Scanning Microscopy

Volume 5 | Number 2 Article 14

6-13-1991

A Technique for Correlative Scanning and Transmission Electron Microscopy of Individual Human Placental Villi: An Example Demonstrating Syncytial Sprouts in Early Gestation

G. J. Burton
University of Cambridge

K. W. Thurley
University of Cambridge

J. N. Skepper
University of Cambridge

Scanning Microscopy, Vol. 5, No. 2, 1991 (Pages 451-459)
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

0891-7035/91$3.00+.00

A TECHNIQUE FOR CORRELATIVE SCANNING AND TRANSMISSION ELECTRON MICROSCOPY OF INDIVIDUAL HUMAN PLACENTAL VILLI: AN EXAMPLE DEMONSTRATING SYNCYTIAL SPROUTS IN EARLY GESTATION

G.J. Burton*, K.W. Thurley and J.N. Skepper

Department of Anatomy, University of Cambridge,
Downing Street, Cambridge, CB2 3DY, U.K.

(Received for publication February 13, 1991, and in revised form June 13, 1991)

Abstract

Correlating the surface appearances of certain features with their internal structure is made particularly difficult in the human placenta by the complex three-dimensional branching pattern of the villous tree. This places a possible limitation on the use of the scanning electron microscope in this field, both for basic research purposes and as a tool in pathological diagnosis. To help overcome this problem, a technique for handling individual placental villi has been devised. By attaching single villi to glass pipette tips it has proved possible to scan the villi, embed them in resin and then section them in a known pre-determined orientation. Exact correlations between the surface appearances and the internal structure, as seen with either the light or transmission electron microscope, can then be drawn. This paper describes the technique and, using an example based on syncytial sprouts in early pregnancy, illustrates the precision afforded by the method.

Key Words: human placenta, scanning electron microscopy, transmission electron microscopy, syncytiotrophoblast, correlative microscopy.

*Address for Correspondence:
Department of Anatomy, University of Cambridge,
Downing Street, Cambridge, CB2 3DY, U.K.

Phone No: 0223 333785

Introduction

Many surface features of the human placental villous tree have been described and illustrated using scanning electron microscopy (SEM), since its application to this field during the late 1960s, (eg. Dempsey and Luse, 1971; Fox and Agrafojo-Blanco, 1974; King and Menton, 1975; Clint et al., 1979; Burton, 1987; Ockleford et al., 1989). Correlating these surface appearances with the cross-sectional profiles traditionally seen by light (LM) and transmission electron microscopy (TEM) is not always easy, however, for the placental villous tree displays a highly complex three-dimensional branching pattern. Consequently, a single histological section through a group of villi will create a wide variety of villous profiles, some villi having been cut in perfect cross-section, others very obliquely but the majority at various intermediate angles (Küstermann, 1981; Burton, 1986; Cantle et al., 1987; Kaufmann et al., 1987). As a result, the internal structure or histological nature of certain surface features remains in doubt, and this limits the potential use of SEM, both for basic research purposes and for any possible role in pathological diagnosis.

In order to rectify this situation a technique has been developed which enables individual villi to be examined by SEM and then embedded in resin, sectioned in a known plane and viewed by TEM. Exact correlations between surface morphology and internal structure are therefore possible. Using an example based on syncytial sprouts during early pregnancy, this paper illustrates the precision of the technique and demonstrates the quality of the images that may be obtained through its use.

Materials and Methods

Fixation

For this example, human placental villi, 8-12 weeks gestational age, were obtained from therapeutic terminations of pregnancy. Small pieces of tissue were fixed by immersion in 2% glutaraldehyde, 2% paraformaldehyde in 0.1 M Pipes buffer made isotonic with sucrose (pH 7.2, vehicle osmolality 310 $mOsm.kg^{-1}$, total osmolality 1150 $mOsm,kg^{-1}$) for 4 hours at room

temperature. Following post-fixation in 1% osmium tetroxide for 1.5 hours, the tissue blocks were dehydrated in ascending concentrations of acetone, and critical-point dried. For ease of handling, they were then mounted on to stubs with conductive paint.

Selection of Villi

The technique allows individual villi to be selected in one of two ways (Fig. 1):-

Method A: Pre-SEM viewing. By viewing the mounted blocks under a binocular dissecting microscope, a villus with an area of potential interest, such as the syncytial sprouts featured in this paper, can be identified. The villus is then removed from the block as follows.

The tip of a glass pasteur pipette is drawn out in a flame so that it possesses a relatively short segment (2-3 cms) approximately 20 µm or less in diameter. The pipette is attached to an X-Y-Z- micromanipulator and, after coating with a small quantity of rapid-setting epoxy adhesive, the tip is brought into contact with the base of the selected villus. Sufficient adhesive is required to cause adequate wetting of the villous surface, but excess results in migration of the glue up the villus and potentially over the area of interest. After the adhesive has set the villus is then removed from the specimen by traction with the micromanipulators or by crushing the villus stem below the pipette with watch-maker's forceps.

In order to be able to view the selected villus in the scanning electron microscope, a small block of epoxy resin is glued eccentrically on to a stub. After coating the upper surface of the block with rapid-setting epoxy adhesive the pipette is lain across it, again under micromanipulator control, so that the tip, with attached villus, projects 0.5 - 1.0 cm (Fig. 2a). When the adhesive has set the proximal end of the pipette is broken away, and the stub coated with 30 nm of gold, in this example with a Polaron Cool Sputter Coater.

