated (6). Although PMV have been observed frequently in specimens from patients with bladder tumors, they are also present on cells occasionally in specimens from patients with benign disorders, particularly those in which a regenerative phenomenon would be anticipated, such as with hydronephrosis, diverticular disease of the bladder (11), or polypoid cystitis (1). All of these studies have been based entirely on the qualitative observation by SEM of the cell surface features.

In a study by Jacobs et al. (7) of biopsy specimens from patients with bladder cancer of different grades, there were differences observed in the type of cells with respect to the grade of the lesions. Preliminary examination of these differences indicated that they might be more quantitative rather than strictly qualitative. However, the studies required extremely tedious and laborious evaluations which would

not be practical in a clinical setting.

With the availability of computerized image analysis, we have evaluated the possibility of studying the cell surface features in cytologic specimens quantitatively rather than only qualitatively to determine if such observations might be of clinical or theoretical significance in the study of bladder cancer. PMV are a useful marker for this purpose since they can be detected and measured quantitatively as "particles" with the electronic computerized image analysis system attached directly to the SEM. Cytologic specimens also have proven to be ideal since the cells are present predominantly as single cell suspensions. It has become obvious in our preliminary observations that the image analysis system is unable to detect cell borders if a number of cells are touching or overlapping each other since there is no identifiable marker distinguishing the cell border from the remainder of the cell in tissue specimens. The boundaries of single cells in cytologic specimens are readily discernible by the system. The present study reports our initial findings with regard to the quantitation of urine and bladder washing cytologic specimens by SEM.

Materials and Methods

Bladder washings and/or voided urine specimens from 23 patients seen at Bishop Clarkson Hospital, Department of Urology, were collected at the time of cystoscopy and processed as described previously (6,11). The specimens were immediately mixed with an equal volume of 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The mixture was centrifuged at 1,000 rpm for 30 minutes in an IEC, model HN-S II, centrifuge (International Equipment Company, Needham Heights, MA). The supernatant was removed and the sediment resuspended in 5 ml of the fixative at 4°C. The mixture was then centrifuged at 1,000 rpm for 10 min. directly onto a 12 mm circular coverslip (Fisher brand Cover Glass, Fisher Scientific Co.) using a cytocentrifuge (Cytospin SCA-003, Shandon Southern Products, Runcorn, Cheshire, England). The coverslip was pre-coated with 0.1% poly-L-lysine (HBr type VI, Sigma Chemical Co., St. Louis, MO). The coverslip was then post-fixed with 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4), and rapidly dehydrated through an ascending series of alcohol, ending with Freon 113. The specimen was then critical-point dried with Freon 13 (Bowman SPC-1500 Critical Point Dryer, Tacoma, WA). The coverslips were mounted on specimen stubs, sputter-coated with gold (Polaron E5100, Polaron, Inc.) and examined at 20 kV in an ETEC autoscan scanning electron microscope (Perkin-Elmer, Hayward, CA) directly interfaced with a LeMont DA-10 computerized image analysis system (Kevex-LeMont Scientific, State College, PA).

Filament current was set to give the maximum emission of electrons. Undersaturation of the filament leads to a loss of resolution and reduces the stability of the image. The choice of magnification also plays an important effect on resolution and was set accordingly. Particles or surface features were located by advancing the electron beam in large precise steps until the video signal was above the threshold. These large steps are classified as the "off point density." While the beam is on the particle, small steps are employed, performing rapid and precise measurements, and are classified as "on point density."

The coarse off point density used to locate particles and the fine on point density for measurements of particles is referred to as the dual point density method. The off point density is probably the most crucial input parameter selected. If the off point density is too large, the particles smaller than the off point density will not be analyzed. The off point density should be set such that all particles of interest are "scanned" at least once during the search process.

To determine the appropriate point density, the following formula is used.

$$\text{Off Point Density} = \frac{\text{Screen Size of Record CRT}}{\text{Magnification} \times \text{Size of Smallest Feature of Interest}} \quad [1]$$

In the above formula the screen size is the size of the record CRT on the SEM. Magnification is determined as well as the smallest feature of interest, in this case, the diameter of a microvillus.

