Indian Journal of Fundamental and Applied Life Sciences ISSN: 2231-6345 (Online)
An Online International Journal Available at http://www.cibtech.org/jls.htm
2012 Vol. 2 (3) July-September, pp.12-19 /Chen et al.
Research Article

IMAGE ANALYSIS SYSTEM FOR AUTOMATIC CANCER CELLCOLONIES COUNTING IN HYPERTHERMIA

***Hsien-Tse Chen[1], Sheng-Fuu Lin[1], Yi-Hsien Lin[2,3] and Jui-Liang Chen[1]**

[1] *Department of Electrical Engineering, National Chiao Tung University, Taiwan. (R.O.C.)*

[2] *Institute of Traditional Medicine, National Yang-Ming University, Taipei, Taiwan. (R.O.C.)*

[3] *Division of Radiotherapy, Cheng Hsin Rehabilitation Medical Center, Taipei, Taiwan. (R.O.C.)*

**Author for Correspondence*

ABSTRACT

Clonogenic assay is the gold standard of *in vitro* cell survival assay in hyperthermia. In this paper, we developed an image analysis system for automatic cancer cell colonies counting in hyperthermia. HA22T cell were seeded in 6-well petri dishes and treated at 41-43 ^{O}C for 0.5-4h. Cells were incubated for 7 days to form colonies. After staining, the numbers of colonies by manual counting and by our image analysis system were compared. As the heating time and temperature increased, the number of colony decreased. The correlation of manual 1 and system counting was 0.987; manual 2 and system counting was 0.983; manual 1 and manual 2 counting was 0.971. Our results showed that automatic cell colonies counting by the image analysis system in hyperthermia is feasible by standard hardware and software. Image analysis system offers efficient and accurate tool in the research of *in vitro* hyperthermia.

Key Words: *Colony-Forming Units Assay, Cancer, Hyperthermia, Image Interpretation, Computer - Assisted Methods*

INTRODUCTION

Hyperthermia (HT) is gaining wider acceptance in clinical treatments due to the substantial technical improvements in achieving selected increase of temperatures in superficial and deep-seated tumors. HT appeared to be effective in cancer treatment (Valdagni, 1990; Falk, 2001 and Moroz, 2001). The combination of HT and ionizing irradiation (Seong, 1994; Hehr, 2003; Feyerabend, 1997; Overgaard, 1989 and Overgaard, 1996) as well as chemotherapy (Herman, 1994 and Teicher, 1988) has been demonstrated to be efficacious in a variety of localized neoplasms. Therefore, the need of pre-clinical studies on HT in treating different cancers is increasing. Rapid and efficient tools of *in vitro* HT studies are necessary.

Clonogenic assay (Kawada, 2002; Maximilian, 2007 and Berger, 1990) is the gold standard of *in vitro* cell (Lokesha, 2011) survival assay in hyperthermia. It is based on the ability of a single cell to grow into a colony. The clonogenic assay showed better correlation of *in vitro* study and clinical response. However, manual counting of cell colonies is time-consuming and laborious.

In this paper, we developed an image analysis system for automatic cancer cell colonies counting in hyperthermia.

METHODS AND MATERIALS

Cell Culture

Human liver cancer cell line, HA22T, was obtained from the Food Industry Research and Development Institute (Taiwan, R.O.C.). HA22T cells are poorly differentiated hepatocellular carcinoma, containing hepatitis B virus integrants. Cells were cultured in DMEM supplemented with 1% glutamine, 1% non-essential amino acids (NEAA), 1% sodium pyruvate, 1% L-glutamine, 2% penicillin/streptomycin (Invitrogen, NY, USA) and 10% fetal calf serum (Chemicon, CA, USA). Cultures were incubated at 37^{O}C under an atmosphere comprising 5% CO_2 / 95% air.

Hyperthermia

Cells with 500 cells/ well were seeded in 6-well dishes and incubated at 37℃ incubator for 16h to allow good cell attachment. The plates were sealed and submerged in a water bath (Kansin Instruments Co., Taiwan, R. O. C.) at three constant temperatures (41^{O}C, 42.5^{O}C or 44^{O}C) for different time intervals (0, 0.5, 1, 2, 4h). Temperature was monitored with a mercury thermometer. The cells were then incubated at 37^{O}C for 7 days for the clonogenic assay.