Our villus was then viewed in a JEOL JSM 35CF SEM microscope and features of interest photographed. Particular attention should be paid to photographing the entire villus at low magnifications so that the features can be located with respect to major landmarks such as villous branching points. Stereo-pairs may be a helpful adjunct, for these records play a key role at later stages when orientating the specimen to create the most advantageous plane of sectioning.

Method B:- Post-SEM viewing. Alternatively, villi may be selected after initial viewing in the scanning electron microscope. Pieces of tissue are fixed and prepared as before, and mounted on to stubs with conductive paint. After coating they are viewed in the microscope and if features of interest are noted during routine scanning, these may be recorded photographically. The villi can then be removed from the sample by the technique described above. They may proceed immediately to the embedding procedure, or if further viewing is considered necessary they can be re-scanned after mounting on to a stub. A second viewing may be useful if previously hidden parts of a villus are revealed after removal, or if a different orientation is preferred. However, it does involve a second coating of gold, which may make the subsequent sectioning more difficult and possibly cause "decoration" artefacts.

Embedding of villi

After viewing, the villi are removed from the stubs and immersed in 100% acetone. To achieve this, the tip of a second pipette is glued perpendicular to the first, close to the point where it is attached to the epoxy block (Fig. 2b). The first pipette is then cracked close to the block, leaving the villus and a short section of the tip of the original pipette attached to the second pipette.

The shaft of the second pipette may then be pushed through a hole made in the polyethylene cap of a $5cm^3$ vial, so that the villus is suspended submerged in the vial's contents (Fig. 2c). By removing the cap and pipette together the vial's contents can be changed, without the villus coming into contact with the glass walls.

In this example the villus was embedded in Taab resin, using a slightly protracted schedule to ensure adequate infiltration. The pipette tip was only broken off during the final stage of embedding into moulds, and the fragment remaining attached to the villus could be used for orientation purposes.

Sectioning

Sections for light microscopy could be cut adequately on glass knives, and stained with toluidene blue/Azure II. For transmission electron microscopy, thin sections (less than 40 nm) were cut using a diamond knife, and were placed on 2 x 1 mm slotted grids, and supported by a Pioloform film using the technique of Wells (1974) as modified by A. Hockerday, Physiological Laboratory, Cambridge. After staining with uranyl acetate and lead citrate the sections were viewed in a Philips EM 300.

For both light and electron microscopy the optimal plane of sectioning could be selected by orientating the resin block or chuck according to either reference features seen on the villus through the block face or the remains of the pipette tip.

Results

The example described here concerns syncytial sprouts, which are one of the most conspicuous features of the placenta during early pregnancy. Syncytial sprouts display a wide variety of forms, ranging from small tags closely associated with the villous surface to the more elaborate structures illustrated in Fig. 3. These commonly possess an expanded distal end supported by a thin neck region, which suddenly increases in diameter close to the point where it is attached to the parent villus. Since syncytial sprouts of the first trimester are generally believed to represent developing villi, these different forms almost certainly reflect various stages in this process. This example illustrates the internal structure of these sprouts in order to determine the validity of this assumption.

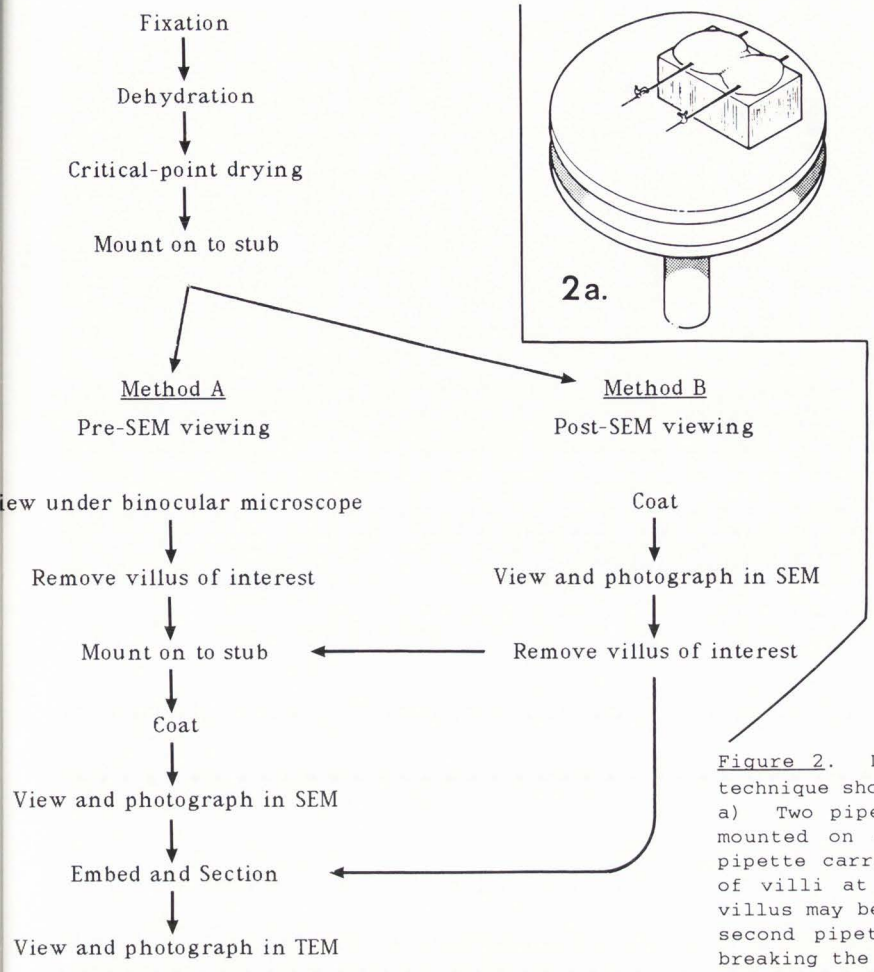


Figure 1. Flow diagram summarising the stages in the technique.

