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Advanced diffusion imaging in the hippocampus of rats with mild traumatic brain injury
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Corresponding Author:	Kim Braeckman Universiteit Gent Ghent, BELGIUM
Corresponding Author's Institution:	Universiteit Gent
Corresponding Author E-Mail:	Kim.Braeckman@ugent.be
Order of Authors:	Kim Braeckman
	Benedicte Descamps
	Christian Vanhove
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1 **TITLE:**
2 Advanced Diffusion Imaging in The Hippocampus of Rats with Mild Traumatic Brain Injury
3
4 **AUTHORS & AFFILIATIONS:**
5 Kim Braeckman[1], Benedicte Descamps[1], Christian Vanhove[1]
6
7 [1]Infinity lab, Medical Imaging and Signal Processing Group, Ghent University, Ghent, Belgium
8
9 **Corresponding Author:**
10 Kim Braeckman   (kim.braeckman@ugent.be)
11
12 **Email Addresses of Co-Authors:**
13 Benedicte Descamps   (benedicte.descamps@ugent.be)
14 Christian Vanhove   (christian.vanhove@ugent.be)
15

16 **KEYWORDS:**
17 traumatic brain injury, magnetic resonance imaging, diffusion tensor imaging, preclinical, rat,
18 hippocampus
19

20 **SUMMARY:**
21 The overall goal of this procedure is to obtain quantitative microstructural information of the
22 hippocampus in a rat with mild traumatic brain injury. This is done using an advanced diffusion-
23 weighted magnetic resonance imaging protocol and region-of-interest based analysis of
24 parametric diffusion maps.
25

26 **ABSTRACT:**
27 Mild traumatic brain injury (mTBI) is the most common type of acquired brain injury. Since
28 patients with traumatic brain injury show a tremendous variability and heterogeneity (age,
29 gender, type of trauma, other possible pathologies, etc.), animal models play a key role in
30 unraveling factors that are limitations in clinical research. They provide a standardized and
31 controlled setting to investigate the biological mechanisms of injury and repair following TBI.
32 However, not all animal models mimic the diffuse and subtle nature of mTBI effectively. For
33 example, the commonly used controlled cortical impact (CCI) and lateral fluid percussion injury
34 (LFPI) models make use of a craniotomy to expose the brain and induce widespread focal
35 trauma, which are not commonly seen in mTBI. Therefore, these experimental models are not
36 valid to mimic mTBI. Thus, an appropriate model should be used to investigate mTBI. The
37 Marmarou weight drop model for rats induces similar microstructural alterations and cognitive
38 impairments as seen in patients who sustain mild trauma; therefore, this model was selected
39 for this protocol. Conventional computed tomography and magnetic resonance imaging (MRI)
40 scans commonly show no damage following a mild injury, because mTBI induces often only
41 subtle and diffuse injuries. With diffusion weighted MRI, it is possible to investigate
42 microstructural properties of brain tissue, which can provide more insight into the microscopic
43 alterations following mild trauma. Therefore, the goal of this study is to obtain quantitative
44 information of a selected region-of-interest (i.e., hippocampus) to follow up disease

45   progression after obtaining a mild and diffuse brain injury.
46
47   **INTRODUCTION:**
48
49   Traumatic brain injury (TBI) has gained more attention in recent years, as it has become clear
50   that these brain injuries can result in lifelong cognitive, physical, emotional, and social
51   consequences[1]. Despite this increasing awareness, mild TBI (mTBI, or concussion) is still often
52   underreported and undiagnosed. MTBI has been referred to as a silent epidemic, and
53   individuals with a history of mTBI show higher rates of substance abuse or psychiatric
54   problems[2]. Several patients with mTBI go undiagnosed every year due to the diffuse and subtle
55   nature of the injuries, which are often not visible on conventional computed tomography (CT)
56   or magnetic resonance imaging (MRI) scans. This lack of radiological evidence of brain injury has
57   led to the development of more advanced imaging techniques such as diffusion MRI, which are
58   more sensitive to microstructural changes[3].
59
60   Diffusion MRI allows in vivo mapping of the microstructure, and this MRI technique has been
61   used extensively in TBI studies[4–6]. From the diffusion tensor, fractional anisotropy (FA) and
62   mean diffusivity (MD) are computed to quantify alteration in the microstructural organization
63   following injury. Recent reviews in mTBI patients report increases in FA and decreases in MD
64   following injury, which can be indicative of axonal swelling[7]. Contrary, increases in MD and
65   decreases in FA are also found and have been suggested to underlie disruptions in parenchymal
66   structure following edema formation, axonal degeneration, or fiber misalignment/disruption[8].
67   These mixed findings can be partially explained by the significant clinical heterogeneity of mTBI
68   caused by different types of impact and severity (e.g., rotation-acceleration, blunt force trauma,
69   blast injury or combination of the former). However, currently there is no clear consensus
70   about the underlying pathology and biological/cellular basis underpinning alterations in the
71   microstructural organization.
72
73   Animal models provide a standardized and controlled setting to investigate biological
74   mechanisms of injury and repair following TBI in greater detail. Several experimental models for
75   TBI have been developed and represent different aspects of human TBI (e.g., focal vs. diffuse
76   trauma or trauma caused by rotational forces)[9,10]. Commonly used animal models include the
77   controlled cortical impact (CCI) and lateral fluid percussion injury (LFPI) models[11,12]. Although
78   the experimental parameters can be well-controlled, these models make use of a craniotomy to
79   expose the brain. Craniotomies or skull fractures are not commonly seen in mTBI; therefore,
80   these experimental models are not valid to mimic mTBI. The impact acceleration model
81   developed by Marmarou et al.[13] makes use of a weight that is dropped from a certain height
82   onto the rat's head, which is protected by a helmet. This animal model induces similar
83   microstructural alterations and cognitive impairments as seen in patients who sustain mild
84   trauma. Therefore, this Marmarou weight drop model is appropriate to investigate imaging
85   biomarkers for diffuse mTBI[14,15].
86
87   This report demonstrates the application of advanced diffusion MRI in an mTBI rat model using
88   the Marmarou weight drop model. First shown is how to induce a mild and diffuse trauma, and

