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Human Embryology

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Additional information is available at the end of the chapter

Abstract

The study of human embryology has a very long history. Modern embryology owes its initial development to the key embryo collections that began in the 19th century. The first large collection was that of Carnegie, and this was followed later by the major 7 collections. The second role of the Carnegie collection was for researchers to establish a defined set of Carnegie stages based on embryo morphological features. Today, embryos are imaged three-dimensionally (3D) by a range of imaging modalities including, magnetic resonance microscopy (MRM), episcopic fluorescence image capture (EFIC), phase-contrast X-ray computed tomography (pCT), and optical projection tomography (OPT). Historically, embryo serial images were reconstructed using wax-plate and model techniques. The above new 3D imaging techniques now allow 3D computer reconstructions, analysis, and even 3D printing. This chapter will describe how the classical embryology collections and techniques have developed into today's imaging and analysis techniques, giving new insights to human embryonic development.

Keywords: Human Embryo, Embryo Collection, Developmental Stages, Imaging, 3D reconstruction, 3D printer

1. Introduction

Human embryology in the 19th century began by using human embryo samples derived from maternal deaths, abortion, or surgery. Nothing has been changed in the 21st century, because animal experimental biology developed in the 20th century could not and should not apply to human embryology on its ethical aspect. However, human embryology has progressed little during the last 100 years, with only recently some limited molecular studies on small numbers of human material. In contrast, recent studies using both nondestructive and destructive

imaging techniques on existing collections have allowed many morphological measurements of these embryos using these novel imaging techniques.

Here we summarize the historic collections of embryos used in the study of human development, explain the criteria used for developmental staging, show sectioned and reconstructed images from newer three-dimensional (3D) imaging in high resolution, and discuss the future directions for the analyses of the human embryo.

2. Human embryo collections

During the history of human embryology the establishment and study of key human embryo collections has greatly contributed to our current understanding. In this section we briefly summarize the history of some of these collections, such as the Carnegie Collection, the Kyoto Collection, the Blechschmidt Collection, and the Madrid Collection (Table 1). More online information can be found on existing historic human collections (http://tiny.cc/Human_Embryo_Collections). The human embryo collections shown in Table 1, along with other collections, form part of the Digital Embryology Consortium (http://human-embryology.org), formed to electronically preserve and make available for research and education these irreplaceable historic collections.

Not included in this chapter will be descriptions of the smaller, less described human embryo collections, species comparative embryo collections, or collections that are of nonembryonic material, such as placenta. An example of one of the best and largest historic comparative embryo collections is the embryological collection of the Natural History Museum in Berlin, which includes many other species in the combined collections of Hubrecht, Hill, Dohrn, Bolk, and Kückenthal. An example of a mainly human placenta and early implanted uterus is the Hamilton-Boyd Collection in Cambridge. More recently, there are smaller collections of embryos used mainly for molecular studies, such as the Human Developmental Studies Network (HuDSeN) in Newcastle and London. Note that many anatomy departments hold their own small collections of human material that are not covered here.

A key factor in understanding the developmental morphological changes is the possession of human embryo samples at sequential developmental stages. The following are the major historic collections used in most research and textbook publications that have aided our understanding of human development.

Collection	Place	Number	Characteristics	Establishment
Carnegie	Washington DC, USA	About 10,000	Human fixed specimens and histology	1887
Madrid	Madrid, Spain	100+	Human histology	1935
Blechschmidt	Göttingen, Germany	About 120	Human histology	1950s
Kyoto	Kyoto, Japan	About 44,000	Human fixed specimens and histology	1961

Table 1. Comparison among major human embryo collections

2.1. Carnegie collection

Franklin P. Mall (1862–1917) began his human embryo at Johns Hopkins University in the early 1900s; these formed the beginnings of the Carnegie collection. He and Franz Keibel (1861–1929) used these embryos in their textbook *Manual of Human Embryology* [1, 2] and also in the Carnegie Institution of Washington Series *Contributions to Embryology* beginning in 1915. Organizing some of these human embryos form the first 8 weeks into a developmental sequence formed the basis of their "23 Carnegie Stages" staging criteria (see Figure 7), described in detail later in this chapter. The same staging criteria have been subsequently applied in the organizing of the other major human embryo collections. These stages will be described in detail from the Kyoto Collection later in this chapter. Reconstructions from histological sections of the collection embryos were the basis of the larger Carnegie models (Figure 1) and this technique has also been used in the development of other collection models, as in the Blechschmidt Collection.

Franklin P. Mall received his medical degree at the University of Michigan in 1883. He traveled to Germany to receive a clinical training, where he met the German embryologist Wilhelm His (1831–1904). This initiated his interest in studying human embryology, and he began collecting human embryos in 1887. His collection had reached several hundreds of specimens by the time he returned to the Anatomy department of the Johns Hopkins School of Medicine in Baltimore, Maryland. He received a Carnegie research grant in 1914 and became the first director of the Department of Embryology at the Carnegie Institution of Washington, in Baltimore, MD. The embryo collection grew at a rate of about 400 specimens a year, donated by clinicians and researchers, and the number of samples reached over 8,000 by the early 1940s. Researchers at the institute then began the complex task of organizing these embryos into a developmental sequence. Note that size alone was a difficult criterion due to the variable effects of fixation shrinkage. The solution was a "staging" system, developed by Mall, based instead on developmental ape embryo morphological features. Internal features were identified histologically from embryos that were serially sectioned, and also formed the basis of hundreds of 3D models and 700 wax-based reconstructions.

During Mall's era, several department members became renowned scientists. George L. Streeter (1873–1948) and Franz J. Keibel were also both former students of the important German embryologist Wilhelm His; Osborne O. Heard worked as an embryo modeler; and James D. Didusch as a scientific illustrator. Mall documented his research in a series of papers compiled in the *Contributions to Embryology* of the Carnegie Institution of Washington, published from 1915 to 1966. These articles even today are considered the core findings for studying human embryology. Mall unexpectedly died in 1917 and was replaced as director by Streeter. Streeter was then the first to define the 23 Carnegie Stages currently used to classify the developmental stages of the human embryo (see Table 2). The collection continued to grow by hundreds of specimens every year and included rare, very young normal specimens. At the time, induced abortions were illegal in the United States and miscarriages usually resulted from embryo abnormalities.

Streeter retired in 1940 and George W. Corner (1889–1981]) became the third departmental director. Corner was a former Johns Hopkins researcher who studied the menstrual cycle and

identified the ovarian hormone progesterone. During his direction until 1956, many advances in human reproductive physiology were made and embryology research continued but came to an end with the succeeding director. Relocation of the collection began in 1973 to the University of California at Davis Medical School and was completed in 1976. Ronan O'Rahilly was the new director of the collection for the next 15 years, publishing many studies, often with Fabiola Müller, on human embryonic development. At the retirement of the director in 1991 the collection was relocated again to its current location at the Walter Reed Army Medical Center in Washington, D.C., forming part of the Human Developmental Anatomy Center 20 historic embryology collections and remains available for researcher study. In 2014, preliminary work began with the current curator on establishing a partnership with the Digital Embryology Consortium to eventually digitize, preserve, and make more widely available this collection. Further details of the embryo collection can be found in earlier publications [3, 4] as well as on the web (http://tiny.cc/HDAC_Collections), see also (http://tiny.cc/Carnegie_Collection).

