Journal of Nuclear Medicine, published on June 22, 2018 as doi:10.2967/jnumed.118.210310

The use of ostrich eggs for *in ovo* research - Making preclinical imaging research affordable and available

Martin Freesmeyer[1], Christian Kuehnel[1], Thomas Opfermann[1], Tobias Niksch[1], Steffen Wiegand[1], Ronny Stolz[2], Ralph Huonker[3], Otto W. Witte[3,4], Thomas Winkens[1]

Affiliations:

[1]Clinic of Nuclear Medicine, Jena University Hospital, Germany

[2]Leibniz Institute of Photonic Technology, Jena, Germany

[3]Biomagnetic Center, Hans Berger Department of Neurology, Jena University Hospital, Germany

[4]Hans Berger Department of Neurology, Jena University Hospital, Germany

Disclaimer and Financial Disclosure

The authors do not have any conflicts of interest to declare. This study was funded exclusively with intramural grants from the Jena University Hospital.

First Author, Corresponding Author and Reprint Requests:

PD Dr. Martin Freesmeyer, M.D.; Head of Department
Clinic of Nuclear Medicine
Am Klinikum 1, 07747-Jena, Germany
Tel.:+0049-3641-9329801
Fax:+0049-3641-9329802
Email: martin.freesmeyer@med.uni-jena.de

Running Title: *In ovo* imaging with routine scanners

Word Count: 4999

ABSTRACT

In ovo research is a valuable option of preclinical research, but imaging studies are severely limited by the costs of dedicated equipment needed for small-size eggs. We sought to verify the feasibility of using larger eggs of ostrich (*Struthio camelus*) for the imaging with PET/CT scanners used for routine clinical investigations.

Methods: Ostrich eggs were incubated until shortly before hatching and prepared for the intra-vitelline venous injection of contrast media or radiotracers. Imaging was performed by native computed tomography (CT), contrast-enhanced CT and positron emission tomography/CT (PET/CT; Biograph-mCT40; Siemens,Germany). Efforts were paid to identify the technical adaptations needed to improve the procedural outcomes.

Results: Of the 34 eggs initially incubated, 12 became fully available for imaging of the embryonal development. *In ovo* imaging with conventional PET/CT was not only feasible, but it also provided images of good quality, including upon dynamic PET imaging.

Conclusion: *In ovo* imaging with ostrich eggs and routine clinical scanners may allow a broader application of this field of preclinical research, bypassing the need of costly dedicated equipment and reducing the number of animals needed for classic animal research. Further experiments are warranted to refine this novel approach, in particular regarding reduction of motion artifacts and improvement of viability monitoring.

Key words: In ovo imaging, functional topography, embryonal development, ostrich

INTRODUCTION

In ovo imaging of chicken embryos is an established preclinical method for research in oncology, toxicology, and pharmacology (*1-3*). Imaging of tumor cell formations and vascularization has been evaluated using chorion-allantois membrane models of chicken eggs as reported previously (4-6). In addition, chicken eggs have been investigated using magnetic resonance imaging (MRI) to visualize developmental processes *in ovo*, predominantly considering brain development (7-9).

Due to the small size of the chicken eggs, however, this method requires the use of expensive, dedicated imaging equipment for small animals (i.e. micro-computed tomography (CT), micro-magnetic resonance imaging (MRI), micro-positron emission tomography (PET), or micro-single photon emission CT (SPECT)), as well as the availability of adequate premises and well-trained personnel (*10*). These prerequisites substantially limit a broad application of *in ovo* research, which is in fact very valuable in terms of reducing the number of animal tests and meeting the requirements of modern animal research (*11*).

We explored the possibility to perform *in ovo* imaging without dedicated techniques by using ostrich eggs (i.e., eggs that are sizably larger than the conventional chicken eggs) along with conventional PET/CT, i.e., a technique routinely used for clinical diagnostics (*10,12-14)*. The goal of the present study was to verify the feasibility of ostrich *in ovo* imaging in terms of viability monitoring, fenestration, anesthesia, vessel catheterization, and acquisition/reconstruction of PET/CT.

MATERIALS AND METHODS

Ethics

This embryo study did not qualify as an animal research study according to the Federal German Animal Protection Act. Registration took place with the Office for Consumer Protection of the Thuringia State, registration number 22-2684-04-02-114/16.

Egg sample

The egg size is the first requisite for a possible use in routine imaging. Ostrich eggs (*Struthio camelus*) appeared good candidates for this purpose, given their average size of 20 x 15 cm (Fig. 1A) and the possibility to obtain a sufficiently precise depiction of the embryonal anatomical structures within the resolution limits of common imaging methods (mm or less for MRI and CT, few mm for PET, mm to cm for SPECT). Ostrich eggs are commonly available in German farms from March to September. For this study, the eggs were obtained either as freshly fertilized (development stage 0, cost: 20 EURO) or as already incubated (development stage day 1-36, cost: 50-130 EURO). They were then placed in an incubator (Grumbach Compact MP GTFS84; ProCon Automatic Systems GmbH&Co.KG, Mücke/Sellnrod, Germany) at 36.4-36.5°C temperature and 30% humidity; the egg position was regularly changed once or twice a day during the incubation period (*15-17*). These conditions allowed a continuous development of the embryo, given that artificial incubation may have a failure rate of 20-50% (*18*). A regular check of viability by candling was performed to avoid that dead

embryos caused spreading of infections. This simple visual monitoring method was used because no other reliable methods are described or commercially available.