After the computer finds a particle as described in the off particle search mode, the program stores the data representing the detailed image in computer memory and systematically makes evaluations and measurements. The logic utilized is too complex to describe briefly, but it is sufficient to state that the algorithm works for nearly all particle shapes. A schematic of the information flow chart of the SEM interfaced automatic image analysis system is shown in Figure 1. The LeMont DA-10 system interfaces a Digital Equipment Corporation (DEC) LSI-11 microprocessor to the SEM through the digital scan generator (DSG) and image/threshold selector. The DA-10 system can analyze approximately 5000 particles per hour. The scan rate is lowered to a level such that the computer can directly control the electron beam positioning allowing a point by point analysis. The DSG has control and can transport the electron beam for threshold determination and image analysis processing, a signal above a preselected video threshold indicates a particle is "off point." The DSG then switches to the "on point" routine and directs the beam to perform specific and precise measurements.

For a given cell in a specimen, the area of the cell is measured using the dual point density approach at a magnification at which the cell nearly fills the Record CRT screen. This was determined to be a magnification of 1000. The area is estimated by the method of multiple triangulation as partially illustrated in Figure 2. To measure width and length of microvilli, a magnification of 8000 is uniformly used, the

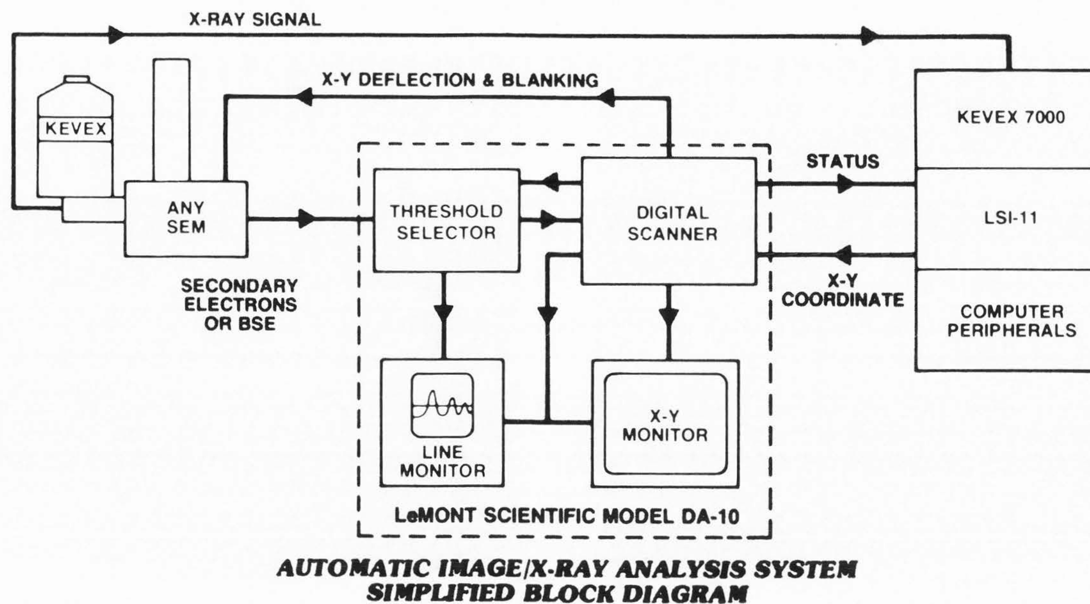


Figure 1. Schematic diagram of the information flow chart for the SEM interfaced with the automatic image analysis system.

angulation of the microvilli being determined by the system in order to estimate these parameters (Figure 3). The ratio of width to length is calculated by the computer. The results for each area measured are averaged so that the mean and standard deviation, range, and distribution of measurements is printed. A histogram for the distribution of each parameter is also printed.

Results

Quantitative analyses were performed on specimens obtained from 23 patients. These included specimens from 10 patients with non-neoplastic urologic disorders and 13 patients with transitional cell carcinoma of the urinary tract which included examples of cases of grade I, II, and III as determined by histopathologic evaluation of the corresponding tissue biopsy specimen. A minimum of 100 consecutive epithelial cells were examined in each of the cases. The criteria used for determining the surface features analyzed have been described (6,7). Unfortunately, no cells in the cases of non-neoplastic specimens had PMV as observed by SEM. Nevertheless, cells were observed which had numerous uniform microvilli on their surfaces. The results of the analysis from one of these patients for width to length ratio is illustrated in the histogram shown in Figure 4.