Figure 1. Carnegie models located at the Carnegie Collection. (Embryos shown in the bottom left-hand corner were laminated from individual layers and then painted.)

2.2. Harvard collection

Originally collected by Charles Minot (1852–1914), sometimes referred to as the Minot Collection, it now forms part of the larger Carnegie Collection. By 1905, the collection consisted of 937 histologically sectioned embryos from human and other species (Figure 2).
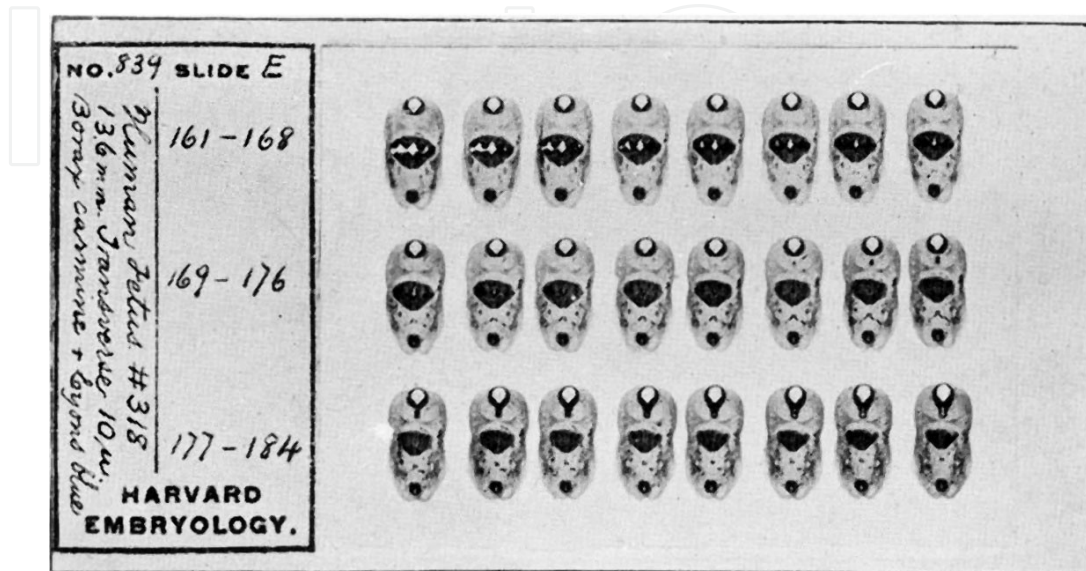

Figure 2. Harvard Collection histology slide No. 839 E, showing 10 micron serial sections from human embryo (No. 318) 13.6 mm in length.

2.3. Blechschmidt collection (University of Göttingen, Germany)

The Blechschmidt Collection is located in the Department of Anatomy and Embryology, Center of Anatomy, University of Göttingen. The University of Göttingen was founded in 1737, and has a long history in research that includes producing 45 Nobel Prize winners.

The human embryo collection is named after Erich Blechschmidt (1904–1992), who directed the Anatomical Institute from 1942 until 1973, and consists of two parts: firstly, a large histology collection of serial sections and, secondly, a model collection based upon these sections.

The histology collection is made up of about 120 human embryos that have been cut in a range of anatomical planes into some 200,000 serial sections. In 1972, some of the embryo serial section sets were temporarily incorporated into the Carnegie Collection and assigned Carnegie Nos. 10315 to 10434. These embryos have since been returned to their original home at the University of Göttingen.

The model collection (Figure 3) "Human embryologische Dokumentations sammlung Blechschmidt" forms a permanent exhibition housed at the Centre of Anatomy and consists of 64 large models, generated from 1946 to 1979. The models are available for viewing upon request and are arranged in perspex cases that allow each model to be observed from all directions. The models range from selected parts or systems of a specific embryo to whole embryos in surface view. In addition, parts of the embryos have been selectively removed or

"windows" generated to observe internal system structures including: circulatory, respiratory, gastrointestinal, neural, and the musculoskeletal system.

The modeling method from the histological material used a technique based upon Blechschmidt's own method, described below. Each model illustrates whole embryo surfaces, some organic systems (including a circulatory organ, respiratory organs, a digestive organ, central nerve, and the skeletal system) in precision, in addition to the right-side out.

The embryo collection has probably the largest number of excellently preserved specimens of the latter half of the embryonic period (covering weeks 5–8 post conception). Detailed documentation on individual specimens of the collection is sparse and some of the specimens are also depicted as color drawings in Blechschmidt [5]. The high quality and standard of the histology material was achieved by a combination of a "state-of-the-art" embryo collection gynecological practice (mechanical curettage or hysterectomy) from operations including termination of pregnancy and development of a special fixation procedure. As a result, the quality of paraffin histological sections mounted on large glass microscope slides is unsurpassed and reveals valuable morphological detail of early organ development in the human embryo.

Figure 3. The Blechschmidt models and histology slides (photo by Saki Ueno).

Like many historic collections, even with optimal storage conditions, the slide histology has gradually deteriorated with evaporation of cover glass glue and bleaching of histological stains. Secondly, the large glass microscope slides are delicate and easily damaged during use. Both these issues highlight the pressing need for generating a "digital copy" of these historic collections.

Photomicrographs of individual histological sections from several specimens were included in Blechschmidt's embryology textbook [5]. At that time, the only way to preserve for posterity morphological information contained in these specimens consisted in building large-scale polymer plastic reconstruction models. These models were made from camera-lucida draw-ings at an intermediate magnification of regularly spaced histological sections [6]. Using the same series of serial sections several times over, Blechschmidt made reconstructions of the surface anatomy and the morphology of several organ systems of the same embryo, thereby enabling direct comparison of topographical characteristics and their dynamic changes during development, even though the cellular detail detectable at high magnification remained unexplored with this method. Currently, the way to preserve the collection in its current condition lies with the scanning and digital preservation of the histological material with the Digital Embryology Consortium.

Figure 4. The Orts-Llorca Madrid Collection. Slides of serially sectioned embryos are stored in individual box sets. (Photo by Mark Hill)

2.4. Madrid institute of embryology human embryo collection

The human embryo histology collection was started in 1935 by the Spanish embryologist Francisco Orts-Llorca (1905–1993) and is located at the Embryology Institute of Complutense University of Madrid [7]. The collection consists of histological serial sections of more than 100 human embryos in thousands of serial sections covering the embryonic and fetal periods (Figure 4). The collection includes both normal and abnormal embryos. The sectioning is in a number of different anatomical planes and includes both normal and abnormal embryonic material. The collection has unfortunately suffered from the rigors of time, handling by many researchers, and fading of histological stains. The collection though still contains many very useful and unexplored embryos of a broad range of stages of development and the current head of department Professor José F. Rodríguez-Vázquez is determined to return this collection to a better condition and preserve this valuable research collection.