Egg preparation

To allow the intravascular administration of contrast media and radiotracers into ostrich egg embryos, it was first necessary to identify suitable vessels in the amniotic membrane (Figs. 1B-D). This was performed by means of a light-intensive candling lamp (Tempo Nr. 119, Brecker Ltd. & Co. KG, Ruethen, Germany; or Powerlux Eggtester 4.5 VDC, Lyon Technologies Inc., Chula Vista, CA, USA), which allows a diaphanoscopy-like depiction through the eggshell (Fig. 1B). After the localization of a suitably large vessel, a rectangular piece of the 2-mm thick shell (2.5 x 5 cm) was removed with a rotating cutter (Dremel® 3000, Dremel Europe - Bosch Powertools B.V., Breda, The Netherlands) (Fig. 1E), paying attention to maintain the integrity of the inner shell membrane, i.e., the chorion-allantois membrane. Thereafter a vitelline vein was punctured with a thin 27G cannula, again with the help of a candling lamp. The cannula was fixed to the eggshell with tape (Figs. 1F-H). This access allowed the injection of CT contrast media or radiotracers by means of a plastic tube (Smiths Medical™ 800/100/100 Smiths, Smiths Medical International Ltd, Ashford, Great Britain). Formation of clots was prevented by flushing with heparin.

Anesthesia

Anesthesia is particularly relevant for nuclear medicine investigations, since motion is associated with imaging artifacts. Immobilization of the embryo is thus a prerequisite for the quality of the images (*13*). When chicken eggs are used for *in ovo*

research, anesthetics in gas form are commonly used (*13*). Isoflurane (Piramal Critical Care Deutschland GmbH, Hallbergmoos, Germany) at a maximum of 4% was thus chosen for this study using a standard vaporizer (Vaporizer Medimorph System, VEB MLW Medizintechnik Leipzig, Germany). To warrant a high concentration and a constant diffusion of the anesthetic gas into the embryonal blood, the egg was placed in a plastic bag for the whole duration of the contrast injection and image acquisition (Fig. 1H).

Investigations

Imaging was performed with a PET/CT scanner (Biograph mCT40, Siemens, Erlangen, Germany) for clinical investigations in humans. For this purpose – in addition to native CT – contrast-enhancement with media and radiotracers were also used (Fig. 2). The number of investigated eggs, the activities used, and other relevant parameters are shown in Table 1. Further experiments were performed using different tracers (e.g. ^{18}F- fluoroethyl-tyrosine; ^{68}Ga-tetraazacyclo-dodecane-tetraacetic-octreotide; ^{68}Ga-prostate-specific membrane antigen); however, these tracers were not investigated systematically in this study. Some eggs were investigated sequentially, either with long-lived radiotracers (one injection, several scans) or with short-lived radiotracers (several injections, several scans; Table 2).

Imaging parameters

The vertical positioning of the eggs was maintained using a base of polyurethane (Necuron 100, Necumer GmbH, Bohmte, Germany) (Fig. 1). First a full-dose CT was

acquired for the purpose of attenuation correction (120kV, 200mAs, slice thickness 0.6 mm, increment 0.3 mm, filter core H20). The CT data were reconstructed via filtered back projection. Finally a PET acquisition was obtained with 1 bed position (21.8 cm trans-axial field-of-view). The reconstruction was performed with a Gauss filter (full width half maximum 2.0) in an iterative manner (4 orders, 12 subsets) with a zoom of 2 and a matrix of 512x512. A dynamic PET in list-mode was also recorded (Supplemental Video 1 and 2).

Disposal of eggs

The hatching of ostrich eggs usually occurs between days 39 and 42, therefore the embryos were sacrificed by freezing on day 38 at the latest (*19*).

RESULTS

A total of 34 eggs were investigated, 15 of which did not contain an embryo, as shown by CT scan between days 6 and 12 (fertilization or incubation failure). These eggs were thus excluded from the analyses. The remaining 19 eggs underwent multiple CT scanning to verify that a continuous development occurred (Fig. 2).

Two of 19 eggs presented a secretion of thick fluid associated with arrest of development. The remaining 17 eggs were investigated at a mean development of 34.6 ± 2.5 days (range: 31-38; median 35).

The chorion-allantois membrane of 3 of 17 eggs was damaged during fenestration, thus no vessel catheterization was possible. The catheterization failed in 2 of the remaining 14 eggs, due to formation of a hematoma that limited the visibility of the vitelline vein. As a result, 12 eggs became fully available for imaging investigations.