Of the 13 patients with transitional cell carcinoma of the lower urinary tract, 3 of the specimens proved to be unsatisfactory because of the presence in the specimen of a gel-like substance which had been used by the urologist for insertion of the cystoscope. Nine of the remaining 10 specimens had cells with PMV present on their surfaces as detected by SEM (Figure 5). The results from a patient with cells having PMV are illustrated in the histogram shown in Figure 6.

In comparing cells with uniform microvilli to cells with PMV, the quantitative measurements readily distinguished these cells. This was evident predominantly in the difference of the length of the PMV compared to the short uniform microvilli, and this was also evident in the width-to-length ratio as illustrated in Figures 4 and 6.

Discussion

Previous studies have clearly documented that PMV are present on neoplastic cells, even those from low grade tumors, in urine and bladder washing specimens from patients with urothelial neoplasms (6). However, PMV are also present on cells occasionally in patients with non-neoplastic disorders, particularly those with regenerative and hyperplastic lesions (1,11). There has been a suggestion from studies of experimental models and in humans that PMV might be quantitatively different in neoplastic and non-neoplastic disorders (3,6,7). Until recently, analyzing cell surface features quantitatively has been extremely tedious if not impossible to do. With the availability of computerized image analysis systems, a quantitative approach is at least feasible. Although most quantitative SEM studies have involved non-biological systems, PMV and uniform microvilli on single cells present in cytologic specimens from the urinary tract provide an ideal system to apply quantitative image analyses to a biological and potentially clinically useful system.

It became apparent early in our quantitative evaluation, that single cells would need to be analyzed since the system was unable to distinguish cell boundaries when cells are in

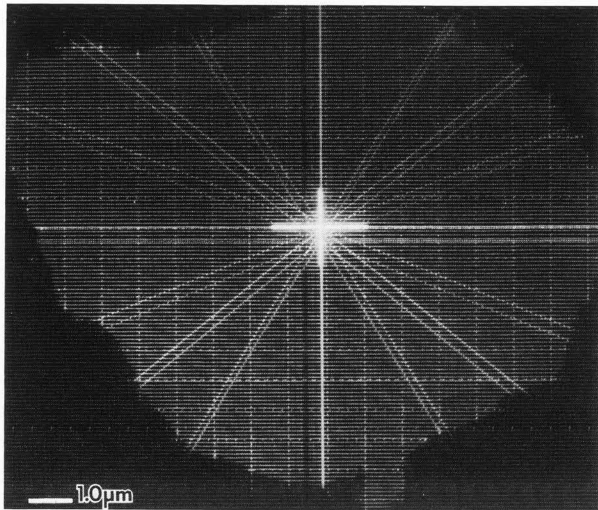


Figure 2. Estimation of cell surface area to be analyzed using multiple triangles, a few of which are illustrated in this photograph.

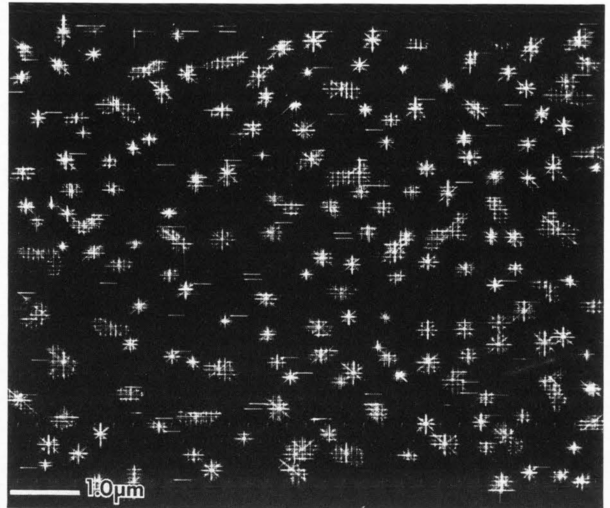


Figure 3. Quantitative estimations of length and width of microvilli by image analysis system.