2.5. Hinrichsen collection (Bochum specimens)

Klaus V. Hinrichsen was a pupil of Blechschmidt and had the chair of Anatomy and Embryology at the Ruhr University Bochum in 1970. Many excellent specimens were collected by Hinrichsen's team between 1969 and 1994 and are now housed in the Department of Anatomy and Molecular Embryology at the Ruhr-Universität Bochum, Germany. The total number of the Hinrichsen Collection reached 70, and details of many of these specimens were published in Hinrichsen's textbook on human embryology [8] and in many original publications [9]. The reconstructions have not been attempted from these specimens and many specimens have likewise remained unexplored, to date.

2.6. Kyoto collection

Hideo Nishimura began this collection in 1961 and currently has over 44,000 human embryo specimens. It was further developed and managed by Kohei Shiota for a long period and is currently managed by Shigehito Yamada and all professors in the Department of Anatomy at Kyoto University School of Medicine.

Under the Maternity Protection Law of Japan, induced abortions were legal and in a great majority of cases pregnancies were terminated for social reasons during the first trimester. These provided Nishimura the beginning of the Kyoto collection. In 1975, he formed the Congenital Anomaly Research Center and the collection had now reached over 36,000 specimens. Currently, this collection is the largest in the world with over 45,000 specimens (Figure 5) and provides a key resource for international embryology researchers.

An important characteristic of the collection is inclusion of both normal and many abnormal embryos with severe malformations [10], including holoprosencephaly. Holoprosencephaly (HPE) is a rare newborn anomaly (1/10,000-20,000) occurring more frequently (1/250 or more) in the embryo, being the most common structural malformation of the human embryonic forebrain due to abnormal midline cleavage of the prosencephalon into cerebral hemispheres. This in turn leads to the characteristically abnormal facial development. [11]. Note that the estimation of embryonic frequency may be lower than the actual prevalence, as milder forms of holoprosencephaly also exist, but are more difficult to diagnose [12, 13].

Another unique feature of the Kyoto Collection is the associated maternal epidemiological data and detailed clinical information on the pregnancies that were collected with each specimen. The epidemiological data has been used for statistical analysis to determine potential causative links between maternal factors and congenital anomalies [14].

The collection has more recently been analyzed using several new advanced imaging technologies that allow 3D embryo imaging and subsequent generation of digital models. Firstly, magnetic resonance microscopy (MRM, see 4.1 in this chapter) of embryos has been carried out [15-18] and analyzed morphologically using 3D reconstruction [19-21]. Secondly, episcopic fluorescence image capture (EFIC) and phase-contrast X-ray computed tomography (pCT) techniques have also been applied to these embryos (18, 22, see 4.2 and 4.3 in this chapter). The current curator, Shigehito Yamada, has now commenced the lengthy process of digitizing all histological sections within this collection and is also a contributing partner in the new digital consortium. The Kyoto Collection is currently one of the largest and best catalogued human embryo collections, containing approximately equal numbers of both normal and abnormal specimens. The collection is also divided into whole wet specimens (see sub-heading 4.4 OPT) as well as about 1,000 serially, histological sectioned embryos (see 5.3, computer reconstructions). More recently, the current curator has digitized and made available online sections from some of the normal embryos in the collection (http://atlas.cac.med.kyoto-u.ac.jp).

Figure 5. Kyoto Collection of human embryos. (Image shows embryo storage, fixed wet whole embryos, histological collection, and digitization process.)

2.7. Hubrecht collection

Ambrosius A.W. Hubrecht (1853–1915) was a Dutch embryologist who held a chair in comparative embryology at the University of Utrecht from 1910 and founded the "Institut Inteniational d'Embryologie" in 1911. This huge collection of comparative embryonic material from 600 vertebrate species consists of 3,000 wet specimens and 80,000 histological sections from many species including human [23]. There is also a significant collection of photographic material and documentation available. This collection along with the Hill Collection and other German collections forms the Embryological Collection at the Museum für Naturkunde in Berlin and is currently curated by Peter Giere (Figure 6). The collection is made available for researchers upon request. (http://tiny.cc/MfN_Berlin_Embryo)
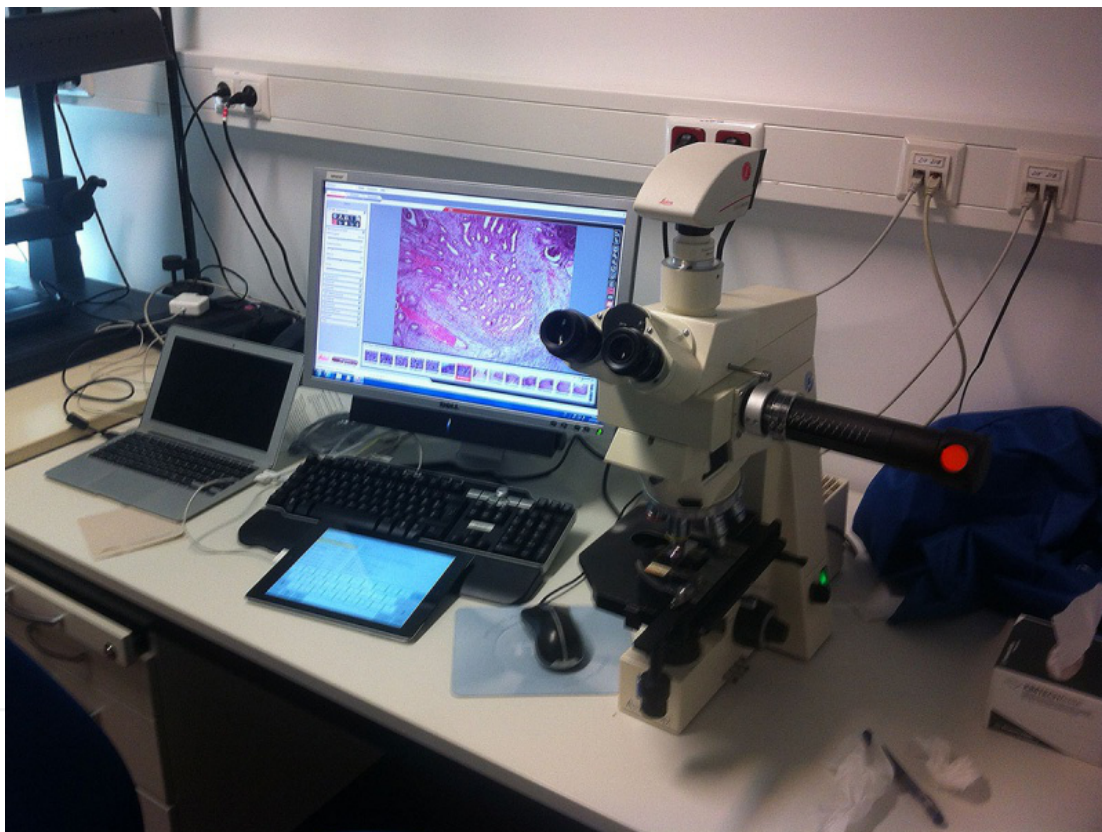
Figure 6. The Embryology Collection photomicroscopy setup at the Museum für Naturkunde. With permission, collection slides can be photographed and used for research purposes. (Photo by Mark Hill)

2.8. HUDSEN collection

The Human Developmental Studies Network (HuDSeN) atlas is based on 12 optical projection tomography (OPT) models covering the range of Carnegie stage 12–23 [24]. The Human Developmental Biology Resource (http://www.hdbr.org/) was established in 1999 in line with the ethical guidelines laid out in the Polkinghorne Report. There are also histological sections (hematoxylin and eosin stained) from human embryos covering these stages of development.