Clonogenic Assay

Preliminary experiments were conducted to optimize cell numbers and incubation time required for colony formation. Cells were plated at 500 cells per well in 6-well dishes and incubated at 37℃ for 16h. Following HT, cells were incubated at 37^{O}C in 5% CO_2 atmosphere for 7 days to form colonies. After removing the media, the cells were washed with PBS and added with 1 ml fixing solution (acetic acid: methanol = 1: 3) per well to fix cells for 2 to 3 hours. The fixing solution was removed and 1 ml 10% Giemsa solution (Sigma, St. Louis, USA) per well was added to stain cells overnight. Then the staining solution was removed. The cells were washed with water and left to dry at room temperature. Finally the cell colonies were counted under a microscope. Only the colonies that contained more than 50 cells were counted. Plating efficiency was the number of colonies counted divided by the number of cells seeded. The plating efficiency varied between the cell lines from 20% to 60%.

Automatic Colony Counting by Image Analysis System

The system's process is divided into three parts. The first part is the rotation and pre-processing of the scanned culture dish image. The second part is the extraction and analysis of cancer cell colony features after staining. The third part introduces the fuzzy inference system to identify the cancer cell colony features and count those with more than 50 cancer cells (See Figure 1). The system was tested on a notebook with AMD Athlon 3200+ 2.0MHz with 1GB DDR SDRAM. The type of scanner was Lexmark X83.

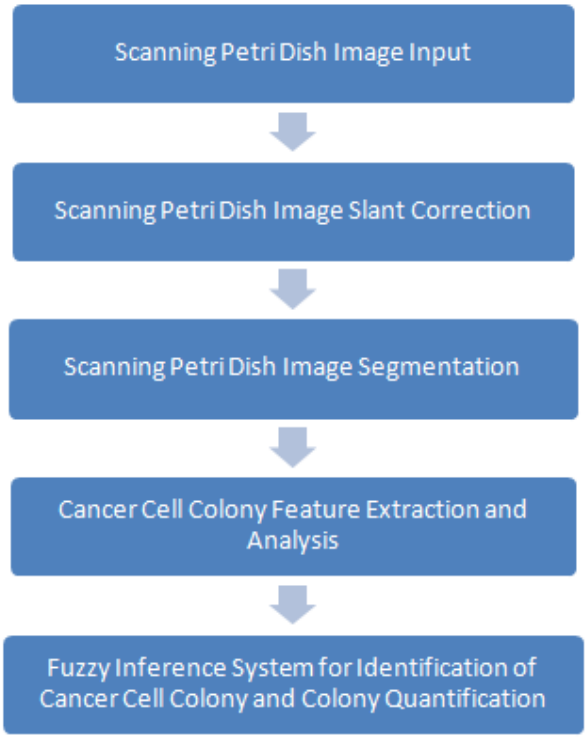

Figure 1: System flow chart

Scan: The image of petri dish was captured via a scanner (Dahle, 2004). As the culture dish was made from transparent acrylic, a stable light source was required to avoid reflection and diffraction. A scanner was therefore used for image capturing since the light came directly from overhead. Another advantage of the scanner is the fixed focal distance. With a scanner, the user would not need to adjust the focus during image capturing.

Image Processing: The system processed the images in three steps. The first step is the rotation and pre-processing of the scanned culture dish images. The shape and size of the culture dishes can be set as the precedent conditions as they are fixed, then image processing techniques (Li, 1986; Kanopoulos, 1988; Lv 2002; Gonzalez, 2002 and Zhang) were used to establish the two longest edges of the culture dish. Since the relative positions of the six wells within the culture dishes are also fixed, the size and location of them can be established as well. The second step is the extraction and analysis of cancer cell colony features after staining. The third step is introducing the fuzzy inference system (Lin, 1996) to identify the cancer cell colony features and count them with more than 50

cancer cells.

In an image of a culture dish, the cancer cell colony only falls within the six circular wells. The position of the six wells must therefore be established through image processing.

To detect the circular wells inside the culture dishes, this paper used Hough transform (Rau, 2003) line detection and bilinear interpolation (Rodrigues, 2002) to rotate the image to the most appropriate angle.

The Hough transform algorithm works as follows:

(1) Determine all of the potential feature points in the image space. These are usually edge or skeleton points.

(2) Find the feature points in the image space:

(2.1) for each point in the image, calculate the (a', b') for all straight lines that pass through (x, y).