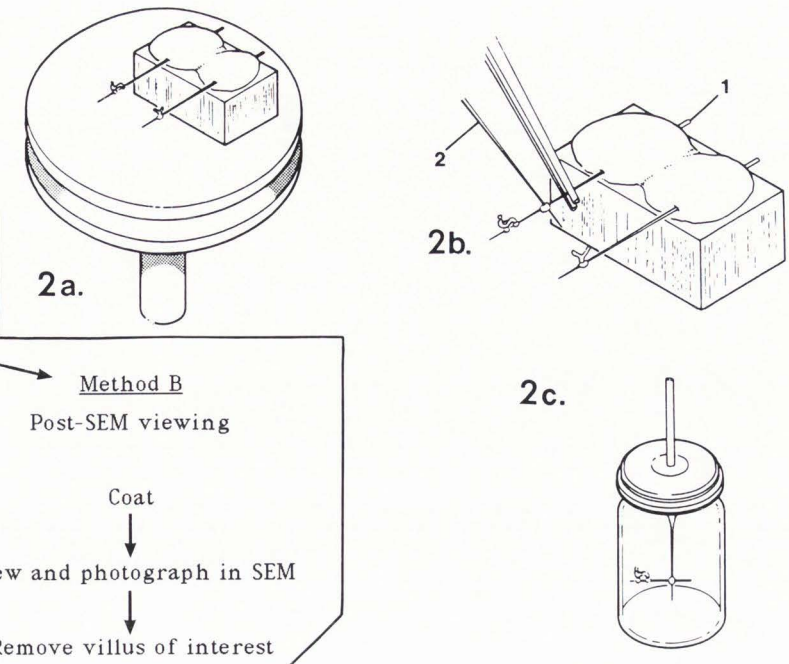


Figure 2. Diagrammatic representation of the technique showing:-
a) Two pipettes attached to a block of resin mounted on a stub ready for viewing. Each pipette carries a single villus or small group of villi at its tip. b) After viewing, the villus may be removed for embedding by glueing a second pipette (2) on to the first (1), and breaking the latter away from the stub. c) The second pipette may be pushed though the cap of a dram vial for ease of handling during embedding of the villus.

The Parent Villus

The parent villus was selected for sectioning since it bore a cluster of sprouts at its tip displaying a variety of forms, as illustrated by Figs 3 and 4. These micrographs were taken at different angles in order to demonstrate the morphology of each sprout clearly. The largest sprout (S1) consisted of a flattened expanded head, a narrow neck and a lengthy shoulder region. Another sprout (S2) appeared broken at the distal end, and consisted only of a neck and shoulder region. Two further sprouts (S3 and S4) possessed more globular heads attached to neck regions which suddenly increased in diameter to form the shoulders. Finally, the smallest sprout available for sectioning (S5) was a paddle-shaped tag arising from the shoulder region of S2. A plane of section was chosen which cut the neck regions of the major sprouts as close to transverse as possible.

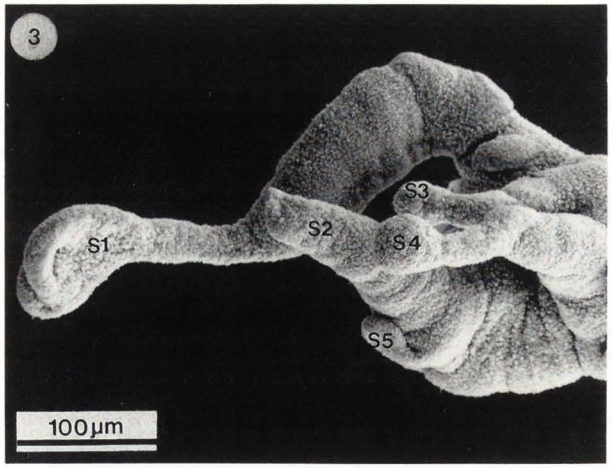
Three sections have been chosen for this example, since they display the characteristic features of the sprouts at various points along their length. Intermediate sections were viewed but are not illustrated here.

