Quantification of Brain Access of Exendin-4 in the C57BL Mouse Model by SPIM Fluorescence Imaging and the Allen Mouse Brain Reference Model

Casper Bo Jensen[1(✉)], Anna Secher[2], Jacob Hecksher-Sørensen[2], Knut Conradsen[1], and Rasmus Larsen[1]

[1] Department of Applied Mathematics and Computer Science, Technical University of Denmark, Kgs. Lyngby, Denmark
{cboa,knco,rl}@dtu.dk
[2] Novo Nordisk, Global Research, Måløv, Denmark
{aasc,jhes}@novonordisk.com

Abstract. With the recent advance in 3D microscopy such as Single Plane Illumination Microscopy (SPIM) it is possible to obtain high resolution image volumes of the entire mouse brain. These data can be used to study the access of several peptides such as the glucagon-like peptide-1 (GLP-1) analogue Exendin-4, into the brain with the aim of developing medication for obesity. To investigate mode of action of the medication it is important to identify the specific anatomical brain nuclei that are targeted by the compound. Such segmentations can be obtained using an annotated digital brain atlas. We construct a SPIM brain atlas based on the Allen mouse brain 3D reference model and use it to analyze the access of peripherally injected Exendin-4 into the brain compared to a negative control group. The constructed atlas consists of an average SPIM volume obtained from eight C57BL mouse brains using group-wise registration. A cross-modality registration is performed between the constructed average volume and the Allen mouse brain reference model to allow propagation of annotations to the SPIM average brain. Finally, manual corrections of the annotations are performed and validated by visual inspection. The study shows that Exendin-4 have access to brain regions such as the arcuate hypothalamic nucleus and the nucleus of the solitary tract, which are areas involved in regulating food intake.

Keywords: Mouse brain · Allen mouse brain 3D reference model · Digital atlas · Atlas segmentation · Image registration

1 Introduction

Obesity is a disease, which has become a worldwide epidemic growing at an alarming rate. For the individual the consequence of obesity is an increased risk of acquiring metabolic disorders like type 2 diabetes and cardiovascular diseases.

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Glucagon-like peptide-1 (GLP-1) analogues such as Exendin-4 have shown a positive effect on weight loss and thus belongs to a class of peptides which might be used for tomorrow's obesity medication. A way to study the distribution of GLP-1 analogues in the brain after administration is using histology, [3]. Due to the numerous histological slides that need to be produced, these types of studies are expensive and time consuming. Additionally, it is difficult to compare results across studies due to the difficulty of precisely denoting anatomical structures in the data.

One technique that might overcome these limitations is the new imaging modality Single Plane Illumination Microscopy (SPIM), which is a non-destructive method to produce well-registered optical sections suitable for 3D reconstruction [7]. SPIM is a type of fluorescence microscopy with the ability to produce 3D data of macroscopic objects in cellular resolution, e.g. of the mouse brain [5].

A popular segmentation strategy in neuro-imaging is the use of computational atlases, used in various imaging modalities both in human and animal models [4]. An atlas typically consists of a reference volume with accompanied annotations. The reference volume can be produced by computation of averages of ensembles of segmented images after image registration, [2]. A new image can then be segmented by image registration to the atlas volume and superimposition of the segmented structures from the atlas. For common imaging modalities some computational atlases of the mouse brain are available, e.g. [6], [9]. To the authors' knowledge no SPIM brain atlases have been published.

We construct a SPIM atlas by group-wise registration of recorded mouse brains. A good initial guess of the annotations are obtained by cross-modality registration to an existing atlas, and finally manual corrections are performed to complete the atlas. The Allen mouse brain reference model, [9], is used as the source of the annotations due to the model's high quality and detailed annotations.

We use the constructed atlas to investigate the access of peripherally injected Exendin-4 into the C57BL mouse brain based on quantification inside the segmented brain structures.

2 Method

2.1 Allen Mouse Brain Reference Model

A 3-D reference model, [9], is available for download using the API of the Allen Brain Atlas Data Portal [1]. The model is based on 132 annotated coronal plates of the adult mouse brain. Annotations are transferred from this reference model onto the constructed SPIM brain atlas.

Reference Volume. The Allen 3-D brain volume was reconstructed from annotated plates using a combination of high frequency section-to-section histology registration with low-frequency histology to (ex-cranio) MRI registration. The reference volume was constructed in 2006, [15], and later updated in 2011. The

volume is based on Nissl stains that highlights brain regions of high nuclei concentration. The volume is available for download in 25 μm isotropic resolution. Prior to usage a smoothing of the reference volume and removal of the outer part of the olfactory bulb were performed.