WIDTH TO LENGTH RATIO DISTRIBUTION FOR ALL TYPES MACR = 0

MOST PROBABLE= 7.50E-02 MEDIAN= 1.50E-01 AVG= 1.90E-01 SIGMA= 1.36E-01

CLASS LIMIT	COUNT	%	0-----0-----0-----0-----0-----0-----0-----0-----0-----0-----0 (0 1 2 3 4 5 6 7 8 9 10)
5.00E-02	11	5.73	[***
1.00E-01	48	25.00	[*************
1.50E-01	37	19.27	[**********
2.00E-01	23	11.98	[******
2.50E-01	16	8.33	[****
3.00E-01	21	10.94	[*****
3.50E-01	15	7.81	[****
4.00E-01	8	4.17	[**
4.50E-01	7	3.65	[**
5.00E-01	1	0.52	[
5.50E-01	0	0.00	[
6.00E-01	1	0.52	[
6.50E-01	0	0.00	[
7.00E-01	1	0.52	[
7.50E-01	2	1.04	[*
8.00E-01	1	0.52	[

Figure 4. Histogram showing the distribution of width to length ratio of microvilli from a cell with unform microvilli.

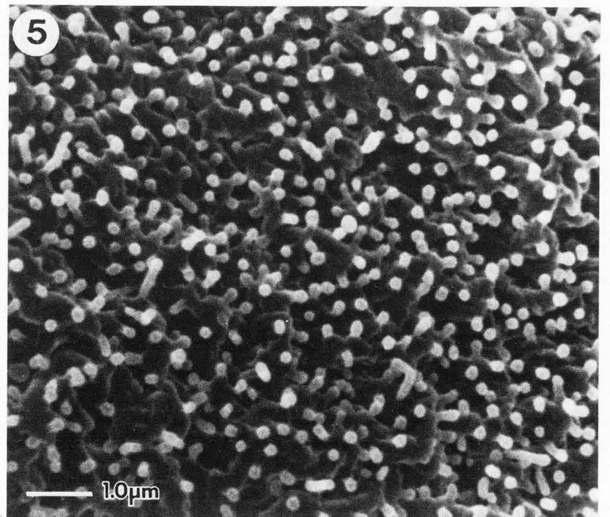
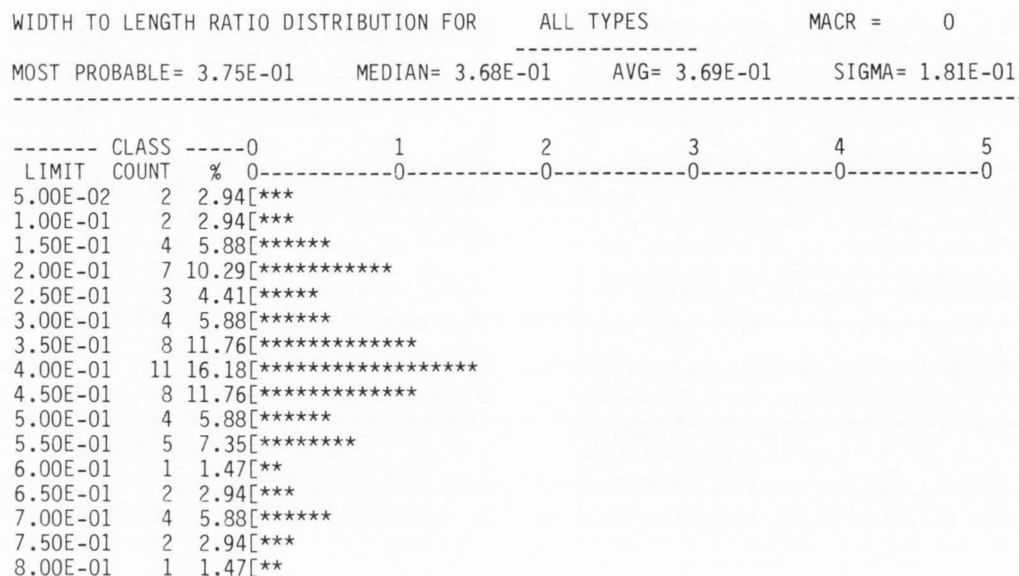


Figure 5. Uniform microvilli with a few pleomorphic microvilli from a patient with transitional cell carcinoma of the bladder, Grade 1.