The prepared access was used for multiple injections in 5 eggs, so that short-lived radiotracers for the investigation of the development stage could be performed with the same or a different tracer. Table 1 shows the radiotracer parameters for ^{18}F-NaF, ^{18}F-fluoro-deoxyglucose (FDG) and ^{124}I-NaI, respectively. Exemplary quantitative PET-data is shown in Table 2. Estimated effective doses range from 2.4 – 6.4 mGv for CT imaging.

The potential of a three-dimensional imaging that combines the high-resolution anatomy of CT images with the functional topography of PET is detailed in Fig. 3. Dynamic list-mode imaging with ^{18}F-NaF and ^{18}F-FDG are shown in Supplemental Video 1 and 2, respectively.

Concerning the issue of viability monitoring, several methods were tested (acoustic detection of cardiac tones via auscultation, cardiotocogram, electrocardiogram, or pressure measurements) but they did not prove suitable for this purpose. Only the cardiac pulsation could be identified via a modified egg-electrocardiogram (Buddy MK2, Avitronics, Cornwall, Great Britain) based on infrared phase delay caused by the blood flow through the embryo's vessels. However, the positioning of the egg until a good signal was obtained was lengthy and error-prone, and an electrocardiogram could be derived only in 3 of 10 eggs. Another promising approach, although not fully implemented, was the use of a magnetogram. This biomagnetic method is based on the detection of magnetic field changes arising from changes of the cardiac activity or embryo movements. This method has the advantage of detecting in parallel the viability and the movement of the embryos.

Ostrich embryos showed a regular motion and change of position from day 2 of development, as could be seen with CT series and dynamic PET investigations. Indeed, in spite of the use of maximum concentrations of isoflurane (3.5-4%), the immobilization of the embryos during PET scanning remained insufficient in 10 of 12 cases (Supplemental Video 1 and 2).

DISCUSSION

The present study showed that *in ovo* imaging with conventional PET/CT is not only a feasible option, but is also associated with a good quality of the images (Figs. 2 and 3). The use of readily-available imaging modalities for *in ovo* research with ostrich embryos offers several advantages are shown in Table 3. Also, given the clarity of images observed with conventional PET/CT, it is conceivable that the use of ostrich eggs is transferable to other common modalities of clinical imaging, e.g., SPECT, CT, MRI, and ultrasonography. As shown in Supplemental Videos 1 and 2, dynamic radionuclide studies using ^{18}F-NaF and ^{18}F-FDG are also feasible, augmenting the area of application.

An additional, major benefit of a larger availability of *in ovo* research with ostrich eggs is also the possibility to reduce the need of animal research, thus achieving a positive societal effect by reducing the number of experiments with fully developed animals (*11*) and by limitating the use of mammals. Indeed, chicken *in ovo* imaging has already been proposed in the past as a feasible alternative to imaging studies with mice (*10*). Even genome modification strategies have been described for avian species, conceivably allowing for *in ovo* knock-out disease models as these are widely used in mice and rats (*20*). Furthermore, *in ovo* research enables studies evaluating processes that occur during embryonal development which are difficult to perform in mammals. Fig. 2 shows sufficient visualization of embryonal structures starting from the second half of development. Imaging studies at earlier development stages are presumably limited by small organ size (*18*).

If *in ovo* research with ostrich eggs is used for imaging, the matter of reproducible quantification needs to be considered. Preliminary PET data analysis regarding sequential ^{18}F-FDG and ^{124}I-NaI studies produced homogenous results (Table 2), indicating that this concept might be suitable for quantification as well. However, this aspect has not been investigated systematically yet and further studies are planned addressing this issue.

At an activity concentration of 13.5 kBq/ml, the recovery coefficients for ^{18}F ranged from 0.15-0.84 for small volume spheres of 3.95-15.43 mm, respectively (own unpublished data). Regarding the image quality, conventional scanners are outperformed by dedicated small animal devices. The PET/CT scanner used in the present study is characterized by a resolution of 4.2 x 4.2 x 4.5mm and the nanoScan PET/CT (PET 8/2, Mediso, Budapest, Hungary) offers a resolution of 0.8x0.8x0.85mm (*21*). Considering that organs of ostrich embryos are naturally larger than those of chicken embryos (e.g. liver size: 15ml vs. 2ml) (*18,22*), it is conceivable that imaging is comparable. This aspect has to be evaluated in future studies. Obviously, due to the small gantry size of 98.6mm the small animal device does not allow the investigation of ostrich eggs (*21*).

The use of ostrich eggs required several technical adaptions compared to the methods described for chicken eggs. While the issues of eggshell access, vessel catheterization, and reconstruction parameters were addressed in this study, several aspects need further investigations. The monitoring of embryo viability by means of conventional commercial methods (Buddy MK2), for example, was not satisfactory. A potential improvement in this case is the use of a magnetometer, which would allow the