3. Human embryonic development

Classification into developmental stages is necessary to accurately describe prenatal growth. Embryonic staging of animals was introduced at the end of the 19th century [25], and was first applied to human embryology by Mall [26]. At first, human embryos were classified based on their length like "3-mm stage," but this approach was quickly obsolete because there are individual variations between each embryo. Subsequently, Streeter developed a 23-stage developmental scheme of human embryos in the 1940s called developmental "Horizons." Finally, stages 1–9 were established by O'Rahilly [1973], stage 10 was summarized by Heuser and Corner in 1957 from Streeter's note [27], and stages 11–23 were described in detail by Streeter [28–31].

3.1. Carnegie stages

The Carnegie stage is commonly known as a staging scheme which remains widely used today. Table 2 shows the relationship between embryonic ages from various researchers and the equivalent Carnegie stages proposed by O'Rahilly and Müller [32]. It is important to note that Streeter's human series included pathological specimens obtained from spontaneous abortion or ectopic implantation.

Carnegie stage (CS)	Embryonic age (days)				
	Streeter (28-31)	Nishimura (33, 34)	Jirásek (35)	O'Rahilly and Müller (32)	O'Rahilly and Müller (36)
11	24	27	23-26	23-25	28-30
12	26	30	26-30	25-27	29-31
13	28	32	28-32	28	30-33
14	29	34-35	31-35	32	33-35
15	31.5	36	35-38	33	35-37
16	33	38	37-42	37	37-40
17	35	40	42-44	41	39-42
18	37	42	44-48	44	42-45
19	39	44	48-51	47-48	45-47
20	41	46	51-53	50-51	47-50
21	43	48	53-54	52	49-52
22	45	50	54-56	54	52-55
23	47	52	56-60	56-57	53-58

Table 2. Embryonic age (days) based on developmental stages (CS) of human embryos, according to various authors. Streeter [28-31], Nishimura [33, 34], Jirásek [35], and O'Rahilly and Müller [32] show the approximate ovulation age (days); O'Rahilly and Müller [36] show embryonic ages calculated from the greater length of embryo and ultrasound findings

3.2. Image and summary of each Carnegie stage (Figure 7)

Carnegie stage 1: Zygote

1 day after fertilization, cell size 120–150 μm in diameter.

At fertilization, the oocyte completes meiosis II, forming the female pronucleus. The sperma-tozoa nucleus in the oocyte cytoplasm decompresses, forming the male pronuclei. These two pronuclei fuse to form the first diploid cell, the zygote. The first mitosis occurs during the 24 h after zygote formation. The term "conceptus" is now used to describe all the cellular products of the zygote.

Carnegie stage 2: Morula.

1.5–3 days after fertilization, conceptus 0.1–0.2 mm in diameter.

The zygote forms two blastomeres. Mitosis of these blastomeres forms a solid ball of 16 cells, then 32 cells, still enclosed by the zona pellucida. This cleavage stage divides the large zygote cytoplasm into sequentially smaller cells. The term "morula" means berry, referring to the appearance of the solid ball of cells.
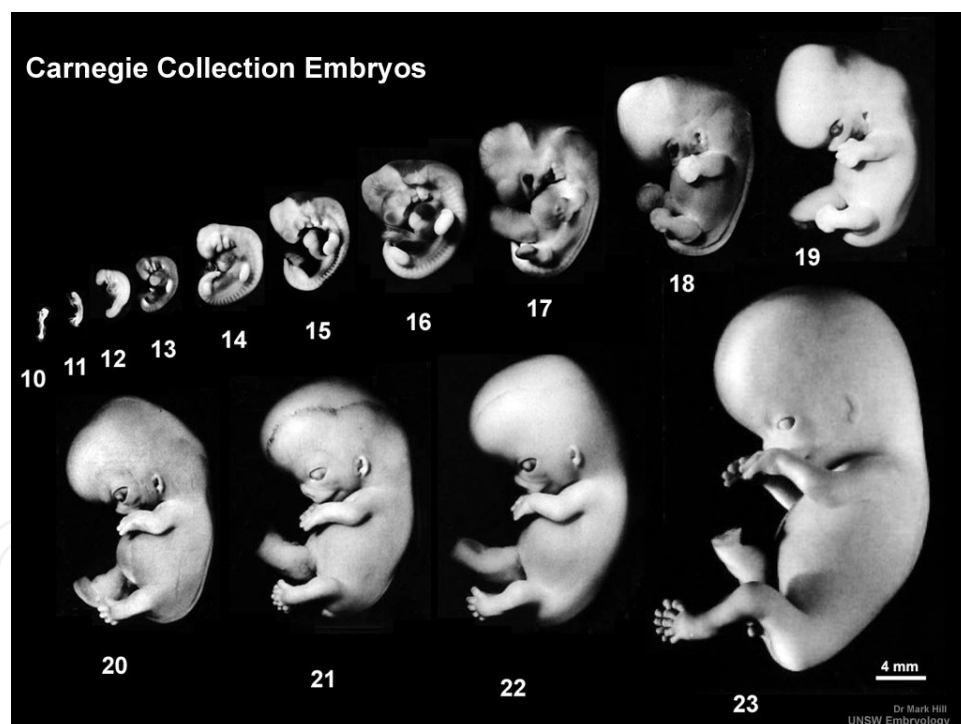
Figure 7. Examples of the Carnegie Collection embryos arranged into the classic Carnegie stages.

Carnegie stage 3: Free blastocyst

4 days after fertilization, conceptus 0.1–0.2 mm in diameter.

Cell division continues after the 32 cell stage occurring more rapidly at the surface and slower in the center cells. This and directional fluid transfer leads to a cavity, the blastocoel, in the

conceptus. The surface cells form an outer squamous trophoblast layer linked by both tight and gap junctions. The larger inner cells form the inner cell mass or embryoblast.

Carnegie stage 4: Attaching blastocyst

5–6 days after fertilization, conceptus 0.1–0.2 mm in diameter.

