Automatic Recognition of Light-Microscope Pollen Images.

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Abstract

This paper is a progress report on a project aimed at the realization of a low-cost, automatic, trainable system "AutoStage" for recognition and counting of pollen. Previous work on image feature selection and classification has been extended by design and integration of an XY stage to allow slides to be scanned, an auto-focus system, and segmentation software. The results of a series of classification tests are reported, and verified by comparison with classification performance by expert palynologists. A number of technical issues are addressed, including pollen slide preparation and slide sampling protocols.

Keywords: pollen recognition, image processing, classification, microscopy.

1 Introduction

Fossil pollen analysis is used to determine flora genus from which climate data, evidence of human activity and oil deposit locations, can be deduced. Honey type, and location of origin, can be indicated by the pollens found in the honey. Allergy sufferers can be advised of high pollen counts in the air. Forensic investigations can be aided by determining if an object has been in a certain general location by identifying the pollen types attached.

The need for an automated pollen counting system has been identified and detailed for many years [1]. A previous paper reported on progress toward such a system [2] and a significant milestone in that project is reached, and reported here, with the complete system designed, built and evaluated as a functioning unit.

The system will:

- reduce the massive amount of laborious counting required by highly skilled people involved in palynological endeavours (30 months in a PhD);
- increase sample quantities allowing more accurate pollen studies, especially in fine resolution sampling [3];
- increase the frequency and locations of pollen counts, which are of use to inhalant allergy and asthma sufferers.

A good description of the problems involved and requirements of a complete automated system have been described recently [4, 5]. The broad requirements are to locate pollens on a microscope slide and classify each into taxonomic categories at reasonable cost, and with a success rate at least that of a skilled person. The saving is labour, and time consumed by people with skills that could be better applied to less mundane tasks.

The steps involved in the AutoStage project are:

1. develop a set of features derived from optical images of pollen that are discriminable. [6]
2. develop a supervised classification system based on the features-set developed in step 1.
3. design a suitable low cost digital microscope [7]
4. develop an image segmentation scheme to isolate images of pollen and exclude detritus
5. develop and build an XY stage to allow slides to be scanned using transmitted or reflected light
6. develop a system to find the location of pollen on a slide and to capture in-focus images
7. integrate the system resulting from steps 1-6
8. evaluate and verify classification and count performance of the system, and compare to trained palynologists.

Steps 1-3 were completed [2]. This project is to develop and build a working microscope, build in an XY stage and focus hardware, develop working segmentation and focus algorithms: steps 4-8. We report development of the final stages and describe the completed system that takes a prepared slide and captures microscopic images from which

Figure 1: AutoStage

pollen are segmented, image features extracted and pollen taxa classified and counted.

2 Automated System Description

The system described here finds pollen grains on a slide and captures images of them together with their location information. Image features are extracted and used for classification of pollen types, enabling a count of the number of grains of each pollen type. The classification of pollen can be manually checked.

Selection of any portion of a slide to be processed is accomplished by the user moving the camera to opposite corners of a rectangular area of interest. The current system is capable of capturing areas shaped with a pixel resolution of $^1/_2$ micron.

The system comprises:

1. a machine to capture the images (§2.1)
2. segmentation, auto-focus and classification algorithms (§2.2)
3. a computer to run the algorithms and control the hardware (§2.3)

In addition to the sub-systems, slide preparation (§2.4) and slide sampling (§2.5) are discussed.

2.1 The Machine

The 'machine', is an XY stage with attached slide holder. Two digital microscopes are solidly mounted above a filtered and cooled light source. As transmission lighting is used, the slide sits on an aperture in the XY stage positioned between the cameras and light source as in Figure 2.

There are two power supplies for lighting and stepper motors. Two motors move the XY stage to locate pollen under the microscope and a third motor adjusts the relative height of the cameras for focussing.

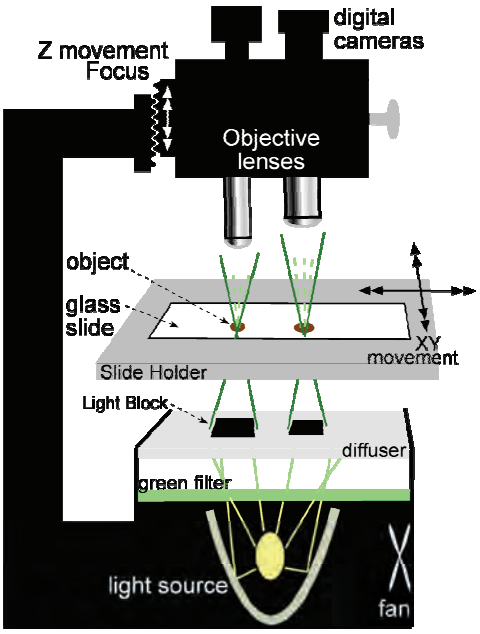

Figure 2: AutoStage elements

2.1.1 The Stage

The slide is held in a standard microscope holder and is moved by a commercial XY precision stage driven by two stepper motors. The motors are micro-stepped to $1/10^{th}$ of their 1.8º step angle, allowing a linear movement of 2.6 microns per step (the smallest pollen of interest is about 10 microns across). The field of view of the high magnification camera is 165 x 123 steps. The speed of movement is set below maximum to about 5mm per second.