monitoring of cardiac activity in a non-invasive manner (*23*). Another issue is the anesthesia, because most of the embryos moved in spite of maximum anesthesia levels using isoflurane. The maximum isoflurane concentration was limited by the specific vaporizer used. We conclude that isoflurane is not suitable for effectively reducing motion, conceivably due to its limited diffusion through the egg shell. Since movement is a major drawback for imaging experiments, this problem requires a systematic investigation for a general solution that guarantees artifact-free images. The use of i.v.-anesthetics as well as muscle relaxants might represent suitable approaches addressing this problem. The negative influence of motion on the image quality has also been described for chicken eggs investigated with small animal MRI scanners which are prone to motion artifacts in a similar way unless ultrafast gradients are used (*8,24*). Both MRI and PET/CT imaging protocols rely on data acquisition over several minutes, emphasizing the need for effective immobilization. From a technical point of view, list mode data acquisition allows for motion correction in PET/CT data sets; however, erroneous attenuation correction caused by incongruence between CT and PET and thus, imprecise SUV measurements, needs to be considered. Effective doses by CT are comparably low considering published doses ranging from 5mGy to 4.5Gy for small animal CT devices (*25*). Therefore, radiation attributable to CT in this concept (6.4 mGy) presumably does not influence the embyro's viability.

Limitations: The present study was a proof-of-concept investigation of the feasibility of an imaging model *in ovo*, and was not designed to cover the arising issues in a systematic way. Also, the imaging investigations were exclusively focused on the

use of ostrich embryos, and the results are thus not transferable to mammals, i.e. humans without restrictions. Furthermore, it is not yet clear that an embryo is a suitable model for imaging processes that occur in adults. Further disadvantages of this model are described in Table 3.

CONCLUSION

The proposed method of *in ovo* imaging of ostrich embryos by means of routine scanners allows a broader application of this field of preclinical research, since the techniques available to date require the use of dedicated and costly imaging scanners. This novel method may also contribute to reduce the number of tests based on classic animal research.

Further experiments are warranted to refine the *in ovo* approach with ostrich eggs, in particular with regard to improvement of viability monitoring motion and reduction of artifacts.

Financial Disclosure

This study was funded exclusively with intramural grants from the Jena University Hospital.

Disclaimer

The authors do not have any conflicts of interest to declare.

Acknowledgements

Dr. Ernesta Palombo-Kinne is gratefully acknowledged for language assistance with this manuscript. Prof. Dr. Hans-Joachim Mentzel, Prof. Dr. Jürgen R. Reichenbach, Dr. Daniel Güllmar and Dr. Karl-Heinz Herrmann (Institute of Diagnostic and Interventional Radiology, Jena University Hospital) are gratefully acknowledged for technical advice regarding MRI imaging. We thank Dr. Susanne Bischoff (Institute of Lab Animal Sciences and Welfare, Jena University Hospital) and Prof. Dr. Christian Hübner (Institute of human genetics, Jena University Hospital) for general advice in animal experiments. Furthermore we would like to express our thanks to Prof. Ekkehard Schleußner and Prof. Dr. Uwe Schneider (Department of Obstetrics, Jena University Hospital) for advice in monitoring embryonal viability.

REFERENCES

1. Czarnecki CM, Sujarit VK. Effects of Furazolidone in the early Chick Embryo. *Poult Sci*. 1979;58:988-990.
2. Deryugina EI, Quigley JP. Chick Embryo Chorioallantoic Membrane Model Systems to study and visualize Human Tumor Cell Metastasis. *Histochem Cell Biol*. 2008;130:1119-1130.
3. Rashidi H, Sottile V. The Chick Embryo: Hatching a Model for contemporary Biomedical Research. *Bioessays*. 2009;31:459-465.
4. Woloszyk A, Liccardo D, Mitsiadis TA. Three-Dimensional Imaging of the developing Vasculature within Stem Cell-seeded Scaffolds cultured in ovo. *Front Physiol.* 2016;7:146.
5. Kivrak Pfiffner F, Waschkies C, Tian Y, Woloszyk A, Calcagni M, Giovanoli P, et al. A new in vivo MRI Method to non-invasively monitor and quantify the Perfusion Capacity of 3D-Biomaterials grown on the Chorioallantoic Membrane of Chick Embryos. *Tissue Eng. Part C, Methods.* 2014;21:1–8.
6. Oppitz M, Pintaske J, Kehlbach R, Schick F, Schriek G, Busch C. Magnetic Resonance Imaging of Iron-Oxide labeled SK-Mel 28 Human Melanoma Cells in the Chick Embryo using a Clinical whole Body MRI Scanner. *MAGMA.* 2007;20:1–9.
7. Peebles, DM, Dixon JC, Thornton JS, Cady EB, Priest A, Miller SL, et al. Magnetic Resonance Proton Spectroscopy and diffusion weighted Imaging of Chick Embryo Brain in ovo. *Brain Res Dev Brain Res.* 2003;141:101–107.

8. Bain MM, Fagan AJ, Mullin JM, McNaught I, McLean J, Condon B. Noninvasive Monitoring of Chick Development in ovo using a 7T MRI System from day 12 of Incubation through to Hatching. *J Magn Reson Imaging* 2007;26:198–201.

9. Boss A, Oppitz M, Wehrl HF, Rossi C, Feuerstein M, Claussen CD, et al. Measurement of T1, T2, and Magnetization Transfer Properties During Embryonic Development at 7 Tesla Using the Chicken Model. *J Magn Reson Imaging.* 2008;28:1510–1514.