89 analysis using diffusion tensor imaging (DTI) model is then provided. Specific biological
90 information is obtained with the use of more advanced diffusion models [i.e., diffusion kurtosis
91 imaging (DKI) and white matter tract integrity (WMTI) model]. Specifically, mild trauma is
92 inflicted and microstructural changes are then evaluated in the hippocampus using
93 conventional T2-weighted MRI and an advanced diffusion imaging protocol.

94

95 **PROTOCOL:**

96

97 The protocol has been approved by the Animal Ethics Committee at the University of Ghent
98 (ECD 15/44Aanv), and all experiments were conducted in accordance with the guidelines of the
99 European Commission (Directive 2010/63/EU).

100

101 **1. Animal preparation and helmet attachment**

102

103 1.1. Weigh a female Wistar H rat (± 250 g or 12 weeks of age) and anesthetize in a small
104 induction chamber filled with a mixture of isoflurane (5%) and O_2 for at least 1 min.

105

106 1.2. Inject the rat with 0.05 mg/kg buprenorphine subcutaneously in the neck, return it to the
107 home cage, and allow pre-emptive analgesia for at least 30 min to take an effect.

108

109 NOTE: During the 30 min wait, the surgical site can be prepared.

110

111 1.3. Place a heating pad kept at 37 °C under the surgical field. Disinfect all surgical instruments
112 and the surgical field with 70% ethanol.

113

114 1.4. Place the rat back in the induction chamber and anesthetize the rat until it is non-
115 responsive to a paw or tail pinch.

116

117 1.5. Place the rat on the surgical field and insert a catheter in the lateral tail vein. Next, wet the
118 rat's fur with 70% ethanol spray, shave the head, remove excess fur, and disinfect the scalp and
119 the rest of the surgical area with ethanol spray.

120

121 1.6. Inject 100 μL of 2% lidocaine locally in the scalp.

122

123 1.7. Make a midline incision to expose the skull, removing any excess membranes with small
124 scissors. Additionally, remove the periosteum by gently rubbing a cotton bud across the skull
125 until the periosteum is no longer present.

126

127 1.8. Put one drop of tissue glue on the skull and one on the metallic disc (diameter of 10 mm
128 and 3 mm thickness), which acts as the helmet. Glue the disc approximately one-third before
129 and two-thirds behind the bregma. Allow the glue to dry for 1 min.

130

131 **2. Induction of traumatic brain injury (TBI)**

132

133   2.1. Place the rat on the custom-made bed with a foam mattress of certain spring constant (see
134   **Table of Materials**). Position the rat directly under a transparent plastic tube with a 450 g brass
135   weight with the helmet as horizontal as possible. Detach the rat from anesthesia.

137   2.2. Pull the weight up to 1 m and release when ready. Ensure that a second experimenter is
138   present to move the rat away from the plastic tube immediately after impact to prevent a
139   second impact.

141   NOTE: The sham injured rats receive the same experimental procedure (steps 1.1–2.7), except
142   for step 2.2.

144   2.3. Re-attach the rat to the anesthesia and inject 1 mL of physiological solution (0.9% NaCl) via
145   the catheter to reduce the hemodynamic shock.

147   NOTE: It is possible that the rat briefly stops breathing because of the impact. Gently compress
148   the thorax if the rat does not spontaneously breathe after 2 s to encourage the breathing
149   reflex.

151   2.4. Remove the helmet by gently pulling it from the skull. Remove any remaining glue from the
152   skull and skin and close the incision with surgical suture. Apply local analgesia gel.

154   2.5. Place the rat on the bed of the CT scanner. Confirm the correct position using a scout scan.
155   Adjust the field of view to enable imaging of the whole head within one bed position.
156   Administer a general purpose, low dose CT scan to rule out skull fractures.

158   NOTE: Skull fracture is a criterium for euthanasia.

160   2.6. Place the rat in a clean cage on a heating pad (37 °C). Monitor the time to regain
161   consciousness. Once the rat is able to sit upright, the rat can be returned to the home cage.