Reference Annotations. The full Allen atlas consists of 1204 symmetric 3-D structures, which are more than needed in this study. For the study of GLP-1 analogues 56 structures were chosen, with the highest concentration of structures in regions relevant for appetite control and obesity such as the hypothalamus. Each structure is color-coded to visually show the hierarchical position in the brain.

2.2 SPIM Brains

In total 16 mice brains were recorded using SPIM. Eight brains were used for atlas construction and eight brains were used to quantify the access of Exendin-4 into the brain.

Tissue Preparation and Data Recording. The conditions of housing and care of the animals as well as performance of the experiments are in agreement with the Danish law of animal experimentation. After sedation the mice were euthanized by cardiac perfusion with heparinized saline followed by neutral buffered formalin. The brains were removed and cleared for SPIM by a stepwise dehydration and clearing process using tetra hydro-flurane and di-benzyl-ether (DBE), respectively.

Data were recorded with a dual side illumination Lavision system utilizing a SuperK white light laser, a 620-nm emission filter, a 700-nm excitation filter, an Olympus microscope, and an Andor Neo sCMOS camera. The brain samples were immersed in a DBE bath during recording to minimize the difference in refractive index. The recordings were performed in 5.16 μm isotropic resolution.

2.3 Atlas Construction

The constructed atlas contains 56 brain structures. Using the naming convention of the Allen reference model, [9], the structures are: Isocortex, AOB, AON, MOB, HPF, CA1, CA2, CA3, DG, BLA, ACB, CEA, CP, LSc, LSr, LSv, MS, SF, SH, TH, PVH, PVHd, ARH, SO, Pvi, PVa, Pvpo, OV, SFO, DMH, SCH, PVp, PH, VMH, LHA, ZI, ME, CLI, DR, IF, SNc, SNr, IPN, RL, VTA, PBl, PBm, AP, NTS, CB, ts, VL, AQ, c, V3, and V4. Two additional annotations are included consisting of other tissue and background, respectively.

Pre-processing. Following an initial cropping, the brain volumes were down sampled to 15 μm isotropic resolution and the axes were reordered to match

the orientation of the Allen reference model with the first axis being dorsal-ventral, the second axis being lateral-medial, and the third axis being rostral-caudal. The data recording process deformed the structure of the olfactory bulb. Therefore this structure was cropped from the data along with the medulla oblongata, which is not part of the Allen reference model. The background voxels were set to zero and additional zero-padding was performed to center the brain inside the volume. Data obtained with the SPIM modality contains a bias field due to continuos attenuation of the laser sheet by the tissue sample, as well as a non-uniform intensity profile in the generated laser sheet depending on the microscope construction. Bias field correction was applied using a toolbox developed in [8]. Outliers originating from air bubbles trapped in the ventricular system during the tissue preparation process were removed.

After the individual pre-processing of the brains, intensity normalization was performed based on matching the median value of the brains. This was done to avoid creating an average volume biased towards scans with higher intensities caused by different microscope settings rather than on real biological differences.

Creation of SPIM Atlas. Creation of the SPIM atlas was based on image registration between recorded SPIM data and the Allen reference model. The main registrations used in this work are based on a hierarchical transformation model, introduced in [11], which captures the global and local deformations of the tissue,

$$\mathbf{T}(x, y, z) = \mathbf{T}_{global}(x, y, z) + \mathbf{T}_{local}(x, y, z) \tag{1}$$

where the global transformation is a standard affine transformation. The local transformation model, a Free Form Deformation (FFD), is in 3D defined by an $n_x \times n_y \times n_z$ mesh of control points $\mathbf{\Phi}$ with spacing (δ_x,δ_y,δ_z). The underlying image is then deformed by manipulating the mesh of control points based on B-splines.

A fast way to regularize the deformation is to apply multi-level B-splines [12]; i.e. given control point resolutions $\Phi_1 ... \Phi_H$, at levels $1, ..., H$, the final local transformation is the sum of the transformations at each level,

$$T_{local} = \sum_{h=1}^{H} T_{local}^{h} \tag{2}$$

All transformations were calculated and applied using the Image Registration Toolkit, which was used under Licence from Ixico Ltd. [11], [12], [14].