The blastocyst hatches from the zone pellucida, still floating in uterine secretions of the secretory phase of the menstrual cycle. The surface trophoblast cells can now initially adhere to the endometrial epithelium at the site of implantation. The trophoblast cells proliferate and differentiate into two layers. The outer cells fusing to form syncytiotrophoblasts, the inner close remain as single cells, cytotrophoblasts.

Carnegie stage 5: Implanted but previllous

7–12 days after fertilization, conceptus 0.1–0.2 mm in diameter.

This stage was originally divided into three (a, b, and c) substages based on trophoblast differentiation status before outgrowth (villi) appears. 5a is the initial solid trophoblast cell layer; 5b, lacunar trophoblast with the appearance of spaces (lacunae) within the trophoblast layer; 5c, maternal blood-filled lacuna as capillaries and uterine glands are opened into the trophoblast spaces.

Carnegie stage 6: Chorionic villi and primitive streak

13 days after fertilization, conceptus 0.2 mm in size.

Trophoblast cells extend into the maternal uterine stroma (decidua) forming chorionic villi. The extra-embryonic mesoderm arises, lining the conceptus cavity and forming the chorionic cavity. Three separate cavities or extra-embryonic coeloms form outside the embryonic disc: the chorionic, amniotic, and yolk sac cavities. Toward the end of this stage, the primitive streak appears on the embryonic disc; this is the site of gastrulation.

Carnegie stage 7: Notochordal process

16 days after fertilization, embryonic disc 0.4 mm in length

The embryonic disc establishes axes and has an initial central primitive node (Hensen's node, primitive pit) with the primitive streak extending caudally to the disc edge where the connecting stalk will later form. Gastrulation occurs here forming endoderm and mesoderm that spread laterally and rostrally from the primitive streak. Above the primitive node, cranially, the notochordal process develops in the mesodermal layer. The length of this process increases from 0.03 to about 0.3 mm. The embryonic disc increases in size and the amniotic cavity enlarges over the yolk sac.

Carnegie stage 8: Primitive pit, neuenteric canal

18 days after fertilization, embryonic disc 1.0 mm in length

The embryonic disc is pyriform, tapering caudally, and now has cranio-caudal axis, measured from this stage onward by crown-rump length (CRL). The stage shows three key features: the

primitive pit, the notochordal canal, and the neurenteric canal. The notochordal canal is marked by the cavity extending from the primitive pit into the notochordal process. The floor of the canal is lost to form a transient passage, neurenteric canal, between the amniotic cavity and the yolk sac. The notochord process will differentiate into the notochord or axial meso-derm. The remainder of new mesoderm layer has not yet segmented and is called the preso-mitic stage.

Carnegie stage 9: 1–3 pairs of somites

20 days after fertilization, embryo 1.5 mm in crown-rump length (CRL)

The mesoderm either side of the notochord now segments into paired somites. Segmentation of paraxial mesoderm only occurs at the level of the trunk, not the head, and proceeds in a cranial-caudal direction. Note that the sequential appearance of somite pairs can also be used as a criterion to stage the embryo. The embryonic disc resembles a shoe-sole, with the broad neural plate in the ectoderm layer positioned in the cranial region. The mid-line neural plate begins to fold forming a neural groove.

Carnegie stage 10: Neural folds begin to fuse, 4–12 pairs of somites

22 days after fertilization, embryo 1.8 mm in CRL

Somitogenesis continues increasing from 4 to 12 somite pairs. The neural groove continues to fold bringing the neural plate edges together to commence fusing. This fusion occurs in both cranial and caudal directions and at several sites. In the head region, the optic sulcus and first pharyngeal (branchial) arch appear. In the underlying trunk region mesoderm the cardiac tube appears.

Carnegie stage 11: Anterior neuropore closes

24 days after fertilization, 2.5–3 mm in CRL

Somitogenesis continues increasing from 13 to 20 somite pairs. The neural groove has formed an open-ended neural tube, and the upper head end (anterior, cranial or rostral) opening (neuropore) commences to close. Optic evagination is produced at the optic sulcus and the optic ventricle is continuous with that of the forebrain. The cardiac tube has formed a loop, with a sinus venosus region appearing. The second pharyngeal arch is visible. A ventral indentation (stomodeum) is present at the level of the first arch. The floor of the stomodeum forms the oral membrane (buccopharyngeal) that commences to degenerate. Dorsally at the level of the second arch, paired otic placodes fold inward to form the otic vesicles.

Carnegie stage 12: Posterior neuropore closes

28 days after fertilization, 4 mm in CRL

Somitogenesis continues with 21–29 somite pairs. The posterior (caudal) neuropore is starting to close or is closed. Three of the pharyngeal arches are now clearly visible. The upper limb buds appear, initially as lateral swellings at the level of the heart. Internally, the heart inter-ventricular septum has begun to form, the liver is present and the lung buds appear.

Carnegie stage 13: Limb buds, optic vesicle

32 days after fertilization, 5 mm in CRL

Somitogenesis continues with more than 30 somite pairs. The numbers of somite pairs are now difficult to determine as staging criteria. Both upper and lower limb buds are visible. The optic vesicle is present, and the lens placode begins to differentiate.

Carnegie stage 14: Lens pit and optic cup

34 days after fertilization, 6 mm in CRL

The upper limb buds elongate and become tapering. Upper limb bud features appear about 2 days before the lower limb. The embryo cephalic and cervical flexures are prominent. Within the head, the future cerebral hemispheres and cerebellar plates are visible. On the head surface, the lens pit invaginates into the optic cup but is not yet closed and the otic vesicle endolymphatic appendage emerges. Within the trunk, pancreatic buds (ventral and dorsal) are present, the mesonephric duct forms the ureteric bud and at its tip is the metanephrogenic blastemal cap.

Carnegie stage 15: Lens vesicles, nasal pit and hand plates

36 days after fertilization, 8 mm in CRL

The upper limb hand plates are now visible. Lens vesicles are closed and covered by the surface ectoderm. The nasal plate invaginates to form a nasal pit. The auricular hillocks on pharyngeal arch 1 and 2 appear. Within the heart, the foramen secundum is present. Lung buds are now branched into lobar buds and the primary urogenital sinus is formed.

Carnegie stage 16: Nasal pit faces ventrally, retinal pigment, foot plate

38 days after fertilization, 10 mm in CRL

The upper limb hand plates are distinct and the foot plate has begun to form. On the trunk between the upper and lower limbs, a distinct mesonephric ridge is visible. On the head, the nasal pits deepen and face ventrally and the eye retinal pigment is visible externally. The nasolacrimal groove begins to form and lies between the frontal and maxillary processes.

Carnegie stage 17: Head relatively larger, nasofrontal groove, finger rays

40 days after fertilization, 11 mm in CRL

The upper limb hand plates have digital rays, and the foot has acquired a rounded digital plate. The head is now larger than previously and the trunk has begun to straighten. On the first and second pharyngeal arches the auricular hillocks are present and the nasolacrimal grooves are distinct.