2.1.2 Two Microscopes

A low magnification microscope with a large field of view (FOV), locates pollen grains quickly while a high magnification microscope captures images with sufficient detail for feature extraction.

A digital camera sensor and a standard microscope objective lens placed 207mm from the camera sensor plane, forms the "high magnification" microscope with an optical magnification of 11·2x. Because the camera sensor elements are 4.65 microns square, the magnification that is required for a human to view the formed image occurs in translation from a 1024x768 pixels in the 6mm diagonal rectangle of the sensor, to 1024x768 pixels on a computer screen. That is about 72x, and 720x including optical magnification.

The small *optical* magnification results in a depth of field greater than for a conventional microscope with the same overall magnification.

The FOV of the main camera is less than half a millimetre square. To image an entire slide more quickly, the low magnification camera with about $1/10^{th}$ the magnification, is used to more quickly cover the slide and locate potential pollen grains. A segmentation algorithm identifies most detritus and the locations of remaining objects found are stored for the high magnification camera to investigate. Segmentation, using the high magnification camera and finding an acceptable object, produces an image slightly larger than the object bounding rectangle. The image is stored for feature extraction and classification (Figure 3).

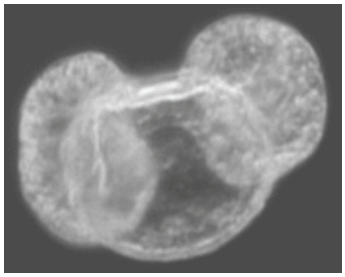

Figure 3: hi-mag segmented image (*Pinus radiata*. ~50µm)

2.1.3 The Lighting

Lighting is provided by a simple arrangement of a quartz halogen lamp directly below the cameras, with filtering, and a fan for cooling. One filter is a band-pass to reduce any chromatic aberrations caused by the objective lens. A green filter was chosen

because the camera is filtered to have a maximum sensitivity in the same area of the spectrum as human vision, $\lambda \approx 550$nm: green.

A diffusion filter is the topmost filter and has a light blocking rectangle below each camera. The diffused light therefore strikes the object oblique to the optical axis, making it a simple form of "dark field" illumination. Little of the light direct from the source enters the objective lens directly so the background is dark and objects are light with darker 'shadows' formed by the surface features. Contrast is increased over light-field transmission microscopy with one study measuring an increase from 10% to 85% contrast [8]. Sub-resolution visualisation is another property of dark-field illumination [9]. This is where objects smaller than the resolution of the optical system are indicated, but not resolved. That this has a positive or negative effect on image features extracted in this case would require further study.

The dark-field effects are helpful for finding pollen in the low magnification camera and creating a better image for feature extraction.

2.2 The Algorithms

2.2.1 Auto-Focus

The low magnification camera is initially focussed manually at the same time the user is setting the limits for a region of interest within the total area of the slide. The auto-focus software then steps the camera through that manually set focus position, to refocus. The auto-focus operates by calculating the standard deviation of all grey levels of each image as it steps through the focal plane. The sequential values are stored as a vector and a suitable peak is located by a "local maximum" algorithm. The camera is moved back to the step where the local maximum was found. Movements of critical placement are always in the upward direction. This focus position is then used for all images taken with the low magnification camera as a high depth of field keeps pollen sufficiently in focus. There are several focus measurement methods in the literature [10-13]. After experimentation, the standard deviation function was chosen for the low magnification microscope as it has a desired smoothing effect and it is not computationally demanding.

The high magnification camera is fixed on the same focus movement so once the low magnification camera is focussed, the high magnification camera can be moved to a near focus position. This position is used to perform an automatic refocus. Auto-focusing is performed on each object because the pollen grains are not necessarily all within the same focal plane and depth of field is less for this microscope.

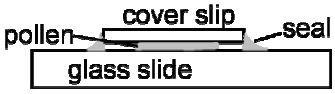

Figure 4: glass slide with cover slip

The auto-focussing algorithm used with the high magnification camera incorporates a squared gradient measure where for each pixel, the maximum grey-scale gradient-squared, between y direction and x direction is chosen and all chosen values summed.