The constructed SPIM atlas, based on the eight recorded SPIM scans, was created based on the following algorithm,

Algorithm 1. SPIM atlas construction

1: Register (rigid) all mouse brains to Allen reference model
2: Template = Intensity average of all registered mouse brains
3: **Repeat**
4: Register (FFD) all mouse brains to template
5: Template = Intensity average of all registered mouse brains
6: **Until** template stops changing
7: Register (FFD) Allen reference model (volume data + annotation data) to SPIM template

In line 1, using normalized mutual information, all scans were linearly (3 degrees of freedom for rotations, 3 degrees of freedom for translations) registered towards a pre-existing atlas, namely the Allen reference model. All scans were then averaged to create the first population atlas, representing the average anatomy of the study samples. An iterative 3 generation multi scale non-linear alignment procedure was then begun, registering each mouse towards the atlas of the previous non-linear generation using cross correlation as similarity measure. In line 6, the root-mean-square error between the voxel intensities of the current atlas and the previous atlas was calculated and an appropriate threshold value chosen to define the state where the atlas stopped changing. In line 7, the FFD transformation was calculated again based on the volume data of the Allen reference model. The calculated transformation was then applied to the annotation data of the model to bring these into alignment with the SPIM atlas volume. The last step in completing the SPIM atlas was manual correction of the computed annotations.

2.4 Exendin-4 Study

A concentration of 240 nmol/kg Exendin-4 was fluorescently labelled with VivoTag-S 750 (Perkin Elmer) and injected into 6-8 weeks old male C57BL mice (Taconic) two hours prior to euthanization.

A two channel SPIM scan was performed of the brains with one channel recording the auto-fluorescence of the tissue, and the other channel recording the specific signal of the labelled Exendin-4. Similarly, four negative control brains were scanned following vehicle injection using the same data recording protocol. Segmentations of the brains were obtained by non-linear registration to the constructed atlas and propagation of the annotations. Detection of signal in the statistical analysis was performed by observing the 95th percentile inside each segmented brain area after removal of any bleed-through signal from the auto-fluorescence channel. The 95th percentile was used to eliminate outliers caused by small air bubbles typically situated on the surface of the tissue.

3 Results

3.1 Overview of Constructed Atlas

A mid-coronal (a-b), mid-transverse (c-d), and mid-sagtial (e-f) view of the constructed SPIM atlas is seen in Figure 1. The left column of images shows the reference volume, and the right column shows the annotations overlaid onto the reference volume.