Carnegie stage 18: Elbows, toe rays, eyelid folds

42 days after fertilization, 13 mm in CRL

The upper limb elbows are discernible and in the hand plates interdigital notches appear. Toe rays are observed in the foot plate. The trunk shape is more cuboidal and both cervical and lumbar flexures are denoted. On the head, eyelid folds appear and auricular hillocks are fusing to form specific parts of the external ear. Ossification commences in some skeletal structures.

Carnegie stage 19: Trunk elongation and straightening

44 days after fertilization, 16 mm in CRL

The upper and lower limbs are parallel, with preaxial borders cranially and postaxial borders caudally. On the head, eyes are now positioned in the front of the face, due to the growth of the brain, and the external ears have their definitive shape. The trunk continues to elongate and straighten. Within the trunk, the intestines have developed and herniated in the umbilical region.

Carnegie stage 20: Longer upper limb bent at elbow

46 days after fertilization, 19 mm in CRL

The upper limbs have increased in length and flexed at the elbows and hand joints. Fingers are curving slightly over the chest. The angle of cervical flexure becomes small, and the direction of the head goes upward. The head has a superficial scalp vascular plexus. The herniated intestines continue to elongate. Embryo spontaneous movements can occur at this stage.

Carnegie stage 21: Fingers are longer, hands approach each other

48 days after fertilization, 21 mm in CRL

The hands are slightly flexed at the wrists and nearly come together over the cardiac prominence. The head becomes round and the superficial vascular plexus has spread and now surrounds the head. The trunk tail now becomes rudimentary.

Carnegie stage 22: Eyelids and external ear are more developed

50 days after fertilization, 23 mm in CRL

The head vascular plexus is now very distinct. The eyelids have thickened and lie over the eyes. The external ear position is higher on the head and the tragus and antitragus regions are more definite. The trunk tail is almost lost.

Carnegie stage 23: End of embryonic period

52 days after fertilization, 30 mm in CRL

The head is now rounded out and the trunk has elongated to a more mature shape. The limbs have increased in length and the forearm is level or above the level of the shoulder. The head scalp vascular plexus is approaching the vertex of the head. The eyelids and ear auricles become definite. The external genitalia are developed but not sex-differentiated. The trunk tail has now gone.

4. Human embryo imaging

Rapid advances in medical imaging are facilitating the clinical assessment of first-trimester human embryos at increasingly earlier stages. To obtain data on early human development,

we have used some micro-imaging modalities such as magnetic resonance microscopy, episcopic fluorescence capture, and phase-contrast X-ray computed tomography. The following sections describe and show the resulting embryo images from each of these imaging techniques.

4.1. Magnetic Resonance Microscopy (MRM)

Magnetic resonance (MR) imaging is now widely used as a tool for diagnostic medical imaging. In research, when scanning small samples this technique is called magnetic resonance microscopy (MRM). MRM was first applied to studying the human embryo in the 1990s [37, 38], and has now become a very powerful tool for 3D measurement of chemically fixed human embryos [15]. This research technique is still being developed and MRM images in higher resolution have been obtained using human embryos and a range of contrast agents [39]. The images shown in Table 3 were obtained using MRM equipped with a 2.34T magnet [15].

4.2. Episcopic Fluorescence Image Capture (EFIC)

Episcopic fluorescence image capture (EFIC) was devised and developed in the early 2000s [40, 41]. With EFIC imaging, tissue autofluorescence is used to image the whole embryo block face prior to histologically cutting each section. These individual sections can then be viewed or reconstructed into a 3D image [18], Figure 8. This technique has now been applied to staged human embryos from the Kyoto Collection. The first and only human embryo atlas developed from Kyoto embryos using EFIC can be accessed at website in University of Pittsburgh (http://apps.devbio.pitt.edu/HumanAtlas/; login ID and password are shown in [18]; the atlas also includes MRM images from similar staged embryos.

4.3. Phase-Contrast X-ray Computed Tomography (pCT)

Phase-contrast X-ray computed tomography (pCT) is a relatively newer technique of imaging. In this technique, the X-rays are used as electric waves characterized by amplitude and phase. Conventional X-ray imaging (radiography) is based on absorption-contrast (i.e., amplitude imaging) and represents the mass-density distribution of X-ray inside the sample.

In comparison, pCT uses the phase-shift, occurring when X-rays pass through samples [42]. The phase shift is converted into a change in X-ray intensity that is collected by a current-detecting device. There are some conversion methods such as interferometry with an X-ray crystal interferometer [42, 43], diffractometry with a perfect analyzer crystal [44-46], a propagation-based method with a Fresnel pattern [47, 48], and Talbot interferometry with a Talbot grating interferometer [49, 50]. Devices based on this principle have been developed [51, 52], and an image of human embryo at CS 17 obtained using a two-crystal X-ray interferometer (Yoneyama et al., 2011) is featured in Figure 9.

4.4. Optical Projection Tomography (OPT)

Optical projection tomography (OPT) was devised in 2002, using the principle of projection tomography [53, 54]. During the embedding process, the samples are dehydrated and cleared
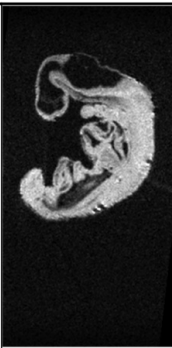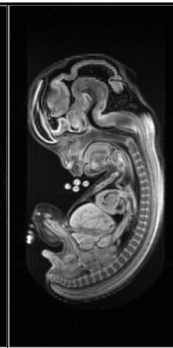
Carnegie stage	13	16	19	22
Days after fertilization (Gestational weeks)	32 (6w4d)	38 (7w3d)	44 (8w2d)	50 (9w1d)
Cranio-rump length	5.0mm	10mm	16mm	23mm
Digital resolution	40μm/pixel	50μm/pixel	100μm/pixel	120μm/pixel