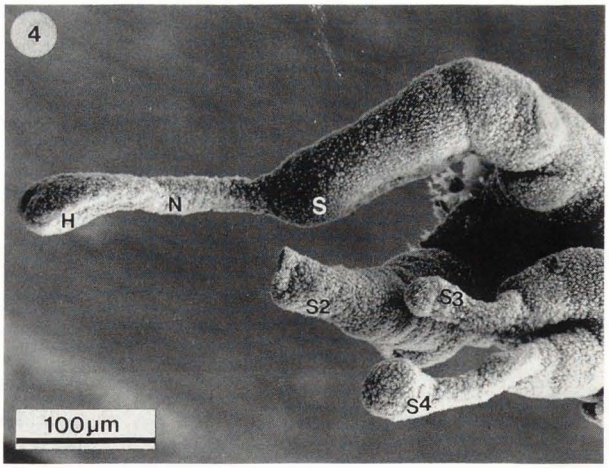
Section 1

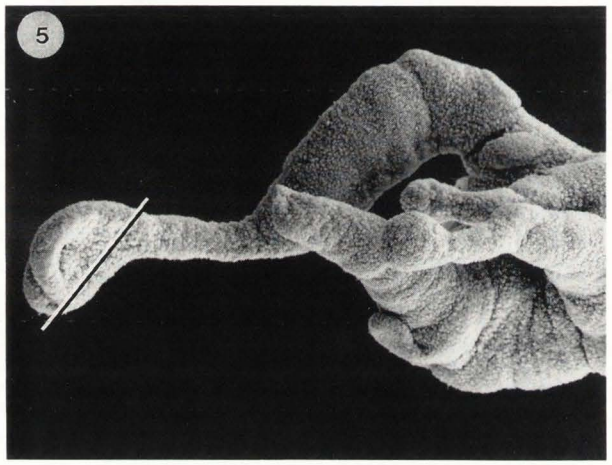
This section passed through the expanded head of the largest sprout S1 (Fig. 5). In the SEM the head could be seen to possess a dense microvillous covering, which displayed the cobblestone arrangement typical of early pregnancy (Fig. 6). This pattern was also evident in the TEM, where it was clear that the underlying villous surface was not smooth but rather possessed a number of short projections at intervals over its extent (Fig. 7). Microvilli arose from all aspects of these projections as well as from the intervening villous surface. At higher magnification, it was confirmed that the head consisted of syncytiotrophoblast alone, and many irregularly-shaped nuclei could easily be discerned, along with osmiophilic vacuoles (Fig. 8). The syncytioplasm contained the normal range of organelles and no overt specialization could be detected.

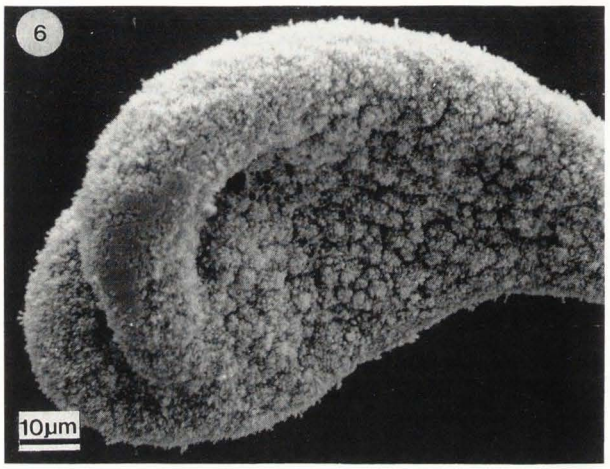
Section 2

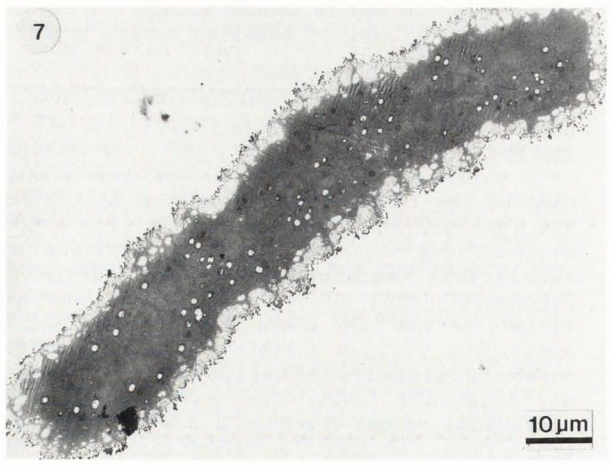
The level of the second section is illustrated in Fig. 9. It passed through the











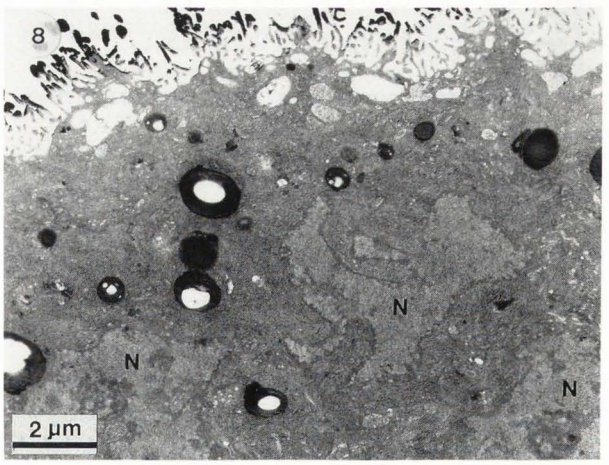
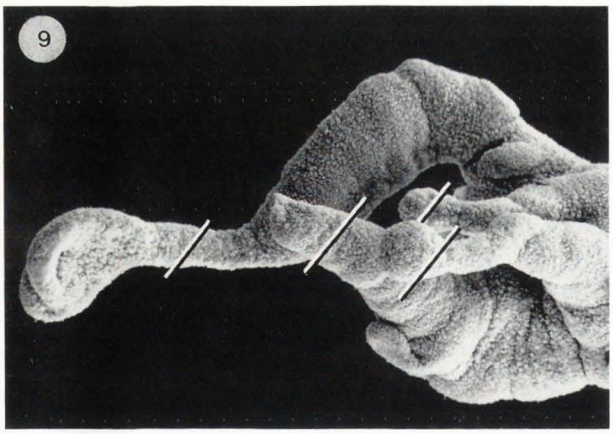
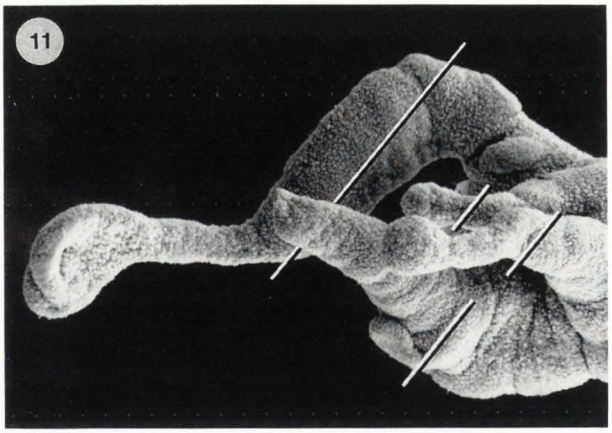