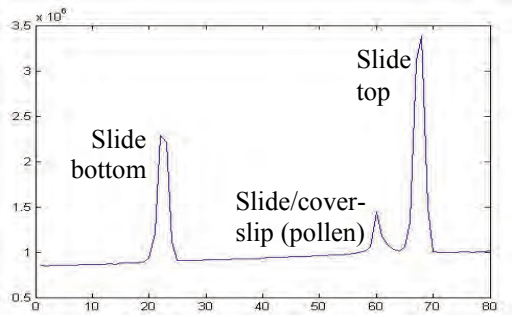

Figure 5: plot of focus image against gradient with a dirty slide giving greater focus values at the outer surfaces. Centre peak is the focus aim.

The values plotted against focus step number, results in a large 'spike' in value for 3 or 4 steps of the focus movement. To improve the auto-focus, the step size would need to be made smaller and an algorithm with greater selectivity might then be used. To reduce computation time and help ensure the object of interest is in focus, the image area is reduced to around the centre of the image where the object may be located.

It takes 15s for one complete pollen grain capture: move stage; auto-focus; capture; segmentation, save image. Auto-focus takes $^2/_3$ of that time at 10s.

2.2.2 Segmentation

Segmentation is difficult and often problem specific. For a review on segmentation techniques see [14].

A stored background image, taken with no slide in place, is subtracted from images captured to remove any image anomalies caused by the system. Objects are located by first finding edges using a Sobel edge operator. As pollen are small objects with well defined outlines, then the edge detection results in a mostly closed loop. Morphological operations follow: dilation, to join any broken edges; filling any closed loops to form solid 'blobs'. Erosion then reduces the blob size to be close to that of the original object.

The blob pixel counts are measured, and any blobs too small or too large to be a pollen grain are removed. The smallest pollen grains of interest (about 10 microns across) have a blob area of 5 pixels in an image from the low magnification camera. Large pollen grains, 100 microns across, are represented by a blob area of about 500 pixels.

For each blob of correct size, a bounding rectangle and its area are calculated. If the rectangle has an aspect ratio too small, or the blob area to rectangle area ratio is too small, then the blob is removed.

The area of a convex hull for each blob is calculated and if the blob area to hull area ratio is too small, the object is removed.

The centres of remaining blobs are found and their positions on the slide calculated and stored. The high magnification camera is moved to each of those positions and performs a segmentation process to find a valid object nearest the centre of the image. Tolerances in movements cause the object to appear with a variable offset.

2.2.3 Classification

To perform taxonomic classification, image features extraction and a multi-layer perceptron [15] are used in line with [16]. The features used are those identified in [17] consisting of 43 shape and texture features.

Texture features are represented by a series of Wavelet transforms that measure localised spatial/spatial-frequency content using Gabor and Orthogonal Wavelet transforms. Orientation sensitivity is reduced by averaging the results corresponding to different directions [6]. Other textural features used are Grey Level Co-occurrence Matrix, and Grey Gradient Co-occurrence Matrix. Shape features are geometric, histogram and second moment.

Linear Discriminant Analysis, together with Principal Components Analysis, were employed to compare discrimination and check for any redundant features [18]. No reduction of feature-set size was found useful. A Support Vector Machine algorithm, with its binary classification capability, was used to discriminate two grass pollens and found to be less effective than the multi-layer Perceptron.

2.3 The Computer

The computer used is a PC with a 2.6GHz processor and 1Gbytes of RAM running Windows XP professional. All the code is written in Matlab including: image acquisition via USB and IEEE1394 (FireWire); control of the stepper motors via a serial port; and the auto-focus, segmentation, and classification algorithms.

2.4 Slide Preparation

To improve the efficacy of the system the slides should be prepared in a prescribed and suitable manner. It is important this should be similar to current practice.

Auto-focus can be adversely affected by objects on surfaces other than the top of the slide and the bottom of the cover-slip. The segmentation algorithms could be compromised and images captured would be degraded if dust or oil were present, even if they were out-of-focus.

The prescription proposed is for the pollen samples to be suspended in some setting gel. Silicon oil is suitable and may be desirable if the slides are to be checked on a conventional microscope, as are agar or glycerol if an aqueous medium is required. The suspension should have a concentration that results in no more than 500 pollen grains per slide to reduce clumping. The sample medium volume and viscosity is such that when dropped onto the slide and the cover slip is placed on top, the medium does not travel past the outer edges of the cover slip.

The slide is placed on a warmer to allow air bubbles to escape the gel. Wax is dropped onto the slide at the edge of the cover slip to 'wick' under the cover slip to seal the pollen suspension in, and hold the cover slip firmly in place. The slide surfaces can now be cleaned without moving the pollen grains within the slide. Adding detergent to a last rinse will help reduce clumping.