163   2.7. Administer a second dose of 0.05 mg/kg buprenorphine one day following TBI induction.

165   **3. Diffusion magnetic resonance imaging (MRI)**

167   NOTE: Diffusion-weighted imaging is performed before and 1 day following trauma induction.

169   3.1. Anesthetize the rat in a small induction chamber filled with a mixture of isoflurane (5%)
170   and O_2. When the rat is non-responsive to a paw or tail pinch reduce the anesthesia to 2% with
171   a flow rate of 500 mL/min. Transfer the animal to the scanner bed in head-first prone position.

173   3.2. Position the rat in the head holder with the teeth bar and nose cone, delivering the
174   anesthesia, and slide the head forwards until the center of the brain is at the level of the center
175   of the quadrature volume MRI coil. Apply lubricating ointment to the eyes in small amounts to
176   prevent any damage to the cornea. Fixate the head with a small piece of tape to avoid

177   movement during scanning.

178

179   3.3. Place a pressure pad under the thorax of the rat to monitor respiration and cover the rat
180   with a circulating warm water heating blanket and bubble wrap to keep the rat warm. Before
181   the scanning, check the respiratory monitor to ensure that the signal is clear without noise and
182   that the respiratory cycle is consistent. Relocate the pressure pad, if necessary.

183

184   NOTE: The respiratory rate should be kept between 1 breath per 1,200–1,700 ms by adjusting
185   the level of anesthesia between 1%–2%.

186

187   3.4. Slide the quadrature volume coil over the head. Adjust the tuning and matching capacitors
188   of the coil to the proper frequency and impedance according to the instructions provided by
189   the coil vendor. Advance the scanner bed into the scanner bore to start scanning.

190

191   3.5. Obtain a default three-plane scout scan ("tripilot") to ensure correct positioning.

192

193   3.5.1. Load the tripilot sequence into the Scan Control by clicking **New Scan** and selecting the
194   tripilot sequence from the protocol list. Next, click the **traffic light** button to start the scan.

195

196   3.5.2. When the scan is finished, load the scan in the image display and ensure that 1) the head
197   is lying straight and 2) the brain is positioned in the center of the magnet and coil. Adjust the
198   position of the head and/or the scanner bed, if necessary, and acquire a new tripilot scan.

199

200   3.6. Adjust the local magnetic field using an automated second-order shimming protocol: load
201   the second-order shim protocol into the Scan Control as described in step 3.5.1. Next, click on
202   the **Acq** tab | **Current Adjustments** | **Method specific adjustment for the Local Field**
203   **Homogeneity** in the Spectrometer Control Tool window to start automated shimming.

204

205   3.7. Load a new T2 Rapid imaging with Refocused Echoes (RARE) sequence into the Scan control
206   as described in step 3.5.1.

207

208   3.7.1. Acquire T2 weighted images using the default settings, except for the following
209   parameters:

210

211   3.7.2. Open the **Edit Scan** tab and adjust the repetition time (TR) and echo time (TE) to 5,500
212   ms and 37 ms, respectively. Also, modify the field of view and matrix size to allow for a higher
213   in-plane resolution of 109 μm x 109 μm (default resolution = 156 μm x 156 μm). Make sure that
214   the slice thickness is 600 μm, number of slices is set to 45, and RARE factor is set to 8.

215

216   3.7.3. Open the **Geometry editor** and place the slice package in the correct position including
217   the bulbus of the brain and the cerebellum.

218

219   3.8. Load three new echo-planar diffusion-weighted spin-echo sequence (DtiEpi) from the
220   B_DIFFUSION folder into the Scan Control protocol as described in step 3.5.1.

221

222 NOTE: Using three different diffusion "shells", the diffusion tensor imaging (DTI) model[4,16],
223 diffusion kurtosis imaging (DKI) model[17], and white matter tract integrity (WMTI) model[18] can
224 all be estimated. It is recommended to use at least three different b-values, with the highest b-
225 value having a maximum of 3000 s/mm^2 with at least 15 evenly spaced directions per imaging
226 shell[17].

227

228 3.8.1. Acquire diffusion weighted images (DWIs) using default settings, apart from the following
229 settings:

230

231 3.8.2. Open the **Edit Scan** tab and adjust the geometrical parameters under the **Geometry** tab.
232 Adjust the field of view and matrix size to 105 x 105 to ensure a resolution of 333 μm x 333 μm.

233

234 3.8.3. Set the slice orientation to axial and the number of slices to 25, resulting in a slice
235 thickness of 500 μm and interslice distance of 600 μm. Amend the readout direction into left-
236 right.

237

238 3.8.4. Click the **Contrast** tab to adjust the echo time to 24 ms and repetition time to 6,250 ms.

239

240 3.8.5. Set the bandwidth to 250,000 Hz and turn the fat suppression on. Adjust the number of
241 averages to one.

242

243 3.8.6. Click on the **Research** tab and change the number of averages (EPI segments) to 4.

244

245 3.8.7. Click on the **Diffusion** tab within the research tab. Perform this step separately for each
246 of the three diffusion shells.

247

248 3.8.7.1. Adjust the number of diffusion directions to 32 for the first shell, 46 for the second
249 shell, and 64 for the third shell.