(2.2) Increment the accumulator's (a', b') by one then look for the next feature point.

Repeat steps (2-1) and (2-2) until all feature points have been processed.

(3) Find the maximum value for each region in the accumulator.

(4) Map each maximum region back onto each representative straight line in the image space.

Efficacy Evaluation: The experiment analysis method proposed by (Dahle and Kakar, 2004) was taken into consideration along with Karl Pearson product-moment correlation. For the product-moment correlation formula, the sample covariance (Zhan, 2005) must first be determined and this is defined by Equation 1. The formula for the Pearson product-moment correlation is then as Equation (2), though in practice it can be simplified to become Equation (3).

$$\sigma_{XY} = \frac{\sum\left(x_i - \bar{x}\right)\left(y_i - \bar{y}\right)}{n-1} \quad \ldots. (1)$$

Here every value x_i corresponds to a value y_i. $\bar{x}$ is the mean of X specimens; $\bar{y}$ is the mean of Y specimens; n is the total number of X specimens and by default the total number of Y specimens as well.

$$\gamma_{XY} = \frac{\sigma_{XY}}{S_X S_Y} \quad \ldots. (2)$$

Here γ_{XY} is the Pearson product-moment relation; σ_{XY} the specimen co-variance; S_X the standard deviation for specimen X ; and S_Y the standard deviation for specimen Y .

$$\gamma_{XY} = \frac{\sum x_i y_i - \left(\sum x_i \sum y_i\right)/n}{\sqrt{\sum x_i^2 - \left(\sum x_i\right)^2/n}\sqrt{\sum y_i^2 - \left(\sum y_i\right)^2/n}} \quad \ldots. (3)$$

Here every value x_i corresponds to a value y_i. n is the total number of X specimens and by default the total number of Y specimens as well.

Statistical Methods

All experiments were performed at least triplicate and repeated once. For the clonogenic assay, survival fractions were calculated as (mean plating efficiency of treated cells/mean plating efficiency of control cells) 100%. The results were expressed as mean ± standard deviation. The correlation analysis was determined by Person correlation. Differences were considered significant when p was less than 0.05. The statistics software used in this study was SPSS 13.0 (SPSS Inc., USA).

RESULTS

Hyperthermia

HA22T cell were seeded in 6-well petri dish and treated at 41-43℃ for 0.5-4h. Cells were incubated for 7

days to form colonies. Cell survival by manual counting was shown in Figure 2. All experimental conditions were coded in Table 1. As the heating time and temperature increased, the number of colony decreased.

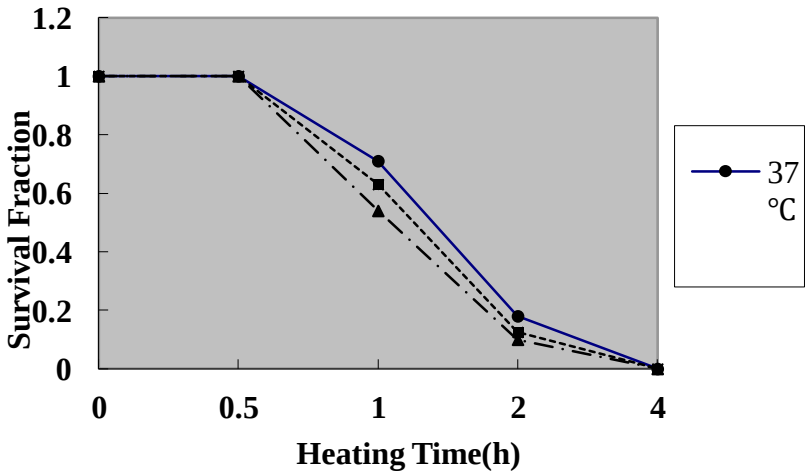

Figure 2: Survival fractions of HA22T cells in different hyperthermia experimental conditions

Table 1: Codes for all experimental conditions

Heating Time	Temperature $37^{\circ}C$	Temperature $41^{\circ}C$	Temperature $42.5^{\circ}C$
0.5h	T1	T5	T9
1h	T2	T6	T10
2h	T3	T7	T11
4h	T4	T8	T12

Correlation of Manual Counting and System Counting

To assess the performance of the image analysis system for automatically quantifying cancer cell colony, the data from the two manual counts and the automatic system count in the experiment were compared as shown in Table 2. The Pearson product-moment correlation values and the data distribution are shown in Figure 3-5. The correlation of manual 1 and system counting was 0.987; manual 2 and system counting was 0.983; manual 1 and manual 2 counting was 0.971.