Table 3. Result of MRM scanning using human embryos

with a mixture of benzyl alcohol and benzyl benzoate, allowing the light to pass through the specimen. This technique has also been applied into human embryo [55, 56], and the atlas regarding gene expression in the developing human brain has been established using OPT [24].
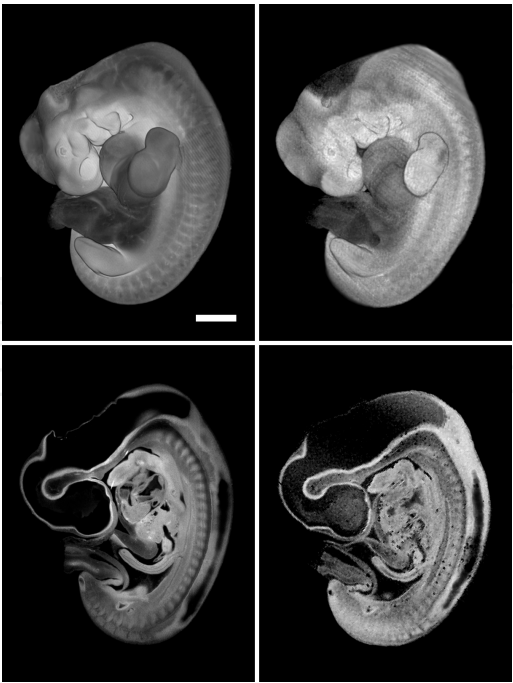
Figure 8. Comparison between imaging of the same stage embryo using two different techniques of EFIC (left) and MRM (right).
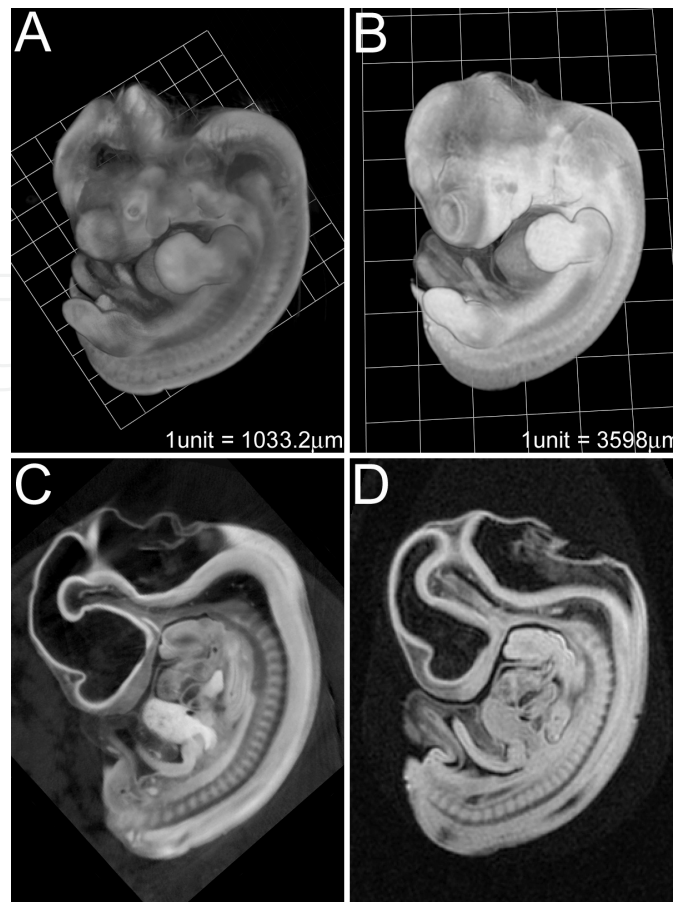
Figure 9. Comparison of human embryos (CS 17) imaged by pCT (A,C) and MRM (B,D). Note that Rathke's pouch can be seen in the embryo by pCT (C) but not detected by MRM (D).

5. Three-dimensional models and analyses of human embryos

In the 19th century, human embryo models were made manually based on macroscopic and microscopic observation. Wax plate technique was introduced into embryology in 1887, and the principle was used continuously until the computer era, although the material of the model has changed from wax to plastics. Computer-assisted reconstruction started at the end of the 20th century. The reconstruction was made from the histological sections at first, followed by reconstruction from 3D image dataset. Recently, as 3D printers become cheaper and widespread, they are being applied in human embryology.

5.1. Ziegler models

By the middle of the 19th century, there had already been 2D illustrations of embryos and 3D embryo models were eagerly awaited, due to the difficulty of obtaining embryos, their fragility and size. Louis Auzoux, a French anatomist, made papier-mâché models in his Normandy workshop (Figure 10A). Later Adolf Ziegler started to render hand-shaped models after he

returned to the University of Freiburg in 1854, and completed his first model series "The Development of the Frog." His modeling was applied in developmental biology, including human embryos. Adolf Ziegler retired in 1883 and his son Friedrich Ziegler took over the modeling operations. The "Ziegler models," including trout, sea urchin, beetle, frog, chick, and human embryos (Figure 10B) were displayed at the 1893 World's Columbian Exposition in Chicago, and there they attracted much attention.
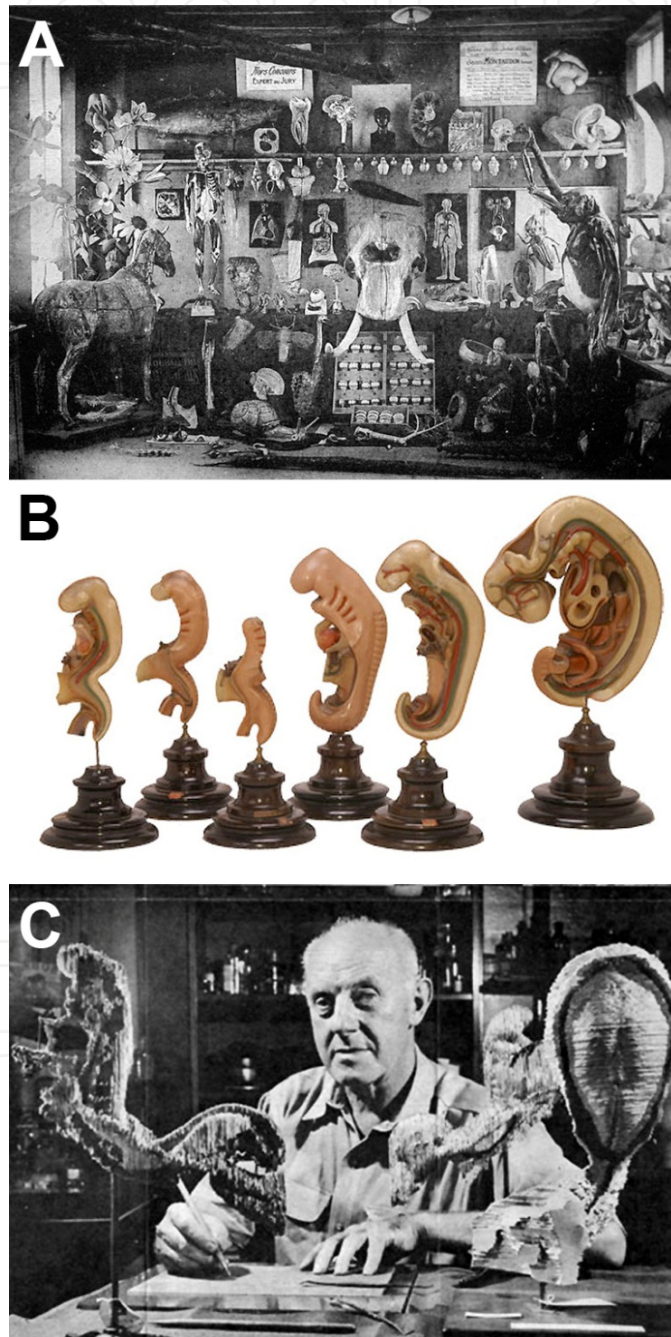

Figure 10. Examples of historic embryo models. Modeling workshop of Louis Auzoux (A), Ziegler human embryo models (B) and Carnegie Laboratory models and Osborne O. Heard (C).