250

251 3.8.7.2. Adjust the gradient directions with custom gradient directions files.

252

253 3.8.7.3. Change the number of B0 images to 5 for the first shell, 5 for the second shell, and 7 for
254 the third shell.

255

256 3.8.7.4. Adjust the b-value per direction to 800 s/mm^2 for the first shell, 1500 s/mm^2 for the
257 second shell, and 2000 s/mm^2 for the third shell.

258

259 NOTE: Adjusting the gradient directions with a custom gradient directions file can be done
260 manually by setting **Enter Diffusion Directions** to yes or automatically by using the
261 DTI_SET_DIRECTIONS macro.

262

263 3.8.8. Open the **Geometry editor** and place the field of view between the bulbus and
264 cerebellum containing only the cerebrum to reduce artifact and scan time. Position six

265 saturation bands of 5 mm outside the brain to reduce artifacts by clicking on **Saturation** and
266 sliding the bands in the preferred position using the scroll bars.
267
268 NOTE: The bulbus and cerebellum can be identified based on anatomical landmarks and the
269 three images of the tripilot scan.
270
271 3.9. Acquire the imported sequences by clicking on the **traffic light** symbol. Using the settings
272 of the parameters described above, the acquisition time of the T2-RARE scan is 12 min, of the
273 first DWI shell 15 min, of the second DWI shell 21 min and of the third shell 30 min. The total
274 acquisition time is approximately 80 min (on a single receiver channel system).
275
276 3.10. At the completion of the scanning protocol, remove the animal from the scanner bed, and
277 place the animal in a clean cage with a heating pad at 37 °C. Return the animal to the home
278 cage when it regains consciousness.
279
280 **4. Image processing**
281
282 NOTE: In the following sections, the processing of the diffusion images is described in MRtrix3,
283 ExploreDTI[19] and Amide software[20] which are open access toolboxes. However, the
284 preprocessing steps can be performed in other toolboxes (e.g., FSL, MedInria, DTIStudio).
285
286 4.1. Transfer the acquired data from the acquisition console by exporting the 2dseq file.
287
288 4.2. Convert the 2dseq files (raw DWI files) into the .mif format, which is the standard
289 formatting of MRtrix3, to allow for further preprocessing steps in MRtrix3. Moreover,
290 concatenate the three diffusion shells using the following commands in the shell:
291 convert_bruker pdata/1/2dseq ratID_T2.mih (for the T2-weighted images)
292 convert_bruker pdata/1/2dseq ratID_dwi1.mih (for the first diffusion shell)
293 convert_bruker pdata/1/2dseq ratID_dwi2.mih (for the second diffusion shell)
294 convert_bruker pdata/1/2dseq ratID_dwi3.mih (for the third diffusion shell)
295 mrcat ratID_dwi1.mif ratID_dwi2.mif ratID_dwi3.mif ratID_dwi.mif
296
297 4.3. Perform noise correction and Gibbs ringing correction on the DWIs in MRtrix3[21,22]. Also,
298 convert the corrected DWI images and T2 image to NIFTI format using the following commands:
299 dwidenoise ratID_dwi.mif ratID_dwi_denoised.mif
300 mrdegibbs ratID_dwi_denoised.mif ratID_dwi_denoised_gr.mif
301 mrconvert ratID_dwi_denoised_gr.mif ratID.nii
302 mrconvert ratID_T2.mif ratID_T2.nii
303
304 4.4. Perform correction for EPI, motion and Eddy current distortions in ExploreDTI:
305
306 4.4.1. Convert the NIFTI images to a .mat file by clicking **Calculate DTI*.mat file | Convert raw
307 data to DTI*.mat file**. Change the diffusion tensor estimation to weighed linear and the b-value
308 to NaN. Adjust the voxel size to 0.333 0.333 0.6, number of non-DWI images to 17, number of

309   DWI images to 142, and matrix size to 105 105 25.

310

311   NOTE: By setting the b-value to NaN, ExploreDTI will regard the dataset as a kurtosis data set.

312

313   4.4.2. Click on the **Settings** tab to adjust the settings for EPI correction (this is turned off by
314   default). Select **SM/EC/EPI correction, also register to other data?** and click on **Yes, to do the**
315   **EPI correction (non-rigid)**. Specify the suffix of the anatomical T2 image corresponding to the
316   diffusion data set.

317

318   NOTE: ExploreDTI corrects for EPI distortions using image registration between the undistorted
319   anatomical image and the diffusion image.

320

321   4.4.3. Click on the **Plugins** tab and select **Correction for subject motion & EC/EPI distortions**
322   and select the preprocessed diffusion data file from step 2.3. Make sure that the T2 image is in
323   the same folder and has the same base as the diffusion data file name (e.g., rat1.nii for the DWI
324   and rat1_T2.nii for the anatomical image). This step will generate a "native" (*native.mat) and
325   "transformed" file (*trafo.mat).

326

327   4.5. Calculate the DTI metrics for each rat by clicking **Plugins** and **Export stuff to *.nii** and
328   selecting the parametric maps of the DTI model: fractional anisotropy (FA), mean diffusivity
329   (MD), radial diffusivity (RD), and axial diffusivity (AD; denoted as "Smallest eigenvalue L1").