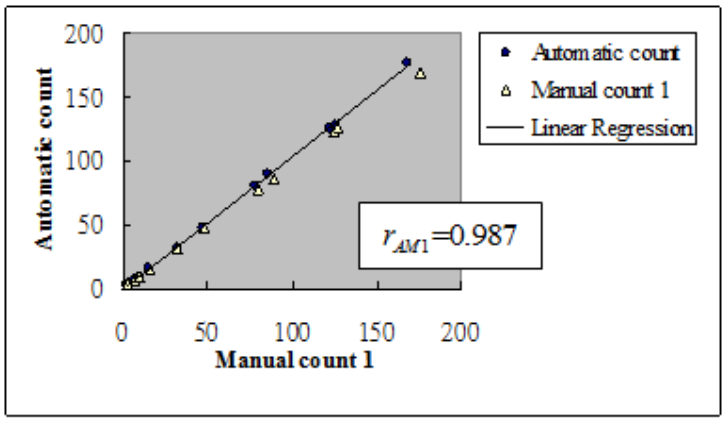

Figure 3: Correlation between system count and manual count 1

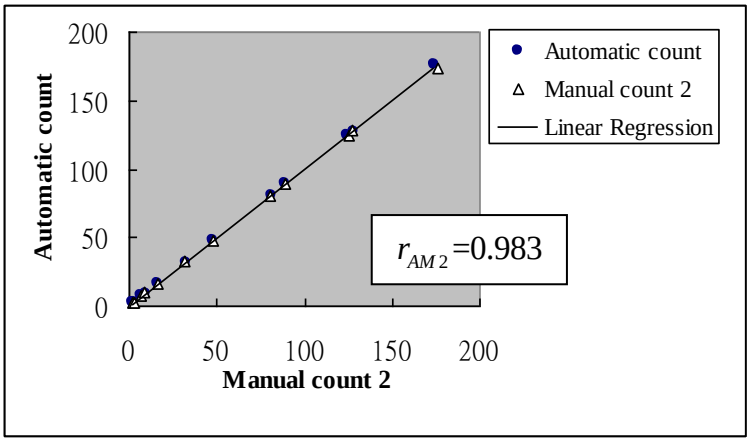

Figure 4: Correlation between system count and manual count 2

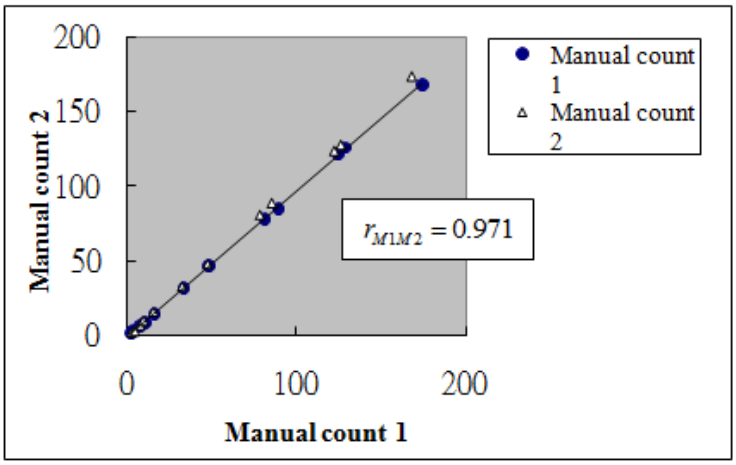

Figure 5: Correlation between manual count 1 and manual count 2

Table 2: Comparison of the colonies number by manual and the system counting

Experimental Conditions	Manual Count 1 (M1)	Manual Count 2 (M2)	System Count (A1)*	Difference (M1-A1/A1)	Difference (M2-A1/A1)
T1	172	174	176	-1.14%	-1.14%
T2	122	124	125	-1.60%	-0.80%
T3	32	34	32	-6.25%	6.25%
T4	7	7	7	0%	0%
T5	126	128	127	-1.57%	0.79%
T6	79	85	82	-7.32%	3.66%
T7	15	16	16	-6.25%	0%
T8	2	2	2	0%	0%
T9	88	89	92	-1.09%	-3.26%
T10	47	48	46	-2.17%	4.35%
T11	9	10	9	-11.11%	11.11%
T12	3	3	3	0%	0%

**, By our method*

The correlation value r was between 0.90 and 0.99, indicating a very strong correlation (Zhan, 2005).