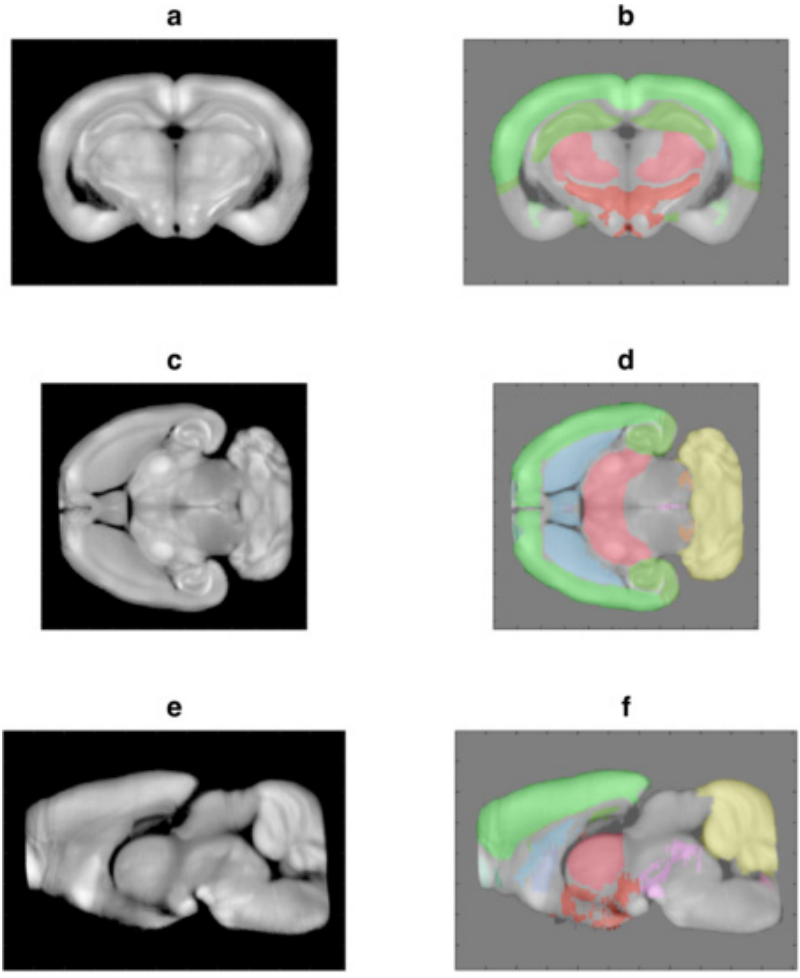

Fig. 1. Overview of constructed atlas. Left column (a,c,e) shows the atlas volume and the right column (b,d,f) shows the atlas with overlaid annotations. Green denotes the isocortex, red colors are part of the hypothalamus and thalamus, yellow is the cerebellum, blue structures belong to cerebral nuclei, and purple denotes midbrain structures.

3.2 Segmentation Example

An example of an automatic segmentation performed using the constructed atlas is seen in Figure 2. The left image shows an example of an auto-fluorescence channel recorded as part of the Exendin-4 study, and the right image shows the corresponding specific channel overlaid with calculated annotations. Strong signals are seen ventrally in the hypothalamic structures and caudally near the hindbrain structures.

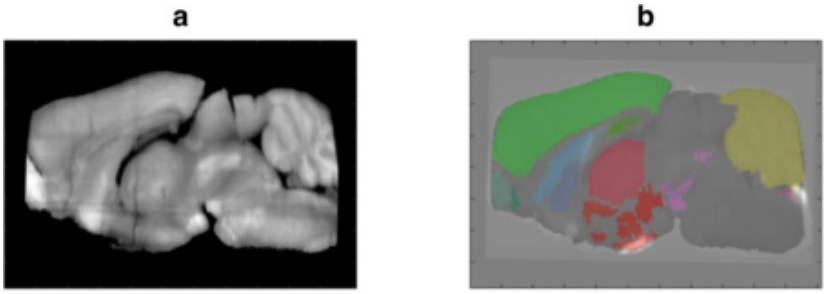

Fig. 2. Example of segmentation of a mouse brain following injection of fluorescent labelled Exendin-4. (a): Auto-fluorescence channel of the recording. (b): Specific fluorescence channel overlaid with calculated segmentaiton. High intensity (white) corresponds to a high fluorescence signal. Green denotes the isocortex, red colors are part of the hypothalamus and thalamus, yellow is the cerebellum, blue structures belong to cerebral nuclei, and purple denotes midbrain structures.

3.3 Quantification Results

Quantification results inside segmented brain regions for the Exendin-4 study are seen in Figure 3. The bar height for each structure corresponds to the mean value of the 95th percentile detected inside that structure in the four mouse brains. The error bars show the standard deviation. Signal is detected inside Nucleus of the solitary tract (NTS), Area postrema (AP), Median eminence (ME), Periventricular hypothalamic nucleus - posterior part (PVp), Vascular organ of the lamina terminalis (OV), Periventricular hypothalamic nucleus - anterior part (PVa), Arcuate hypothalamic nucleus (ARH), Septofimbrial nucleus (SF), Lateral septal nucleus, caudal part (LSc). Signal is defined as a p-value less than 0.05 when performing a two sample t-test between the Exendin-4 group and the negative control group, with the null hypothesis of equal means in the groups, and the alternative hypothesis being the Exendin-4 group having a larger mean value than the negative control group. The sample size is four mice in each group. The scale of the x-axis on the plot is in arbitrary unit.