330

331   4.6. Additionally, export the parametric maps for the kurtosis model (MK, AK, and RK) and
332   WMTI model (AWF, AxEAD, RadEAD and TORT). Processing of the diffusion images will result in
333   12 parametric maps (**Figure 1, Figure 2**, **Figure 3**) that can be used for further microstructural
334   analysis.

335

336   4.7. Create a mask file for the hippocampus of each rat using MRtrix3.

337

338   4.7.1. Load the FA image of the rat in the MRtrix viewer by clicking **Tool** and **ROI editor**.

339

340   4.7.2. Create a new ROI by clicking the "**+**" button and press **Edit** to draw the ROI on each slice
341   that includes the hippocampus (**Figure 4**). To erase unwanted areas from the ROI drawn, press
342   the right mouse button.

343

344   4.7.3. When the drawing of the ROI is completed, save the mask image by clicking the **Save**
345   button.

346

347   NOTE: This mask file will be a binary NIFTI image file with voxels of value 1 containing the
348   hippocampal tissue, and remaining voxels will have values of 0. To standardize the region of the
349   hippocampus across rats, the parametric maps can be co-registered with a study specific
350   template with predefined regions of interest delineated[23] or a rat brain atlas.

351

352   4.8. To extract the diffusion metrics of the hippocampus of the rat, use the created mask file of

353 step 4.6 and open the Amide software.

355 4.8.1. Open the parametric maps and mask image of the rat.

357 4.8.2. To add the ROI of the mask file into Amide, select the mask file image, click **Edit | Add**
358 **ROI | 3D Isocontour** and click on the ROI displayed in the mask image. Give the ROI a
359 meaningful name and confirm that this volume should only contain voxels having a value of
360 one.

362 4.8.3. To calculate the mean values of the diffusion metrics in the hippocampus, click **Tools |**
363 **Calculate ROI Statistics** and indicate the images and ROI that should be included. After clicking
364 **Execute**, another screen will pop up with computed values that can be used for further
365 statistical analysis. This file can either be saved or copied into a preferred data format (e.g., .xlsx
366 or .csv file).

368 **5. Statistical analysis**

370 NOTE: In the following sections, we describe processing of the diffusion images in SPSS
371 Statistics 24; however, the statistical analysis can be performed in other statistical toolboxes.

373 5.1. Load the data in the wide format in an SPSS *.sav file.

375 5.2. To test for statistical differences between the two groups for each timepoint (i.e., baseline
376 or 1-day post-injury), click **Analyze | Nonparametric tests | Legacy Dialogs | 2 Independent**
377 **Samples Tests**. Load the variables that need to be tested and specify the groups (i.e., TBI and
378 sham groups). Indicate the Mann-Whitney U as the test type.

380 5.3. To test for statistical differences between the 2 time point within each group the data file
381 needs to be split. Go to Go to **Data, Split File** and indicate Compare groups. Next, click on
382 **Analyze, Nonparametric tests, Legacy Dialogs, 2 Related Samples Tests**, load the variables that
383 need to be compared and indicate Wilcoxon as test type.

385 NOTE: To correct for multiple comparisons, p-values are adjusted for each diffusion model
386 using Bonferroni correction [i.e., p-value divided by the number of parameters compared (DTI
387 4, DKI 3, and WMTI 4)]. More specifically, $p < 0.0125$ is considered significant for the DTI and
388 WMTI models, and $p < 0.016$ is considered significant for the DKI model.

390 **REPRESENTATIVE RESULTS:**

392 In the study, all TBI rats (n = 10) survived the impact and were able to recover from the impact
393 and anesthesia within 15 min after detachment from anesthesia[23]. On the CT images, there was
394 no evidence of skull fractures and the T2 images did not show any abnormalities such as
395 bleeding, enlarged ventricles, or edema formation at the contusion site 1 day after trauma
396 (**Figure 5**). Thus, based on these visual inspections of the anatomical images, large focal lesions