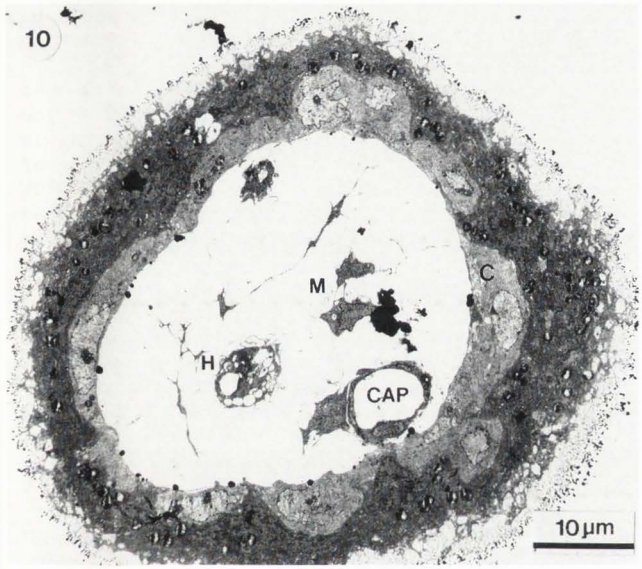
Figure 3. Low magnification view of the parent villus demonstrating the presence of five villous sprouts (S1, S2, S3, S4, S5) in various stages of development.

Figure 4. The same group of villous sprouts photographed from a different angle. Note the clear demarcation of S1 into an expanded head region (H), a more slender neck region (N) and a much larger shoulder region (S). Similar regions can be distinguished in S3 and S4. Note that the tip of S2 appears damaged, suggesting that it was broken in the neck region.

Figure 5. Illustration showing the level of the first section, passing through the head region of S1.







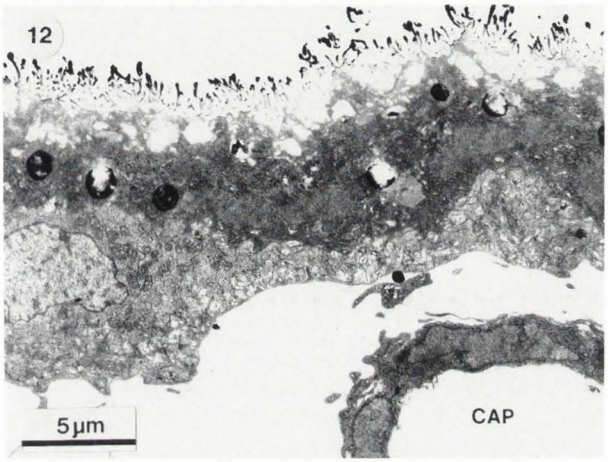


Figure 6. Higher magnification scanning electron micrograph of the head of S1 showing the cobblestone arrangement of the microvillous covering. Note that this pattern continues over the neck region.

Figure 7. Low magnification correlative transmission electron micrograph view of the head, confirming that it is composed of syncytiotrophoblast only. Note how the villous surface is irregular, giving rise to the appearances in Figure 6, and that despite the use of a sputter coater only the tips of the microvilli appear covered with gold. This is borne out by the problem of specimen charging often experienced in SEM viewing of placental villi.

Figure 8. Higher magnification of the above section showing the presence of several syncytial nuclei (N) and numerous osmiophilic vacuoles, which most likely represent lipid droplets.

Figure 9. Illustration showing the level of the second section, passing through the neck region of S1, the shoulder region of S2, and the heads of S3 and S4.

Figure 10. Thin section through the shoulder region of S2 showing the presence of a stromal core containing a capillary profile (CAP), a Hofbauer cell (H) and mesenchymal cells (M). Note the almost complete layer of cytotrophoblast cells (C) lying deep to the syncytiotrophoblast, and the presence of numerous electron-dense inclusions within or just external to the trophoblastic basement membrane (Jones and Fox, 1991).

Figure 11. Illustration showing the level of the third section passing through the shoulder regions of S1 and S4, the neck region of S3 and the origin of S5.

Figure 12. Thin section through the shoulder region of S1 demonstrating the state of preservation of the syncytiotrophoblast and underlying cytotrophoblast cells. CAP, capillary.

neck region of S1, and this too consisted only of syncytiotrophoblast. The syncytial nuclei appeared densely-packed, and displayed a similar chromatin pattern to those in the head of the sprout. Osmiophilic vacuoles were still present and were distributed throughout the syncytioplasm.

The section also passed through the broader shoulder region of the broken sprout S2. This had all the internal features of an early villus, for it consisted of an outer covering of syncytiotrophoblast beneath which was a complete layer of cytotrophoblast cells and in the centre a stromal core (Fig. 10). Within the core, there was a single capillary profile with accompanying pericyte, along with a Hofbauer cell and mesenchymal cells. All of these components were well preserved, and the usual complement of cytoplasmic organelles could be distinguished.

Finally, the section passed through the tips of S3 and S4. These both displayed an identical structure to that of S1, consisting of numerous densely-packed syncytial nuclei.