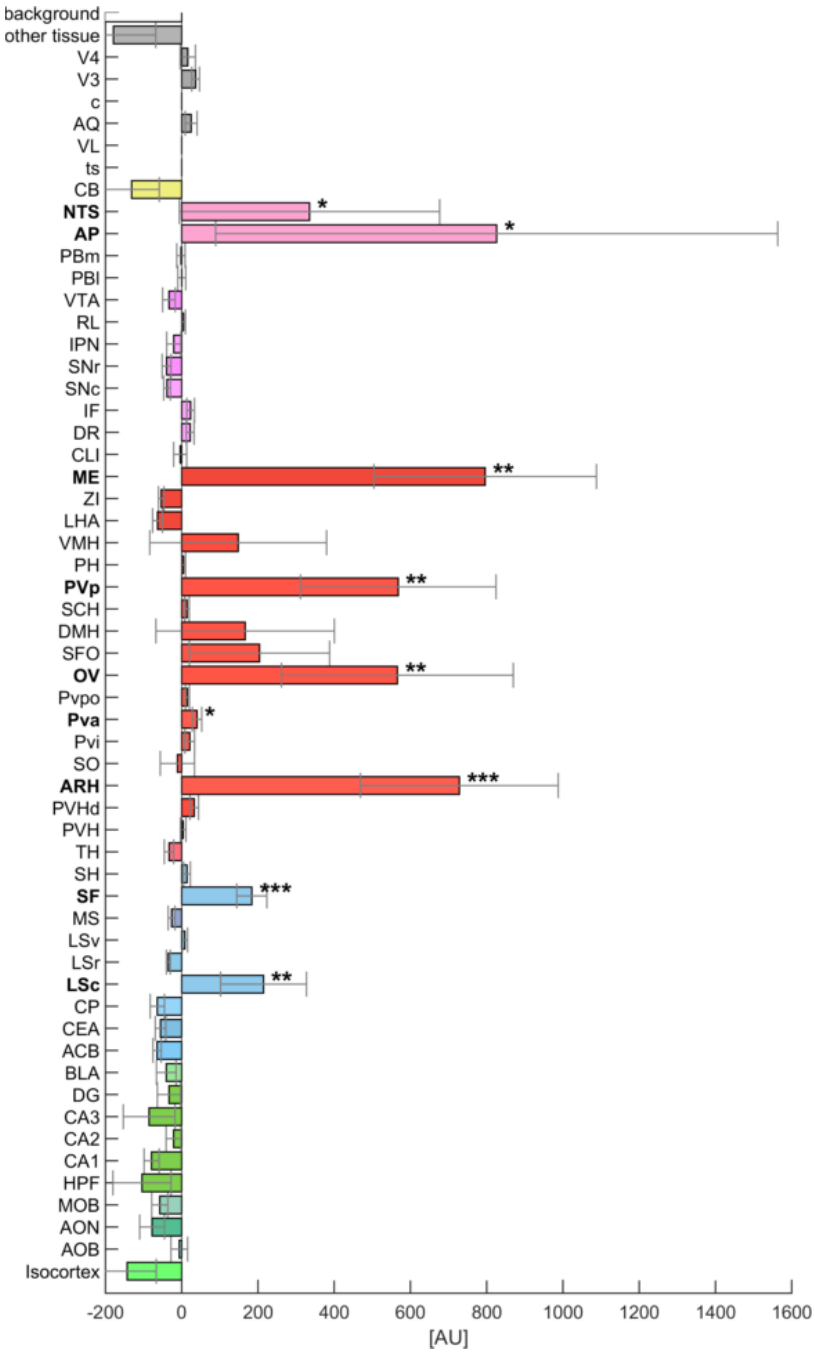

Fig. 3. Signal detection inside the automatically segmented brain regions following injection of Exendin-4. *p<0.05; **p<0.01; ***p<0.001 in a right tailed two sample t-test between the Exendin-4 group and the negative control group. Colors and acronyms follow the convention of the Allen 3D reference model.

4 Discussion

Access of Exendin-4 is seen in circumventricular structures such as ME, OV, and AP, which are brain areas not protected by the blood-brain-barrier and therefore expected to show a signal if any GLP-1 receptors are present. Access is also observed in other areas known to contain GLP-1 receptors such as the ARH and NTS. These areas have previously been identified as being part of the complex system that regulates food intake [13].

Observing the quantitative results in Figure 3, it is seen that some bars have a negative value. This is not expected in a scan setup where the value zero corresponds to no measured signal. The negative values appear due to the method of subtracting bleed-through signal of the auto-fluorescence channel. In future studies this can be avoided by using a better algorithm to remove the bleed-through signal. Although 58 t-test were made, no correction for multiple comparisons has been applied since the nature of the study was explorative.

Due to the lack of a ground truth a measure such as the Dice score can not be computed to evaluate the quality of the atlas, but by visually comparing the constructed atlas to other resources such as [10] the annotations of the structures appear to be correct. Basing a new atlas on a well established resource such as the Allen reference model has shown to reduce the dependency on neuro-anatomical expertise in order to construct the atlas. This is useful when working with a new image modality such as the SPIM system, and a similar approach can also be useful for constructing atlases for tomorrow's imaging systems.