Comparison of System Counting by Different Methods

The results counted by our system are compared using the methods proposed by Dahle and Kakar (2004) as well as Maximilian and Ismat (2007). The difference between the three counting methods and manual count 1, the expert count, is shown in Table 3. Our method showed less difference than the others.

Table 3: Comparison of system counting by different methods

Experimental Conditions	Manual Count 1 (M1)	System Count 1 (A1) *	System Count 2 (A2) #	System Count 3 (A3) $	Difference (M1-A1/M1)	Difference (M1-A2/M1)	Difference (M1-A3/M1)
T1	172	176	177	163	-2.33%	-2.91%	5.23%
T2	122	125	128	135	-2.46%	-4.92%	-10.66%
T3	32	32	28	40	0	12.50%	-25.00%
T4	7	7	8	5	0	-14.29%	28.57%
T5	126	127	120	133	-0.79%	4.76%	-5.56%
T6	79	82	73	81	-3.80%	7.59%	-2.53%
T7	15	16	18	17	-6.67%	-20.00%	-13.33%
T8	2	2	2	2	0	0	0
T9	88	92	97	93	-4.55%	-10.23%	-5.68%
T10	47	46	41	51	2.13%	12.77%	-8.51%
T11	9	9	8	12	0	11.11%	-33.33%
T12	3	3	3	3	0	0	0

**, By our method*
#, By the method of J. Dahle and M. Kakar
$, By the method of N. Maximilian and N. Ismat

DISCUSSION

Our study proposed an efficient method of automatically counting colonies in hyperthermia. This image analysis system was designed for identifying whether a cancer cell colony contains over 50 cells as well as for counting how many cancer cell colonies contain more than 50 cells in a culture dish.

By means of digital image process, image differencing is then used to find the cancer cell colonies image. Our study uses the feature of color model, the area and the fuzzy inference system designed to identify if the cancer cell colonies contain over 50 cells. The experimental results showed that there is a very strong correlation between the quantities counted by the system and by manual counting γ values all exceeding 0.9. Therefore system counting has the potential to replace manual counting, reduce the manpower required for clonogenic assays and accelerate the development of new drugs in hyperthermia. Accordingly the fuzzy inference system in this paper can be used to distinguish cancer cell colonies to improve system efficacy in order to avoid missing counts or misjudgments.

CONCLUSION

This system could not achieve a 100% identification rate because the counts were based on the empirical observation. This meant that uncertainties tended to emerge where two colonies overlapped. Some considered them as two colonies, while others treated them as one. In our experience, a joined region with no more than 300 cells (or 150 image pixels) can't be visually separated into two colonies as one single colony.

As for the Maximilian and Ismat (2001) method, the greatest difference between the results was on average in experimental environments Tc1, Tc2, Tc4, Tc5 and Tc11. This was because the use of gray level image

processing led to the lost of color data, so surface area was used directly to determine if a candidate region exceeds 50 cells. The problem with this method is with two special cases previously mentioned. The first is a high density of cells, leading to a very small surface area that contains over 50 cells and therefore should be counted; another case is where the cells are distributed too sparely so there aren't 50 cells but it is still counted anyway. These cases can all be handled by the method proposed in this paper.

Our technique demonstrated that automatic counting colonies are achievable by standard desktop scanner and image analysis system. Using image analysis system will make basic research of *in vitro* hyperthermia more efficiently.

ACKNOWLEDGEMENTS

This work was supported partially by the National Science Council under grant NSC 99-2221-E-009-148.

REFERENCES

Berger DP, Henb H, Winterhalter BR and Fiebig HH (1990). The Clonogenic Assay with Human Tumor Xenografts: Evaluation, Predictive Value and Application for Drug Screening. *Annals of Oncology* **1** 333-341.

Dahle J, Kakar M, Steen HB and Kaalhus O (2004). Automated Counting of Mammalian Cell Colonies by Means of a Flat Bed Scanner and Image Processing. *Cytometry Part A* **60** 182-188.