10. Haller S, Ametamey SM, Schibli R, Muller C. Investigation of the Chick Embryo as a potential Alternative to the Mouse for Evaluation of Radiopharmaceuticals. *Nucl Med Biol*. 2015;42:226-233.

11. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving Bioscience Research Reporting: the ARRIVE Guidelines for Reporting Animal Research. *PLoS biology*. 2010;8:e1000412.

12. Gebhardt P, Wurbach L, Heidrich A, Heinrich L, Walther M, Opfermann T, et al. Dynamic Behaviour of selected PET Tracers in embryonated Chicken Eggs. *Rev Esp Med Nucl Imaging Mol*. 2013;32:371-377.

13. Heidrich A, Wurbach L, Opfermann T, Saluz HP. Motion-artifact-free in vivo Imaging utilizing narcotized Avian Embryos in ovo. *Mol Imaging Biol*. 2011;13:208-214.

14. Wurbach L, Heidrich A, Opfermann T, Gebhardt P, Saluz HP. Insights into Bone Metabolism of Avian Embryos in ovo via 3D and 4D 18F-fluoride Positron Emission Tomography. *Mol Imaging Biol*. 2012;14:688-698.

15. Hassan SM, Siam AA, Mady ME, Cartwright AL. Incubation Temperature for Ostrich (Struthio camelus) Eggs. *Poul Sci*. 2004;83:495-499.

16. van Schalkwyk SJ, Brand Z, Cloete SWP, Blood JR. The Influence of different Disinfection Protocols on the Hatching Performance of Ostrich Eggs. In: Huchzermeyer FW, ed. *Ratites in a competitive World Proceedings of the 2nd international Ratite Congress*. Oudtshoorn, South Africa: 1998:158.

17. Deeming DC. Factors affecting Hatchability during commercial Incubation of Ostrich (Struthio camelus) eggs. *Br Poult Sci*. 1995;36:51-65.

18. Deeming, DC, Ar A. Factors affecting the Success of commercial Incubation. In: Deeming, DC, ed. *The Ostrich: Biology, Production and Health, CAB International*. Wallingford, United Kingdom:159-190.

19. Leary S, Underwood W, Antony R, Cartner S, Corey D, Grandin T, et al. AVMA Guidelines for the Euthanasia of Animals: 2013 Edition. In: Association AVM, ed. *AVMA Guidelines for the Euthanasia of Animals: 2013 Edition. 1: American Veterinary Medical Association*. Schaumburg, IL, USA:2013:63.

20. Lee HJ, Kim YM, Ono T, Han JY. Genome Modification Technologies and their Applications in Avian Species. *Int J Mol Sci.* 2017;18:2245.

21. Gaitanis A, Kastis GA, Vlastou E, Bouziotis P, Verginis P, Anagnostopoulos CD. Investigation of Image Reconstruction Parameters of the Mediso nanoScan PC Small-Animal PET/CT Scanner for Two Different Positron Emitters Under NEMA NU 4-2008 Standards. *Mol Imaging Biol*, 2017;19:550-559

22. Maatjens CM, van Roovert-Reijrink IAM, Engel B, van der Pol CW, Kemp B, van den Brand H. Temperature during the last Week of Incubation. I. Effects on

Hatching Pattern and Broiler Chicken embryonic Organ Development. *Poult Sci*, 2016;95:956–965.

23. Goernig M, Liehr M, Tute C, Schlosser M, Haueisen, J, Figulla HR, et al. Magnetocardiography based spatiotemporal Correlation Analysis is superior to conventional ECG Analysis for identifying myocardial Injury. *Ann Biomed Eng.* 2009;37:107-111.

24. Waschkies C, Nicholls F, Buschmann J. Comparison of Medetomidine, Thiopental and Ketamine/Midazolam Anesthesia in Chick Embryos for in ovo Magnetic Resonance Imaging free of Motion Artifacts. *Sci Rep*. 2015;5:15536.

25. Meganck JA, Liu B. Dosimetry in Micro-computed Tomography: a Review of the Measurement Methods, Impacts, and Characterization of the Quantum GX Imaging System. *Mol Imaging Biol*. 2017;19:499-511.