397 were not detected, confirming the diffuse and mild nature of the injury.
398
399 The quality of the coregistration (non-rigid) step between the T2 image and diffusion data set
400 (step 4.4) was examined by adding an overlay of the T2 image to the color-encoded FA map
401 (**Figure 6**). Then, the FA, MD, AD, and RD parametric maps were calculated (**Figure 1**) and
402 loaded into the Amide software. Based on the FA map, a ROI including the hippocampal
403 structure was drawn (**Figure 4**). Statistical values of the diffusion metrics were calculated
404 averaged over all voxels within the region of interest and the mean values of each DTI metric
405 were exported for further analysis. Another quality check of the diffusion data can be
406 performed by inspecting the outliers in the DTI metrics. For example, FA values in the
407 hippocampus should be around 0.15; therefore, values of <0.10 (denoting isotropic diffusion) or
408 >0.30 (values are seen in white matter) can be regarded as biologically implausible values.
409 These datapoints should be rejected from further analysis. Also, the mean values for AK, RK,
410 and MK of the diffusion kurtosis model as well as the AWF, AxEAD, RadEAD, and TORT of the
411 WMTI model were calculated (**Figure 2, Figure 3**).
412
413 In our study, analysis of the DTI metrics revealed significant increased FA values (p = 0.007), and
414 decreased diffusivity values (MD and RD) (p = 0.007 and p = 0.007, respectively) following
415 impact in the mTBI group (**Figure 7**). These decreases in RD and MD were significantly different
416 from the sham group (p = 0.005 and p = 0.004, respectively). Diffusion kurtosis metrics showed
417 a significant decrease in RK (p = 0.005) following impact but no changes in AK or MK (**Figure 8**).
418 Using the WMTI model, RadEAD (p = 0.007) and TORT (p = 0.007) displayed a significant
419 decrease and increase, respectively, in the mTBI group 1 day after the impact (**Figure 9C,D**). The
420 values in the sham group did not show any significant changes.
421
422 **FIGURE AND TABLE LEGENDS:**
423
424 **Figure 1: Representative parametric maps for fractional anisotropy (FA), mean diffusivity**
425 **(MD), axial diffusivity (AD), and radial diffusivity (RD).**
426
427 **Figure 2: Representative parametric maps for mean kurtosis (MK), axial kurtosis (AK), and**
428 **radial kurtosis (RK).**
429
430 **Figure 3: Representative parametric maps for axonal water fraction (AWF), axial and radial**
431 **extra axonal diffusivity (AxEAD, RadEAD), and tortuosity (TORT).**
432
433 **Figure 4: Creating a mask in MRtrix3**. A ROI is drawn around the hippocampus on all slices
434 containing the volume of the hippocampus, and the volume is saved as a mask file. This can
435 either be done for each rat individually or by using a study specific template mask file to which
436 each of the parametric maps can be co-registered.
437
438 **Figure 5: CT and T2 weighted images of a representative mTBI animal 1 day after impact.** The
439 CT images (top row) do not show any skull fractures. On the T2-weighted images (bottom row)
440 no bleeding, enlarged ventricles, or edema formation were demonstrated. Of note, edema

441 formation is clearly visible as a hyperintense area around the wound area from the surgical
442 intervention.
443
444 **Figure 6: Color encoded FA map of diffusion data set overlaid with the anatomical image after**
445 **correction for EPI, motion, and Eddy current correction in ExploreDTI**. Shown is a bad
446 correction and co-registration on the left and good examples on the right. It should be ensured
447 that the color encoding is correct: left-right direction in red (e.g., corpus callosum), anterior-
448 posterior direction in green, and inferior-superior direction in blue (e.g., cingulum).
449 Additionally, the color encoded FA image should be perfectly aligned with the anatomical
450 image.
451
452 **Figure 7: Changes in diffusion tensor metrics of hippocampus for sham (n = 10) and mTBI**
453 **animals (n = 10).** Following impact, there was a significant increase in FA (**A**) and significant
454 decreases in mean diffusivity (**B**) and radial diffusivity (**D**) in the mTBI animals (**B,D**). No
455 significant differences were observed for axial diffusivity (**C**) in the mTBI rats. The sham animals
456 did not show any significant DTI changes (*$p < 0.0125$).
457
458 **Figure 8: Changes in diffusion kurtosis metrics of hippocampus for sham (n = 10) and mTBI**
459 **animals (n = 10).** Following impact, there was a significant decrease in RK (**C**) of the mTBI
460 animals but no changes in AK (**B**) or MK (**A**). The sham animals did not show any changes (*$p <$
461 0.0166).
462
463 **Figure 9: Changes in white matter tract integrity metrics of hippocampus for sham (n = 10)**
464 **and mTBI animals (n = 10).** Following impact, there was a significant decrease in RadEAD (**C**)
465 and significant increase in TORT (**D**) of the mTBI animals but no change in AWF or AxEAD (**A,B**).
466 The sham animals did not show any changes (*$p < 0.0125$).
467
468 **DISCUSSION:**
469
470 Since mTBI often is the result of a diffuse and subtle injury that shows no abnormalities on CT
471 and conventional MRI scans, the evaluation of microstructural damage after a mild trauma
472 remains a challenge. Therefore, more advanced imaging techniques are needed to visualize the
473 full extent of the trauma. The application of diffusion magnetic resonance imaging in TBI
474 research has gained more interest during the last decade, where diffusion tensor imaging is
475 most frequently used[5]. A limitation of the DTI model is the assumption of a Gaussian diffusion
476 process that is not a precise assumption for brain microstructure (consisting of a complex
477 network of axons and cells with membranes acting as barriers), resulting in DTI metrics non-
478 specific to the underlying biological microstructure[24]. Diffusion kurtosis imaging is an extension
479 of the DTI model and attempts to characterize the degree of non-Gaussian diffusion[17]. This may
480 provide additional information about tissue heterogeneity or complexity.
481
482 Though, a drawback of DTI and DKI models is that they are only a representation of the
483 diffusion signal, which characterizes the probabilistic water displacement profile but is not
484 specific to microstructure[6]. On the other hand, the white matter tract integrity model based on