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WIDTH TO LENGTH RATIO DISTRIBUTION FOR     ALL TYPES           MACR =     0
                                         ---------------
MOST PROBABLE= 3.75E-01    MEDIAN= 3.68E-01    AVG= 3.69E-01    SIGMA= 1.81E-01
-------------------------------------------------------------------------------

------- CLASS -----0           1           2           3           4           5
 LIMIT  COUNT    %  0-----------0-----------0-----------0-----------0-----------0
5.00E-02    2  2.94[***
1.00E-01    2  2.94[***
1.50E-01    4  5.88[******
2.00E-01    7 10.29[***********
2.50E-01    3  4.41[*****
3.00E-01    4  5.88[******
3.50E-01    8 11.76[*************
4.00E-01   11 16.18[******************
4.50E-01    8 11.76[*************
5.00E-01    4  5.88[******
5.50E-01    5  7.35[********
6.00E-01    1  1.47[**
6.50E-01    2  2.94[***
7.00E-01    4  5.88[******
7.50E-01    2  2.94[***
8.00E-01    1  1.47[**
```

Figure 6. Histogram showing the distribution of width to length ratio of microvilli from a cell with pleomorphic microvilli.

contact with each other. In epithelial systems, such as the urothelium, the cells frequently have microvilli present in large numbers at cell junctions which could greatly skew the data. Based on initial observations in specimens not reported in the present study, we were able to standardize the magnification necessary for the analyses so that a magnification of 1000 could be used for determination of cell surface areas, and a magnification of 8000 was used for measurements of the microvilli.

In each specimen, the cells could be screened qualitatively as described previously (6,11) for the presence of transitional cells, squamous cells, or other cells, and also for the presence or absence of microridges, uniform microvilli and/or PMV on the cell surfaces. Cells were then selected for quantitative analysis. The cells containing PMV were analyzed first and then cells with uniform microvilli were also analyzed. Microridges, although identifiable by the analysis system, provide a markedly distorted image since the microridge network on the cell surface is read as a single "particle" by the system. Fortunately, these cells can be excluded from quantitative analysis readily by simple SEM observation.

The data presented in this paper clearly show that PMV and uniform microvilli can be analyzed with the image analysis system being utilized. Unfortunately, none of the specimens from patients with non-neoplastic disorders contained PMV so that a comparison could not be made between quantitative results in such patients compared to those with neoplastic lesions. With the analysis system now established, additional specimens will be required for quantitation to determine if a quantitative distinction can be found between patients in which PMV are present on the cell surface of neoplastic cells as compared to non-neoplastic cells.

Acknowledgements

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References

1. Anderstrom, C., Ekelund, P., Hansson, H-A., Johansson, S.L. (1984) Scanning electron microscopy of polypoid cystitis - a reversible lesion of the human bladder, J. Urol. 121, 242-244.
2. Cohen, S.M. (1984) Pathology of experimental bladder cancer in rodents. In: The Pathology of Bladder Cancer, Vol. II, Eds., Bryan, G.T., Cohen, S.M. - CRC Press (Boca Raton, FL), 2-35.
3. Fukushima, S., Arai, M., Cohen, S.M., Jacobs, J.B., Friedell, G.H. (1981) Scanning electron microscopy of cyclophosphamide-induced hyperplasia of the rat urinary bladder, Lab. Invest. 44, 89-96.
4. Fukushima, S., Cohen, S.M., Arai, M., Jacobs, J.B., Friedell, G.H. (1981) Scanning electron microscopic examination of reversible hyperplasia of the rat urinary bladder, Amer. J. Pathol. 102, 373-380.
5. Holmberg, V., Nilsson, B. (1984) Short-term prognosis of bladder cancer. Evaluation with urinary CEA, Anticancer Res. 4, 121-122.

6. Jacobs, J.B., Cohen, S.M., Friedell, G.H. (1984) Scanning electron microscopy of the lower urinary tract. In: The Pathology of Bladder Cancer, Vol. II, Eds., Bryan, G.T., Cohen, S.M. - CRC Press (Boca Raton, FL), 142-178.
7. Jacobs, J.B., Cohen, S.M., Farrow G.M., Friedell, G.H. (1981) Scanning electron microscopic features of human urinary bladder cancer, Cancer 48, 1399-1409.
8. King, E.B. (1984) Cytology of bladder cancer. In: The Pathology of Bladder Cancer, Vol. 1, Eds., Bryan, G.T., Cohen, S.M. - CRC Press (Boca Raton, FL), 91-148.