Section 3

The level of the third section is illustrated in Fig. 11. It now passed through the shoulder region of S1, which again consisted of a stromal core similar to that depicted in Fig. 10, although several capillary profiles were present in this case. The syncytiotrophoblast contained many osmiophilic vacuoles and the nuclei appeared identical to those seen in the previous sections (Fig. 12).

The section also passed through the neck region of S3, and the shoulder region of S4, confirming the presence of a stromal core at this point too.

The main feature of interest, however, and the reason for choosing this level was that the section also passed through the origin of the smallest sprout S5. In the SEM, it was clear that the sprout already seemed to be differentiating into distinct head and neck regions (Fig. 13), although in the TEM it appeared to consist simply of a small protrusion of syncytiotrophoblast (Fig. 14). No direct involvement of either cytotrophoblast cells or elements of the stromal core was visible at this level, or in adjacent sections. Nuclei, displaying a normal chromatin pattern, were already present in the sprout, along with the ubiquitous osmiophilic vacuoles (Fig. 15). The distribution of microvilli and other membrane specializations, such as coated pits, over the sprout appeared identical to that over the remainder of the villous surface.

"Decoration" artefacts

Occasionally over the villous surface "decoration" artefacts were observed where the tips of adjacent microvilli were joined together by a continuous film of gold coating (Fig. 16).

Discussion

Correlative microscopy has found many applications in biology, and varied examples are presented in Hayat (1987). The technique can not only assist in the correct interpretation of microscopic images, as illustrated by the present example, but can also be of value in selecting areas of interest for sectioning. One of the great advantages of the scanning electron microscope is that large areas of tissue can be viewed at high resolution with relative speed. Using this instrument, surface features of potential interest, whether these be, for example, the earliest syncytial sprouts, aggregations of cells on the villous surface or defects in the microvillous covering can therefore be located easily and quickly. The correlative technique described here enables these features to be subsequently sectioned for examination by TEM with complete certainty of success. This two-step procedure could be of particular value when investigating small, discrete features which occur infrequently in the placenta, for the chance of catching such a feature in a single random thin section would be very low indeed.

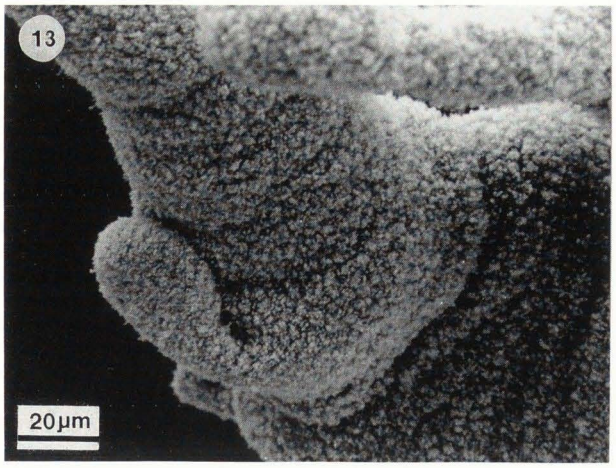
The technique

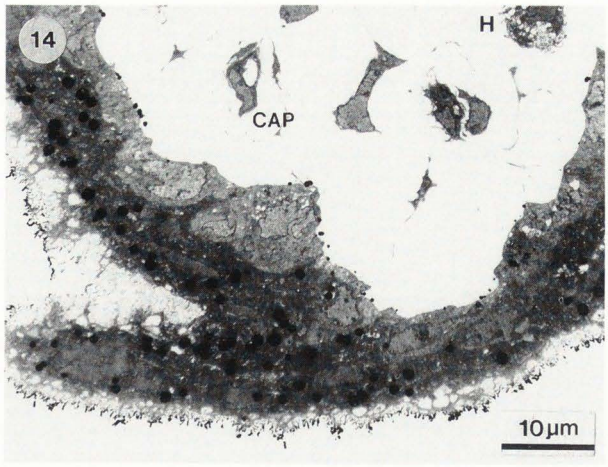
Preservation of the tissue, despite the dual electron bombardment appeared good. The most significant adverse effect noted by TEM was the greater than normal staining intensity of the syncytiotrophoblast. Rehydration to 70% alcohol/water or further prior to embedding has been advocated by some workers in an attempt to reverse the shrinkage caused by critical-point drying (eg. Laschi et al., 1987). However, this additional step did not appear to alter the staining characteristics of placental material in our hands, and was therefore not pursued. A more effective way of dealing with the problem was to reduce section thickness to a minimum by using a diamond knife. This was of particular advantage when attempting to discriminate between cytoplasmic organelles within the syncytium.

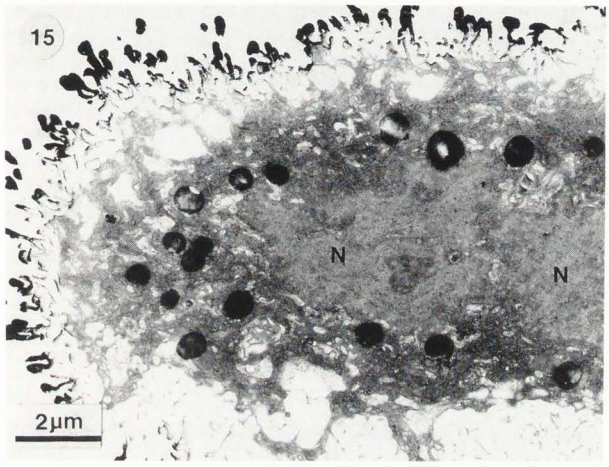
Removal of the gold coating prior to sectioning is also possible (Sela and Boyde, 1977; Dickson et al., 1989), and was again considered here. However, in practice it was found that for most purposes thin sections could be cut adequately on a glass knife, although for serial sections a diamond knife was again more successful. It was felt that removal of the gold coating may have dislodged the villus from the pipette and so was not attempted. Indeed the presence of the gold proved a useful marker in the TEM, and an examination of the extent of the covering over the microvilli provided information in itself. The discontinuity of the coating and its inability to penetrate the clefts between microvilli provided clear evidence of why specimen charging should be such a persistent problem in SEM of placental tissue. It also exposed "decoration" artefacts of coating, when the tips of individual microvilli were observed joined together by a film of gold with no apparent underlying biological structure. On the basis of SEM alone such features might be misinterpreted as areas of microvillous loss.