485 the kurtosis tensor is a microstructural mapping technique that incorporates *a priori* biological
486 information (assumptions) into the model[18]. It attributes the diffusion signal to tissue
487 compartments and can assess biological attributes more directly. These biophysical models may
488 thus offer new information for describing abnormalities after mTBI and overcome this non-
489 specificity issue[6]. Using these three different models, microstructural alterations and biological
490 processes were able to be visualized following mTBI in more detail, specifically by using the
491 Marmarou weight drop model.
492
493 The Marmarou weight drop model is easy to use and requires only minor surgery; however, a
494 second experimenter is recommended to move the rat away from the glass tube immediately
495 after the first impact to avoid a second one. Additionally, it is sometimes required to help the
496 rat regain its breathing reflex following the impact. The rather long MRI protocol, with a total
497 acquisition time of around 80 min, is well-tolerated by both the sham and mTBI rats. Though,
498 during scanning, it is important to monitor breathing cycle and adjust the anesthesia if the
499 animal is sleeping too deeply or lightly. It is also important to keep the animal warm both
500 during and after the acquisition until the rat is fully awake to avoid hypothermia.
501
502 In advanced diffusion MRI, movement artifacts should be avoided as much as possible. A simple
503 solution to reduce movement during scanning is to make use of a teeth bar and fixate the head
504 with a small piece of tape or two ear bars, if available. This ensures that the head will not move
505 up and down every time the rat takes a breath.
506
507 Using advanced diffusion MRI protocols, the acquired images must pass through several (pre-)
508 processing steps, mostly using different software tools, before they can be used for further
509 analysis. A drawback of using different software tools to process the diffusion-weighted images
510 is that (often) each tool uses its own data format to encode the gradient directions table.
511 MRtrix3 stores the gradient information together with the diffusion weighted image in a .mif
512 file, while ExploreDTI makes use of a separate file (B-matrix) to store the gradient directions.
513 Therefore, it is important to check that the gradient directions are correctly transferred from
514 MRtrix3 to ExploreDTI. This can be done by checking that color encoding is correct on color
515 encoded FA images [i.e., left-right direction in red (e.g., corpus callosum), anterior-posterior
516 direction in green, and inferior-superior direction in blue (e.g., cingulum)]. The color encoded
517 FA images can also be used to check the quality of the non-rigid co-registration process
518 between the diffusion-weighted images and structural T2- weighted images.
519
520 Using ExploreDTI, parametric maps were extracted using the DTI, DKI, and WMTI models. The
521 DTI model provided parametric maps for MD, AD, RD, and FA, while the DKI model provides
522 parametric maps for MK, AK, and RK. Although four metrics of the WMTI model were calculated
523 (i.e., AWF, AxEAD, RadEAD, TORT), it was not possible to extract intra-axonal diffusivity (IAD)
524 within ExploreDTI. IAD can be obtained using a MATLAB tool provided by the developers of the
525 WMTI model[25]. To do so, the diffusion-weighted images and gradient information must be
526 transferred again from ExploreDTI into Matlab. This step is again prone to errors concerning the
527 encoding of gradient information. Additionally, the kurtosis tensor and the WMTI parameters
528 must be estimated and calculated again.

529

530 Preprocessing of the acquired images, estimation of the tensors, and calculation of the
531 parametric maps requires a long period of computing time. Corrections for EPI, motion, and
532 eddy current required ~40 min per data set on a server with eight cores and 16 GB RAM. Using
533 a ROI analysis, mean values within the hippocampus were calculated before and 1 day after
534 impact. Changes in the DTI, DKI and WMTI metrics were then quantified in the mTBI group.
535 However, in the DKI metrics and AWF of the WMTI model, large inter-subject variability was
536 observed, which resulted in an unexpected difference in baseline values between the sham and
537 mTBI groups. This is most likely the result of voxels containing biologically implausible values
538 (outliers) within the investigated region and may be filtered out in future studies before
539 calculation of the mean values in Amide.

540

541 In conclusion, this protocol demonstrates the feasibility of advanced diffusion MRI for
542 investigating and quantifying microstructural alterations in the hippocampus in a rat model of
543 mTBI. Using three different diffusion models, complementary information can be obtained
544 about the underlying biological processes that contribute to the conditions after mTBI. This
545 represents a step forward in the development of biomarkers for mTBI that may be sensitive
546 enough to identify specific microstructural changes in the early phase after mild impact.

547

551

552 **DISCLOSURES:**
553 The authors have no conflicts of interest to disclose.