Required manual corrections, and the overall quality of the atlas, is heavily influenced by the registration between the Allen reference model and the constructed SPIM average brain. The registration is challenging since it is both cross-modality and cross-subject. We have obtained a satisfactory result by varying registration parameters, but improvements can be obtained by manually adding landmarks. This could especially be useful around the ventricular system where the largest differences are observed between the reference model and the SPIM scans.

5 Conclusion

Access of Exendin-4 in brain structures known to regulate food intake is successfully demonstrated. Based on visual inspection the constructed brain atlas contains correct annotations of the included structures. Evaluating brain access of GLP-1 analogues, such as Exendin-4, will be important to investigate mode of action of the analogues with hope of developing future obesity medication. Since the constructed atlas is based on the Allen mouse brain reference model additional brain structures can easily be added if found relevant for study of other substances than GLP-1-analogues.

References

1. Allen institute for brain science. allen brain atlas [internet]. http://www.brain-map.org
2. Balci, S.K., Golland, P., Shenton, M., Wells, W.M.: Free-form b-spline deformation model for groupwise registration. In: MICCAI... International Conference on Medical Image Computing and Computer-Assisted Intervention, vol. 10, p. 23. NIH Public Access (2007)
3. Balland, E., Dam, J., Langlet, F., Caron, E., Steculorum, S., Messina, A., Rasika, S., Falluel-Morel, A., Anouar, Y., Dehouck, B., et al.: Hypothalamic tanycytes are an erk-gated conduit for leptin into the brain. Cell Metabolism **19**(2), 293–301 (2014)
4. Castro-González, C., Ledesma-Carbayo, M.J., Peyriéras, N., Santos, A.: Assembling models of embryo development: Image analysis and the construction of digital atlases. Birth Defects Research Part C: Embryo Today **96**(2), 109–120 (2012)
5. Dodt, H.U., Leischner, U., Schierloh, A., Jährling, N., Mauch, C.P., Deininger, K., Deussing, J.M., Eder, M., Zieglgänsberger, W., Becker, K.: Ultramicroscopy: three-dimensional visualization of neuronal networks in the whole mouse brain. Nature Methods **4**(4), 331–336 (2007)
6. Dorr, A., Lerch, J.P., Spring, S., Kabani, N., Henkelman, R.M.: High resolution three-dimensional brain atlas using an average magnetic resonance image of 40 adult c57bl/6j mice. Neuroimage **42**(1), 60–69 (2008)
7. Greger, K., Swoger, J., Stelzer, E.: Basic building units and properties of a fluorescence single plane illumination microscope. Review of Scientific Instruments **78**(2), 023705 (2007)
8. Larsen, C.T., Iglesias, J.E., Van Leemput, K.: N3 bias field correction explained as a bayesian modeling method. In: Cardoso, M.J., Simpson, I., Arbel, T., Precup, D., Ribbens, A. (eds.) BAMBI 2014. LNCS, vol. 8677, pp. 1–12. Springer, Heidelberg (2014)
9. Lein, E.S., et al.: Genome-wide atlas of gene expression in the adult mouse brain. Nature **445**(7124), 168–176 (2006)
10. Paxinos, G., Watson, C.: The rat brain in stereotaxic coordinates: hard cover edition. Academic press (2006)
11. Rueckert, D., Sonoda, L.I., Hayes, C., Hill, D.L.G., Leach, M.O., Hawkes, D.J.: Non-rigid registration using free-form deformations: Application to breast mr images. IEEE Transactions on Medical Imaging **18**, 712–721 (1999)
12. Schnabel, J.A., et al.: A generic framework for non-rigid registration based on non-uniform multi-level free-form deformations. In: Niessen, W.J., Viergever, M.A. (eds.) MICCAI 2001. LNCS, vol. 2208, pp. 573–581. Springer, Heidelberg (2001)
13. Secher, A., et al.: The arcuate nucleus mediates glp-1 receptor agonist liraglutide-dependent weight loss. The Journal of Clinical Investigation **124**(10), 4473 (2014)
14. Studholme, C., Hill, D.L., Hawkes, D.J.: An overlap invariant entropy measure of 3d medical image alignment. Pattern Recognition **32**(1), 71–86 (1999)
15. Yushkevich, P.A., Avants, B.B., Ng, L., Hawrylycz, M., Burstein, P.D., Zhang, H., Gee, J.C.: 3D mouse brain reconstruction from histology using a coarse-to-fine approach. In: Pluim, J.P.W., Likar, B., Gerritsen, F.A. (eds.) WBIR 2006. LNCS, vol. 4057, pp. 230–237. Springer, Heidelberg (2006)