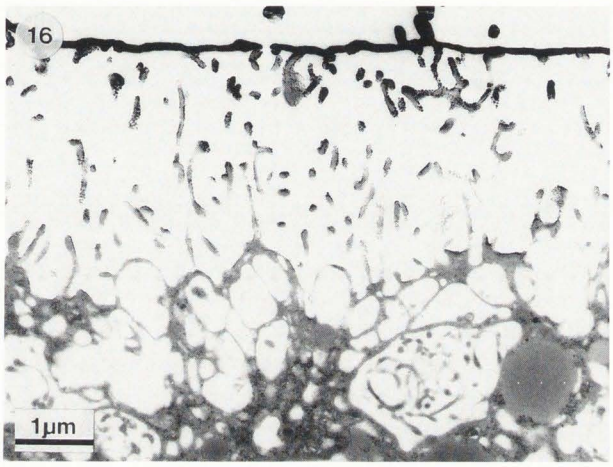
Syncytial Sprouts

Syncytial sprouts are most common in early gestation but can still occasionally be seen in the mature placenta. They are generally









considered to be indicative of new villous growth, and have been used as an index of placental well-being (eg Alvarez, 1964; Boyd and Hamilton, 1970). Although the earliest sprouts consist of trophoblast alone, in order to play a functional role in placental exchange they must be vascularised by the fetal capillaries.

The correlative data presented here indicate that both the head and neck regions of early sprouts are composed solely of syncytiotrophoblast, and that using TEM alone no distinctions can be made between the two regions. They also confirm that the point at which the neck region suddenly enlarges to form the shoulder corresponds to the limit of mesenchymal invasion. It is apparent from Figure 11 that at this stage of gestation capillary ingrowth may take place contemperaneously with mesenchymal invasion, and so the transformation of sprout to functional villus is very abrupt.

As yet the mechanism underlying sprout formation is not known. It seems likely from the illustrations presented here that the motive force must lie in the syncytiotrophoblast, although no overt specialisations are evident, for there is no stromal or capillary involvement in the earliest stages. However, the trophoblast does possess a well developed cytoskeleton, and changes in the pattern of tubulin distribution have been noted around early sprouts (Ockleford and Wakely, 1982). Hypoxia appears to be a potent stimulus inducing sprout formation, for the highest densities of

Figure 13. Higher magnification scanning electron micrograph view of the early sprout S5. Even at this stage it appears to be differentiating into head and neck regions.

Figure 14. Correlative thin section through the origin of S5, confirming that the sprout is composed of syncytiotrophoblast only and that there is no involvement of cytotrophoblast cells or elements of the stroma at this stage of its development. CAP, capillary; H, Hofbauer cell.

Figure 15. Thin section through the sprout S5, showing the presence of syncytial nuclei (N), and the normal distribution of membrane specialisations such as microvilli and coated pits.

Figure 16. Thin section demonstrating a "decoration" artefact, where the gold coating has formed a sheet over the tips of the microvilli concealing their presence.

sprouts are found in the peripheral parts of the lobule and under the chorionic plate (Alvarez et al, 1970; Sala et al, 1983). How this stimulus influences the arrangement of the cytoskeleton is not known.

It is possible, however, that two forms of sprout exist for not all are invaded by a mesenchymal core. In those that are not invaded the attachment to the parent villus becomes increasingly attenuated, until finally the sprout breaks free and enters the maternal bloodstream (Boyd and Hamilton, 1970). Why this takes place or what factors determine the fate of an early sprout are also not known. This correlative technique which enables these structures to be selected for sectioning with precision and reliability may help researchers address some of these questions.

References

Alvarez H (1964) Morphology and physiopathology of the human placenta. I. Studies of morphology and development of chorionic villi by phase-contrast microscopy. Obstet. Gynecol. 23, 813-825.

Alvarez H, Benedetti WL, Morel RL, Scavarelli M (1970) Trophoblast development gradient and its relationship to placental hemodynamics. Am. J. Obstet. Gynec. 106, 416-420.

Boyd JD, Hamilton WJ (1970) The Human placenta. Heffer, Cambridge. pp 178-182.

Burton GJ (1986) Intervillous connections in the mature human placenta: instances of syncytial fusion or section artifacts? J. Anat. 145, 13-23.

Burton GJ (1987) The fine structure of the human placental villus as revealed by scanning electron microscopy. Scanning Microsc. 1, 1811-1828.

Cantle SJ, Kaufmann P, Luckhardt M, Schweikhart G (1987) Interpretation of syncytial sprouts and bridges in the human placenta. Placenta. 8, 221-234.