FIGURES

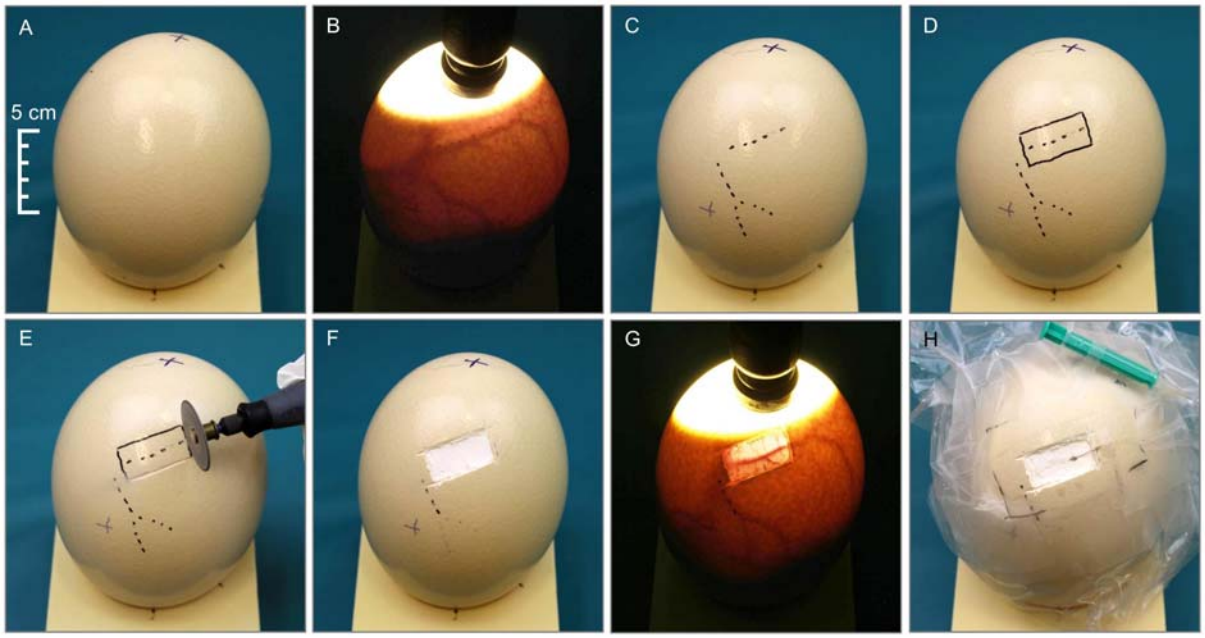

Fig. 1 Preparation of an ostrich egg (day 36 of incubation). **A:** Vertically-placed ostrich egg stabilized on a polyurethane foam base. **B:** Light-intensified candling lamp enhancing the air cell, below which the egg content and vessels become visible. **C:** The vessel profiles are drawn on the eggshell and a suitable place for fenestration is marked (**D). E:** Fenestration by means of a rotating cutter. **F:** After removal of the eggshell, the chorion-allantois membrane is now visible. **G:** Repeated candling of the vessels. **H:** Puncture and insertion of a 27G cannula. A 3.5% mixture of isoflurane/air contained in a plastic bag sealed around the egg is used to immobilize the embryo, whereas the operating window is kept open to enable full interventional access.