5.2. Wax-plate model and its derivatives

In 1865, Wilhelm His Sr. invented the microtome [57], and he applied it to embryology. In 1883, Gustav Born devised the wax plate technique; 3D reconstruction from serial histological sections was made by wax plate [58]. This technique was applied to embryology [59] and later modified in the Carnegie Laboratory in Baltimore [60]. The material of model originally used was mainly wax (Figure 10C), and changed into plastic or its derivatives [61] in the 20th century. These new models were a significant improvement in detail and accuracy over the earlier Ziegler models. The technique was later further developed, with larger scale and detail, by Blechschmidt in his model series (see above, section 2.3].

5.3. Computer graphics from serial sections

Recent advancement in image technology and computer science has made computer-assisted reconstruction of embryonic structures more effectively, and the reconstructed images can be manipulated as desired on the viewing screen. This technique has been applied to human embryos in 1994, using the Madrid Collection [62], and 3D reconstruction of human embryo has also been established using the Kyoto Collection [62]. In the 21st century, the 3D models were colorized and elaborated [63, and see Figure 11]. In combination with the advance of web technique, some attractive web-based human embryo atlases have been constructed using the Kyoto Collection [18], http://apps.devbio.pitt.edu/HumanAtlas/), and the Carnegie Collection has been established and available freely [64], (http://www.ehd.org/virtual-human-embryo/).

5.4. 3D printer and scanner

A 3D printer is a tool for making 3D solid objects from digital data. Stereolithography was a technique developed at the end of the 20th century; in recent years, it has enabled the creation of inexpensive 3D models in engineering, medical and dental fields, as well as the academic area [65] and has been applied to human embryology [66], (see Figure 12).
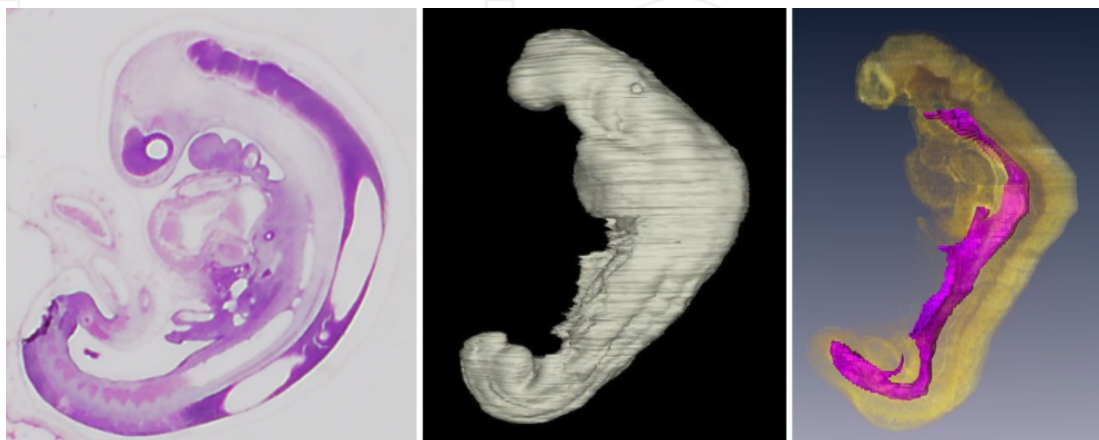

Figure 11. Histological section (left), embryo surface reconstruction (center) and a 3D reconstruction labeling of the gastrointestinal system (right) from a human embryo.
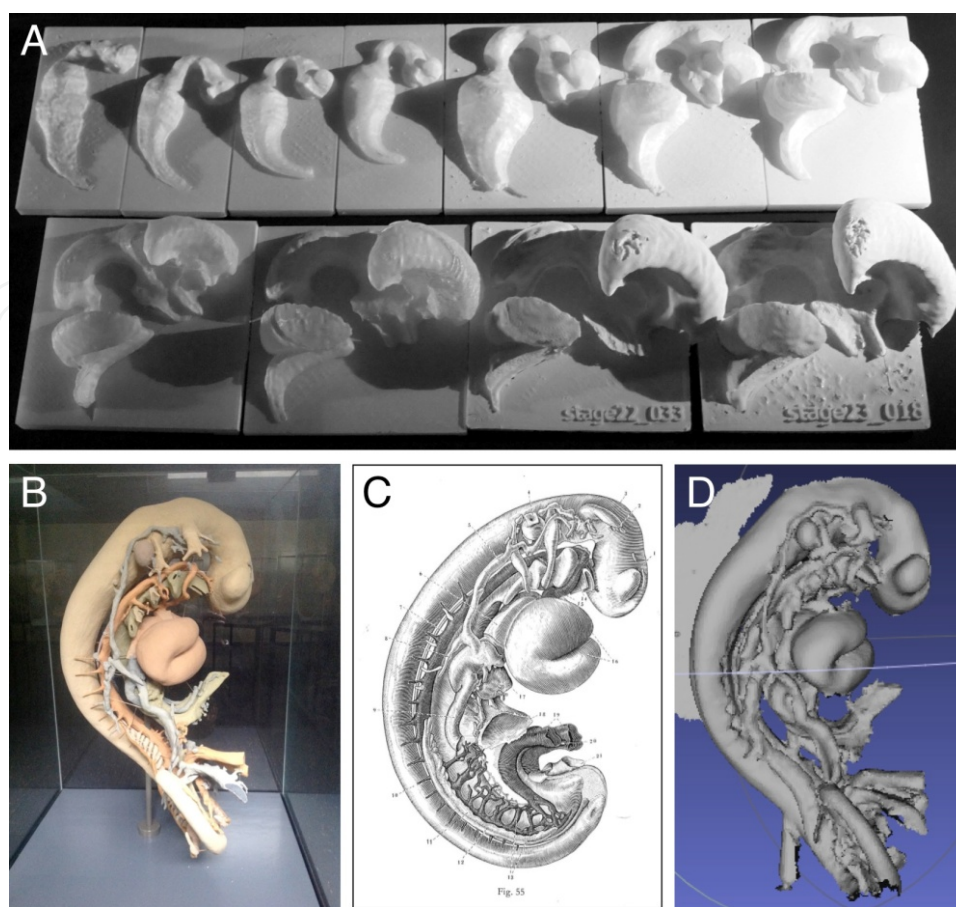
Figure 12. Images related with 3D printer and scanner. (A) The brain ventricle of human embryo ranging from CS13 to 23. (B) Solid reconstruction in the Blechschmidt Collection. (C) Drawing derived from B. (D) Image data of B converted by a commercially available 3D scanner (http://cubify.com/en/Products/Sense).

A 3D scanner is a tool for digitizing the surface of an object as data. Several groups are currently investigating its application to human embryology.

Acknowledgements

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