9. Pauli, B.U., Alroy, J., Weinstein, R.S. (1984) The ultrastructure and pathobiology of urinary bladder cancer. In: The Pathology of Bladder Cancer, Vol. II, Eds., Bryan, G.T., Cohen, S.M. - CRC Press (Boca Raton, FL), 42-126.
10. Summers, J.L., Falor W.H., Ward, R. (1980) A 10-year analysis of chromosomes in non-invasive papillary carcinoma of the bladder, J. Urol. 125, 177-178.
11. Suzuki, T., Cano, M., Cohen, S.M. (1985) Scanning electron microscopic exfoliative urinary cytology in patients with malignant and non-malignant diseases of the lower urinary tract, Urology, in press.

Discussion with Reviewers

Reviewer I: How much time does it take to analyze 100 cells (time at the SEM and time for image analysis)?
Authors: The time factor varies on each specimen due to the presence of either a high concentration of red blood cells (hematuria), renal cells, or bacteria if the patient has bacterial cystitis. However, the average time to analyze a cytological specimen is about 1 1/2 hours, approximately half of which is image analysis time.

Reviewer I: How do you evaluate the number of cells lost during preparative procedures, especially CPD?
Authors: The pre-coated poly-L-lysine coverslips virtually eliminate the loss of cells during dehydration and critical point drying. (See Jacobs, et al., Ref. 6).

Reviewer II: If the method can demonstrate differences between PMV on malignant and non-malignant cells, I would like to know if diagnostic studies can be performed on cytologic preparations for a reasonable cost?
Authors: The possibility of diagnostic studies is good, and as the technology advances the cost effectiveness could allow the procedure to be done in specific clinical situations.

Reviewer II: What does the instrumentation cost and who can operate the instrument? In other words, what does it cost to do this test, and in this era of health care cost-containment, is it worth our time and money to do the test?
Authors: The actual cost of the instrumentation varies with manufacturers based on the optional accessories and software and should be based on your needs. Obviously, such equipment will only be available in certain specialized laboratories where the volume of specimens is high. Under those circumstances, we would anticipate the cost to be approximately $100 per specimen.

Reviewer III: In that several morphological grades may exist in a single tumor, how do you know that the cells recovered for SEM for this study are selected from the predominant histological type?
Authors: For higher grade lesions, SEM cytology is not necessary since the lesions are detectable usually by routine light microscopic cytology. For the qualitative or quantitative interpretation of an SEM specimen, the changes are not dependent directly on grade. A determination of grade is made only by light microscopic determination.

Reviewer III: How is cellular polarity identified?
Authors: In general, the luminal surface of cells is identified by the presence of microridges if they are present on the cell. Also, superficial cells are readily determined by the flat nature of the cell. Evaluation by SEM is not dependent on cellular polarity.

Reviewer III: Do you think that quantitative SEM of urine washings will have a primary role for cytologic diagnosis, or is it to be considered as an adjunct investigative tool of diagnostic value in limited situations?
Authors: Qualitative and quantitative SEM of urine and bladder washings, because of the cost, will only be useful in limited situations, not as a primary screening method. The major setting in which we have investigated SEM cytology is during the follow-up examinations of patients who have already had a previous diagnosis and resection of a low-grade, low-stage carcinoma of the bladder.

Reviewer III: It is well established that dimensional changes take place during the processing of soft tissue biological specimens for scanning electron microscopy and that these changes can vary with the type of biological material. To what extent may your SEM data be a reflection of a differential response to the preparation procedures? Light microscope image analysis computer studies of cell volume change in wet preparation could possibly provide a clearer picture of the level of distortion subsequently attained following dehydration and CPD.
Authors: Undoubtedly there are quantitative changes occurring during the processing of these specimens. However, qualitatively and quantitatively, the evaluation of the cell features is on a relative basis, not absolute.

Reviewer I : E. de Harven
Reviewer II : J.B. Jacobs
Reviewer III : G.M. Hodges