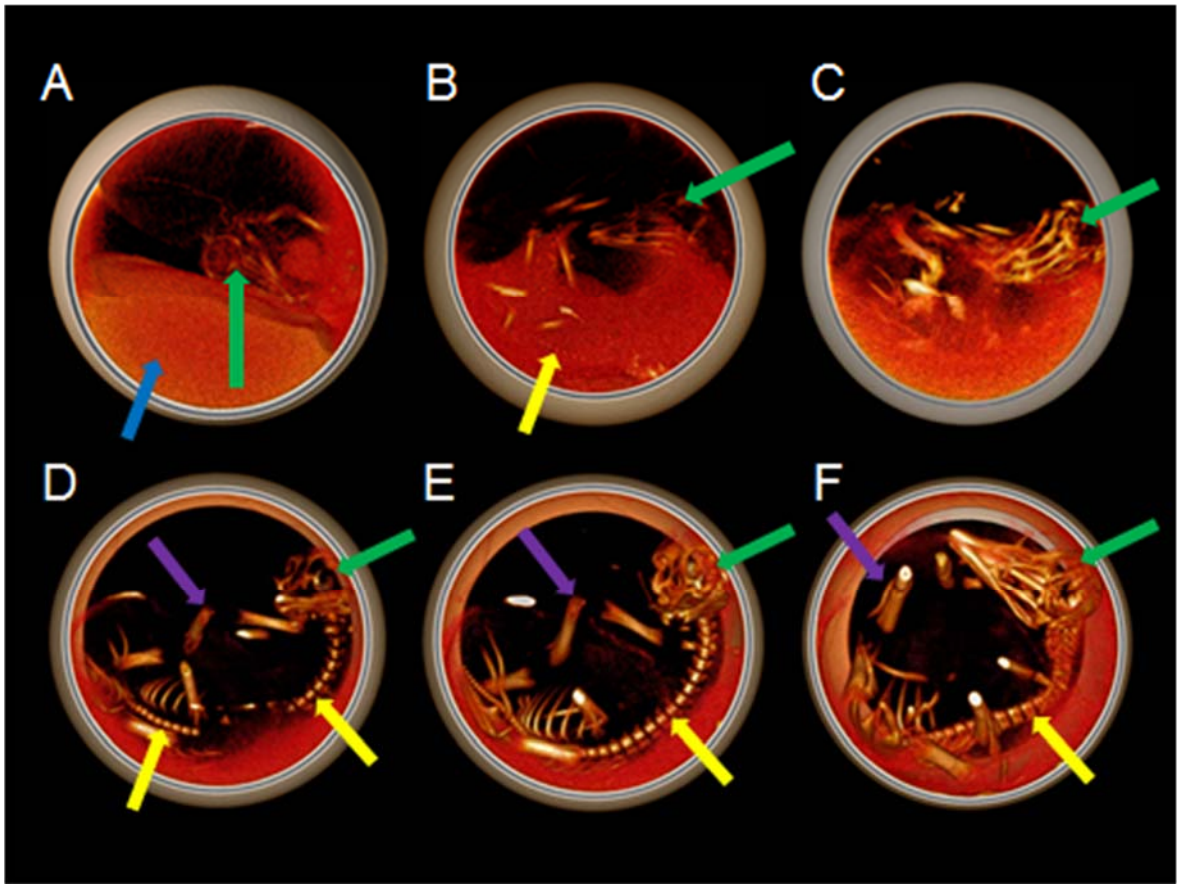

Fig. 2 Serial computed tomography (CT) images of a developing ostrich embryo, three-dimensional volume rendered CT (3D-VRT) with trans-axial view and visualization of bone tissue. The size is measured from the vertex to the end of the tail. **A:** Day 24 of incubation; size 121 mm: Skull (green arrow) and yolk (blue arrow) are depicted. **B:** Day 29; size 149 mm: Faint osseous structures with vertebrae (yellow arrow). **C:** Day 32; size 186 mm: Larger bone structures as compared to B, bone calcification increases with increasing incubation time. **D:** Day 34; size 207 mm: The embryo occupies more than half of the egg. Vertebrae (yellow arrows) and head (green arrow) are now clearly visible, the lower extremities (purple arrow) are also identified. **E:**

Day 36; size 228 mm: Progressive embryonal growth. **F:** Day 38; size 241 mm: Gradually decreasing surrounding space and further embryonal growth.