Clint JM, Wakely J, Ockleford CD (1979) Differentiated regions of human placental cell surface associated with attachment of chorionic villi, phagocytosis of maternal erythrocytes and syncytiotrophoblastic repair. Proc. R. Soc. Lond. B. 204, 345-353.

Dempsey EW, Luse SA (1971) Regional specializations in the syncytial trophoblast of early human placentas. J. Anat. 108, 545-561.

Dickson GR, McKenna S, McColl K, Carr KE (1989) Examination of mucosal surfaces by scanning electron microscopy before and after removal of debris. J. Microsc. 153, 75-79.

Fox H, Agrafojo-Blanco A (1974) Scanning electron microscopy of the human placenta in normal and abnormal pregnancies. Europ. J. Obstet. Gynec. Reprod. Biol. 4, 45-50.

Hayat MA (1987) Correlative Microscopy in Biology. Instrumentation and Methods. Academic Press, Orlando. pp 437.

Jones CJP, Fox H (1991) Ultrastructure of the normal human placenta. Electron Microsc. Rev. 4, 129-178.

Kaufmann P, Luckhardt M, Schweikhart G, Cantle SJ (1987) Cross-sectional features and three-dimensional structure of placental villi. Placenta. 8, 235-247.

King BF, Menton DN (1975) Scanning electron microscopy of human placental villi from early and late in gestation. Am. J. Obstet. Gynec. 122, 824-828.

Küstermann Von W (1981) Uber "proliferationsknoten" und "syncytialbrücken" der menschlichen plazenta. (Syncytial sprouts and intervillous bridges in human placenta). Anat. Anz. 150, 144-157.

Laschi R, Pasquinelli G, Versura P (1987) Scanning electron microscopy application in clinical research. Scanning Microsc. 1, 1771-1795.

Ockleford CD, Wakely J (1982) The skeleton of the placenta. In: Progress in Anatomy. RJ Harrison & V Navaratnam, Cambridge University Press, Cambridge. Vol 2, p. 19-47.

Ockleford C, Barker C, Griffiths J, McTurk G, Fisher R, Lawler S (1989) Hydatidiform mole: an ultrastructural analysis of syncytiotrophoblast surface organization. Placenta. 10, 195-212.

Sala MA, Matheus M, Valeri V (1983) Volume density of syncytial sprouts and its regional variation in the human mature placenta. Gegenbaurs morph. Jahrb. 129, 489-493.

Sela J, Boyde A (1977) Cyanide removal of gold from SEM specimens. J. Microsc. 111, 229-231.

Wells B (1974) A convenient technique for the collection of ultra-thin serial sections. Micron. 5, 79-81.

Discussion with Reviewers

W.H. Wilborn: It is well-known that the placenta is fragile and very sensitive to different fixatives, solvents, etc. Do you believe the placenta can be prepared for SEM by critical point drying, etc., and be optimal for detailed analysis by TEM? The placenta you showed by TEM appeared somewhat condensed.

Authors: Correlative microscopy generally requires striking a compromise between those fixation techniques which yield optimum results for SEM and those which provide the minimum of tissue distortion for TEM. The technique described here is particularly useful for looking at disturbances in the normal pattern of microvillous distribution that may have a pathological basis. Critical point drying is desirable for the maintenance of normal microvillous architecture in the SEM, and so one has to accept some condensation of the tissue, particularly of the syncytiotrophoblast, as an inevitable consequence. Partial rehydration during embedding did not allieviate the problem.

B.F.King: In the Discussion you state that "hypoxia appears to be a potent stimulus inducing sprout formation, for the highest densities of sprouts are found in the peripheral parts of the lobule and under the chorionic plate". Do the authors agree, based on their

own observations, with the described regional distribution in sprouts?
Authors: The work of Alvarez et al. (1970) and Sala et al. (1983) describing this distribution was caried out on term placentae which possess well defined lobules. The material examined in the present study came from early gestation, before the lobules are formed, and so we cannot comment on this point.

P. Kaufmann: In the discussion the authors noted that "hypoxia appears to be a potent stimulus inducing sprout formation, ..." Following our experience, it is necessary to clearly discriminate between those really sprouting sprouts described by the authors in the first trimester placenta and the "so-called sprouts" of the term placenta as described eg. by Alvarez et al. (1970). The latter type seems to represent mostly trophoblastic flat sections as described by Küstermann (1981) as well as degenerative trophoblastic protrusions (Cantle et al., 1987). Did the authors study also mature placentas and if so, did their results corroborate our findings that the "sprouts" are a very heterogeneous family of structures?
Authors: We did not examine sprouts in mature placentae, but again this is an interesting point that could be addressed in future studies.

B.F. King: Is there any experimental evidence to support the conclusion that hypoxia induces sprouting? It seems likely many other stimuli are possible in this complex developmental situation.
Authors: To our knowledge no one has successfully cultured villi long enough to observe sprouting in vitro, and so been able to test this hypothesis.

P. Kaufmann: From histological serial sections we got the impression that the villous tips just below syncytial sprouts of the first trimester are sometimes characterized by accumulations of cytotrophoblast which may be double or triple layered. It was followed by villous stroma with nearly epitheloid connective tissue cells. Features like that depicted in Fig. 10 were only typical for the next, deeper sectional plane. Did you make similar observations using your sophisticated method?
Authors: The plane of section illustrated in Fig. 10 is certainly well into the stromal core, but intermediate sections did pass through the cytotrophoblast layer. However, the purpose of the present study was to evaluate the technique, rather than to answer specific questions. The point raised is an interesting one that could be addressed using the technique, but further studies involving many more sprouts would be required.