2.5 Spatial Sampling of Slides

If sampling the slide is applicable, the high magnification camera only might be utilised. It may perform sampling better than in the current methods of manual counting.

It is proposed that the area of interest of the slide be divided up into rectangles, a sample of those rectangles randomly selected, and that the camera capture an image of each selected rectangle. The images would be segmented, classified and counted for each rectangular sample. A statistical analysis would estimate the slide populations of each pollen type.

By running trials on slides with known populations, a suitable sample size could be calculated.

This should prove a better method than the present manual methods, as the randomness of the present slide sampling approach is suspect [19].

3 Experiments and Results

Three image data bases were compiled:

1. CM: captured using a conventional microscope
2. AS: captured using AutoStage
3. BR: images used by France et al. [4]

A selection of the data base images was made of 50% for training, 25% for validation and 25% for the final tests reported here. The validation set was used with the training set to adjust neural net parameters for optimum results and verify the system working. The training and validation sets were then combined for training and the test set used for the final test. The feature sets extracted from the images, were presented

in random order to the classification software. Results are expressed as total correctly classified pollens as a percentage of all pollens, and the means and standard deviations over 5 tests recorded.

3.1 Compare AS with CM

The aim of this experiment is to compare classification results using images taken from the same slides by AutoStage and by a conventional microscope.

Test description: Take 40 training, 10 test and 7 types of images from AS and CM data bases. Classify both sets and compare mean results and check for difference with a Students t test.

Results: The AS mean was **98%** correct (sd = 1.2) and the CM mean was **94%** correct (sd = 0.6). Using a 95% confidence t-test, the means are significantly different.

3.2 Classification of Grass Pollens

The aim of this experiment is to check performance of the AutoStage when classifying grass pollens which are commonly counted as one type as they are very difficult to distinguish manually under a light microscope.

Test description: take 3 grass pollen image sets from the AS data base, using 150 training and 50 test images. Classify the sets.

Results: Mean = **90%** correct (sd = 0.3).

3.3 Large Pollen Type Count

The aim of this experiment is to check the performance of the AutoStage using a wider range of pollen types in a single test.

Test description: 19 types were used for the experiment including all types available, however 2 of the 3 grass pollens were excluded. 150 training and 50 test images were used.

Results: Mean = **89%** correct (sd = 0.5).

3.4 AS Compared With another Project

The aim of this experiment is to compare AS classification results, to results recorded by France et al [4].

Test description: France, recorded results using 3 pollen types with 60/60/84 images made available on the internet. Here, 45 of each set of these images were used for training and 15 images for testing. Validation was not done as the neural network configuration and weights were not altered from other tests.

Results: France achieved overall **82%** correctly identified in the final classification stage with 3% being misclassified and 15% being rejected. The AS was, on average, **95%** successful in distinguishing 15 of the same images with 5% misclassification.

3.5 AS Compared with Experts

The aim of this experiment is to compare the total process of pollen counting from a slide by the AutoStage, with the count of the same slide by experts.

Test description: A slide with 6 pollen types is prepared. Five 'experts' including two professors, a post doctoral student, a technician working in palynology and an honours student, count the slide. The AutoStage then counts the slide.

Result. The table below shows statistics of the human count and one AutoStage count.

Pollen type	5 People			AutoStage
	Mean	StdDev	Range	Raw Count
1	65.6	13.4	43 - 77	64
2	14.2	4.8	9 - 20	13
3	21.8	8.7	16 - 37	18
4	86	17.9	58 - 102	75
5	0.8	0.4	0 - 1	1
6	8.6	1.5	7 - 11	7

Table 1: The performance of AutoStage was compared to five human experts.

4 Conclusions

1. Most importantly, for a *complete working system* and functional test described in §3.5, AutoStage has matched the result of experts. The *variability* of AutoStage has yet to be determined with multiple counts by AutoStage on more slides and a comprehensive statistical analysis.
2. The AutoStage system is giving classification results improved upon known published results.
3. The system is completed, functions well with promises of the ability to meet the requirements to be useful to a palynologist.
4. Images from the AutoStage used for classification performed better than images from a conventional microscope.
5. The lighting system described gives images of excellent contrast.
6. The auto-focus system performs well. The digital microscope, having a greater depth of field than a conventional microscope, makes focussing less critical.
7. The XY stage, with movement limits larger than a slide, a repeatability of position of 20 microns, speed in excess of 10mm per second, and a spatial resolution of 2.6 microns, would be satisfactory for a manufactured product.
8. The component costs of the prototype system were under $NZ15,000 including the computer.

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