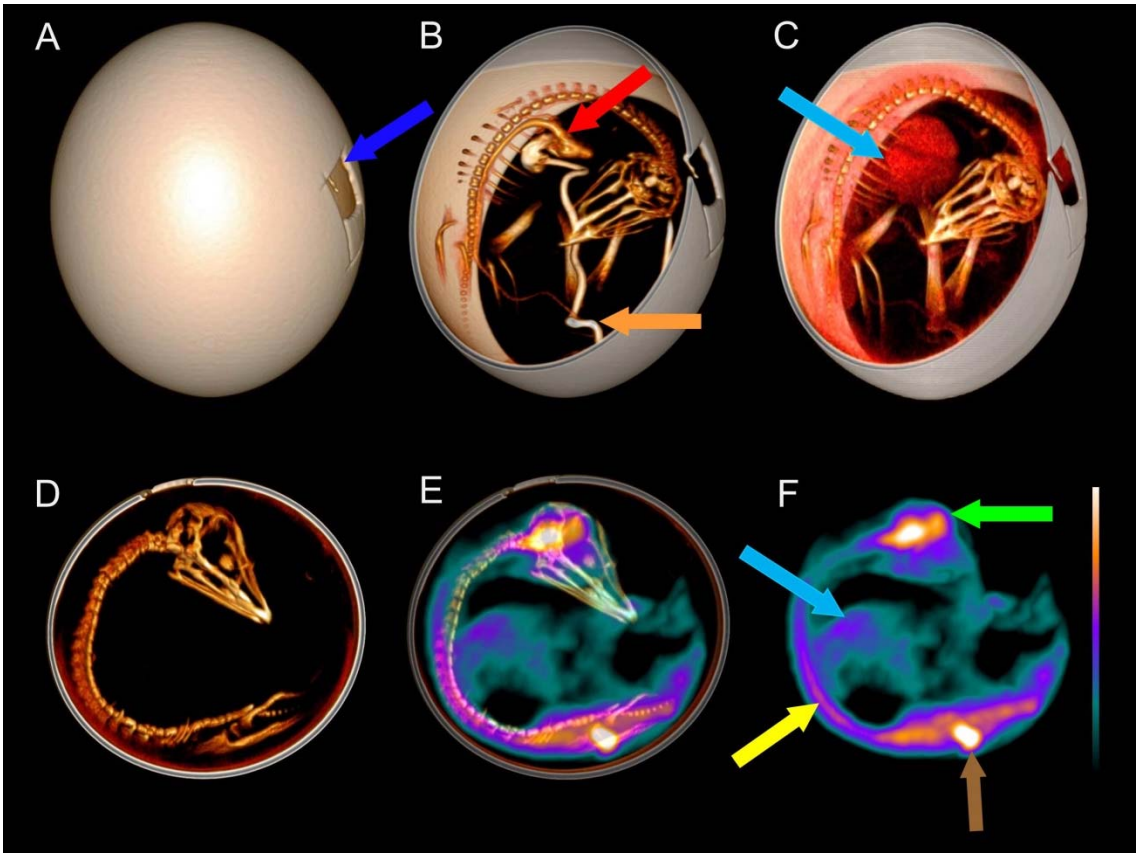

Fig. 3 ^{18}F-FDG-PET/CT of an ostrich embryo (day 32 of incubation). **A:** Three-dimensional volume rendered CT (3D-VRT) of an egg with partially removed eggshell (access window; dark blue arrow). **B:** 3D-VRT with a dual clipping plane revealing the inner structures. Texture mapping was applied for visualization of bone tissue. This early-sequence image was obtained after administration of 1 ml of standard contrast agent (Ultravist 300; Bayer, Leverkusen, Germany) into a vitelline vein. As the vitelline circulation empties into the omphalomesenteric vein, these vessels (orange arrow) and both atria are clearly visible. Heart ventricles and aorta are also faintly enhanced (red arrow). **C:** Same scanning conditions as in B, but now showing a later sequence with faint imaging of the liver (light blue arrow) and blood pool. **D** to **F:** 3D-VRT with a slim dual clipping plane and texture mapping, showing the eggshell and the axial skeleton of the embryo, and combining morphologic and metabolic information: CT image (**D**), ^{18}F-FDG-PET/CT image (**E**) and ^{18}F-FDG PET image (**F**) with intense homogeneous tracer

accumulation within the brain (green arrow) and allantois (excretion, brown arrow), as well as in the bone marrow (yellow arrow) and liver (light blue arrow; low intensity).

TABLES

Table 1 Number of performed imaging tests and parameters used.

Parameter	n	Mean ± SD (range; median)
Eggs	34	-
CT scans, native	204	-
CT scans, contrast-enhanced	2	injected volume 2.5 ± 0.7 ml (2.0 - 3.0)
PET examinations	18	injected volume 1.3 ± 0.8 ml (0.6 - 3.0; 1.0)
^{18}F-FDG-PET/CT	8	injected activity 32.8 ± 4.4 MBq (29.1 - 40.1; 30.3)
^{18}F-NaF	1	injected activity 38.0 MBq
^{124}I-NaI	6*	injected activity 0.8 ± 0.3 MBq (0.54 - 0.99)

FDG-PET/CT: Fluoro-deoxyglucose positron emission tomography/ computed tomography. * ^{124}I-NaI was injected once into two eggs, afterwards 4 datasets were acquired for the first egg and two datasets for the second egg at different time points, considering a relatively long half-life of 100 hrs.

Table 2. Sequential quantitative PET data

Tracer	Day of incubation	Injected activity [MBq]	Time difference between injection and acquisition	Scan duration [min]	Organ activity concentration [kBq/ml]					
^{18}F-FDG					**Liver**		**Brain**		**Yolk**	
					max	**mean***	**max**	**mean****	**max**	**mean*****
	27	30.3	28 min	10 min	160.4	133.4	151.0	136.3	0	0
	28	36.7	29 min	10 min	163.8	139.4	155.3	140.3	0.2	0.1
	29	40.1	30 min	10 min	178.8	159.1	159.8	136.2	0	0
	32	34.8	30 min	10 min	5225.3	729.6	0	0	0	0
^{124}I-NaI					**Thyroid**		**Brain**		**Yolk**	
					max	**mean******	**max**	**mean****	**max**	**mean*****
	29	0.99	2 hrs	10 min	4.3	2.0	1.1	0.5	0.01	0
	30	0.99	20 hrs	10 min	3.0	1.4	0.6	0.2	0.6	0.1
	31	0.99	44 hrs	10 min	7.9	2.4	0.4	0.2	0.9	0.2
	33	0.99	96 hrs	10 min	10.9	6.2	0.2	0.1	1.6	0.4

FDG: Fluoro-deoxyglucose. NaI: Sodium-iodine. VOI: volume of interest. * Spherical VOI 1.5 ml. ** Spherical VOI 1.0 ml. *** Spherical VOI 5.0 ml. ****Isocontour VOI, threshold 35% of maximum activity.

Sequential ^{18}F-FDG PET/CT with four injections of FDG on different days produces homogenous results of representative VOIs for the first three studies. The fourth study without brain uptake is associated with the embryo's death between day 29 and 32 of incubation. High liver uptake is contributable to adjacent blood pool activity in the heart and thus represents an artifact.

Sequential ^{124}I-NaI PET/CT with one injection of NaI and four scans on different days produces increasing thyroid activity over time, attributable to continuous iodine trapping within the thyroid. Gradually decreasing brain activity, presumably due to reduction of blood pool activity over time. Increasing yolk activity over time, most likely attributable to unspecific tracer distribution and partial excretion within this compartment.

Table 3 Advantages and disadvantages of *in ovo* imaging with ostrich eggs.

Advantages	Disadvantages
• Availability (no dedicated small animal imaging device necessary)	• Rather high costs per ostrich egg (20-130 EURO) compared to chicken eggs
• Low costs regarding equipment, premises and personell (if a clinical scanner used for humans is available on site)	• Lower availability of ostrich eggs (March – September) compared to chicken eggs
• No need for appropriate mice or rat husbandry	• Lower spatial resolution compared to dedicated small animal devices
• Not considered animal testing, simplifying study design planning	• Experiments are usually in the afternoon/evening (after all patients have been examined)
• Large organ size compared to chicken embryos	
• Lower radiation exposure compared to small animal CT devices	
• Reduced CT acquisition time compared to flat-panel small animal CT devices → higher temporal resolution	