Falk MH and Issels RD (2001). Hyperthermia in oncology. *International Journal of Hyperthermia* **17** 1-18.

Feyerabend T, Steeves R, Wiedemann GJ, Richter E and Robins HI (1997). Rationale and clinical status of local hyperthermia, radiation and chemotherapy in locally advanced malignancies. *Anticancer Research* **17**(4B) 2895-2897.

Gonzalez RC and Woods RE (2002). Digital Image Processing, Upper Saddle River, (NJ: Prentice Hall).

Hehr T, Wust P, Bamberg M and Budach W (2003). Current and potential role of thermoradio therapy for solid tumours. *Onkologie* **26**(3) 295-302.

Herman TS and Teicher BA (1994). Summary of studies adding systemic chemotherapy to local hyperthermia and radiation. *International Journal of Hyperthermia* **10** 443-449.

Herman TS, Teicher BA, Jochelson M, Clark J, Svensson G and Coleman CN (1988). Rationale for use of local hyperthermia with radiation therapy and selected anticancer drugs in locally advanced human malignancies. *International Journal of Hyperthermia* **4**(2) 143-158.

Kanopoulos N, Yasanthavada N and Baker R (1988). Design of an Image Edge Detection Filter Using the Sobel Operator. *IEEE Journal of Solid State Circuits* **23** 358-367.

Kawada K, Yonei T, Ueoka H, Kiura K, Tabata M, Takigawa N, Harada M and Tanimoto M (2002). Comparison of Chemo-sensitivity Tests: Clonogenic Assay versus MTT Assay. *Acta Med Okayama* **56**(3) 129-134.

Lokesha R and Naik MK (2011), Selection of Somaclones Resistant to Alternaria Blight in Sesame (*Sesamum Indicum* L.) Through *in vitro* Cell Line Method. *Indian Journal of Fundamental and Applied Life Sciences* **1**(4) 388 -346.

Li H, Lavin MA and LeMaster RJ (1986). Fast Hough Transform: A Hierarchical Approach, Computer Vision. *Graphics and Image Processing* **36**(2-3) 139-161.

Lv F, Zhao T and Nevatia R (2002). Self-Calibration of a Camera from Video of a Walking Human. Proc. *International Conference of Pattern Recognition* **1** 562-567.

Lin CT and Lee CSG (1996). Neural Fuzzy Systems: A Neuro-Fuzzy Synergism to Intelligent Systems, Upper Saddle River, (NJ: Prentice Hall).

Maximilian N, Ismat N and Claus B (2007). Counting Colonies of Clonogenic Assay by Using Densitometric Software. *Radiation Oncology* **2** 2-4.

Moroz P, Jones SK and Gray BN (2001). Status of hyperthermia in the treatment of advanced liver cancer. *Journal of Surgery Oncology* **77**(4) 259-269.

Overgaard J (1989). The current and potential role of hyperthermia in radiotherapy. *International Journal of Radiation Oncology Biology Physics* **16**(3) 535-549.

Overgaard J, Gonzalez GD, Hulshof MC, Arcangeli G, Dahl O, Mella O and Bentzen SM (1996). Hyperthermia as an adjuvant to radiation therapy of recurrent or metastatic malignant melanoma. A multicentre randomized trial by the European Society for Hyperthermic Oncology. *International Journal of Hyperthermia* **12** 3-20.

Rau JY and Chen LC (2003). Fast Straight Lines Detection Using Hough Transform with Principal Axis Analysis. *Aerial Survey and Telemetering Measurement Academy Periodical* **8** 15-34.

Rodrigues L, Leandro DB and Marcos LG (2002). A Locally Adaptive Edge-preserving Algorithm for Image Interpolation. *In Proc. IEEE International Conference of Computer Graphics and Image Processing* 300-305.

Seong J, Lee HS, Han KH, Chon CY, Suh CO and Kim GE (1994). Combined treatment of radiotherapy and hyperthermia for unresectable hepatocellular carcinoma. *Yonsei Med Journal* **35** 252-259.

Valdagni R (1990). International Consensus Meeting on Hyperthermia: Final report. *International Journal of Hyperthermia* **6** 839-877 and 879-880.

Zhan ZY and Lai SP (2005). The Fundamental Statistic Application with Excel. *Publication of National Library*.

Zhang WC. Human International Collaborative Project on Micronucleus Frequencies in Human Populations.