554

555 **REFERENCES:**
556 1.   Carroll, L. J. et al. Systematic Review of the Prognosis After Mild Traumatic Brain Injury
557 in Adults: Cognitive, Psychiatric, and Mortality Outcomes: Results of the International
558 Collaboration on Mild Traumatic Brain Injury Prognosis. *Archives of Physical Medicine and*
559 *Rehabilitation*. **95** (3), S152–S173, doi: 10.1016/j.apmr.2013.08.300 (2014).
560 2.   Buck, P. W. Mild Traumatic Brain Injury: A Silent Epidemic in Our Practices. *Health &*
561 *Social Work*. **36** (4), 299–302, doi: 10.1093/hsw/36.4.299 (2011).
562 3.   Bodanapally, U. K., Sours, C., Zhuo, J., Shanmuganathan, K. Imaging of Traumatic Brain
563 Injury. *Radiologic Clinics of North America*. **53** (4), 695–715, doi: 10.1016/j.rcl.2015.02.011
564 (2015).
565 4.   Basser, P. J., Mattiello, J., LeBihan, D. MR diffusion tensor spectroscopy and imaging.
566 *Biophysical Journal*. **66** (1), 259–267, doi: 10.1016/S0006-3495(94)80775-1 (1994).
567 5.   Hulkower, M. B., Poliak, D. B., Rosenbaum, S. B., Zimmerman, M. E., Lipton, M. L. A
568 Decade of DTI in Traumatic Brain Injury: 10 Years and 100 Articles Later. *American Journal of*
569 *Neuroradiology*. **34** (11), 2064–2074, doi: 10.3174/ajnr.A3395 (2013).
570 6.   Hutchinson, E. B., Schwerin, S. C., Avram, A. V, Juliano, S. L., Pierpaoli, C. Diffusion MRI
571 and the detection of alterations following traumatic brain injury. *Journal of Neuroscience*
572 *Research*. **96** (4), 612–625, doi: 10.1002/jnr.24065 (2018).

573   7.   Wallace, E. J., Mathias, J. L., Ward, L. Diffusion tensor imaging changes following mild,
574   moderate and severe adult traumatic brain injury: a meta-analysis. *Brain Imaging and Behavior*.
575   1–15, doi: 10.1007/s11682-018-9823-2 (2018).
576   8.   Rutgers, D. R. et al. White Matter Abnormalities in Mild Traumatic Brain Injury: A
577   Diffusion Tensor Imaging Study. *American Journal of Neuroradiology*. **29** (3), 514–519, doi:
578   10.3174/ajnr.A0856 (2008).
579   9.   Bondi, C. O. et al. Found in translation: Understanding the biology and behavior of
580   experimental traumatic brain injury. *Neuroscience & Biobehavioral Reviews*. **58**, 123–146, doi:
581   10.1016/j.neubiorev.2014.12.004 (2015).
582   10.   Shultz, S. R. et al. The potential for animal models to provide insight into mild traumatic
583   brain injury: Translational challenges and strategies. *Neuroscience & Biobehavioral Reviews*. **76**,
584   396–414, doi: 10.1016/j.neubiorev.2016.09.014 (2017).
585   11.   Osier, N. D., Dixon, C. E. The Controlled Cortical Impact Model: Applications,
586   Considerations for Researchers, and Future Directions. *Frontiers in Neurology*. **7** (AUG), doi:
587   10.3389/fneur.2016.00134 (2016).
588   12.   Lyeth, B. G. Historical Review of the Fluid-Percussion TBI Model. *Frontiers in Neurology*.
589   **7** (DEC), 1–7, doi: 10.3389/fneur.2016.00217 (2016).
590   13.   Marmarou, A., Foda, M. A. A. -E., Brink, W. van den, Campbell, J., Kita, H., Demetriadou,
591   K. A new model of diffuse brain injury in rats. *Journal of Neurosurgery*. **80** (2), 291–300, doi:
592   10.3171/jns.1994.80.2.0291 (1994).
593   14.   Heim, L. R. et al. The Invisibility of Mild Traumatic Brain Injury: Impaired Cognitive
594   Performance as a Silent Symptom. *Journal of Neurotrauma*. **34** (17), 2518–2528, doi:
595   10.1089/neu.2016.4909 (2017).
596   15.   Zohar, O., Rubovitch, V., Milman, A., Schreiber, S., Pick, C. G. Behavioral consequences
597   of minimal traumatic brain injury in mice. *Acta Neurobiol Exp (Wars)*. **71** (1), 36–45 (2011).
598   16.   Pierpaoli, C., Basser, P. J. Toward a quantitative assessment of diffusion anisotropy.
599   *Magnetic resonance in medicine : official journal of the Society of Magnetic Resonance in
600   Medicine / Society of Magnetic Resonance in Medicine*. **36** (6), 893–906, doi:
601   10.1002/mrm.1910360612 (1996).
602   17.   Jensen, J. H., Helpern, J. A. MRI quantification of non-Gaussian water diffusion by
603   kurtosis analysis. *NMR in Biomedicine*. **23** (7), 698–710, doi: 10.1002/nbm.1518 (2010).
604   18.   Fieremans, E., Jens H., Jensen, J. A. H. White matter characterization with diffusional
605   kurtosis imaging. *NeuroImage*. **58**, 177–188 (2011).
606   19.   Leemans, A. Explore DTI.
607   20.   Loening, A. M., Gambhir, S. S. AMIDE: A Free Software Tool for Multimodality Medical
608   Image Analysis. *Molecular Imaging*. **2** (3), 131–137, doi: 10.1162/153535003322556877 (2003).
609   21.   Veraart, J. et al. Denoising of diffusion MRI using random matrix theory. *NeuroImage*.
610   **142**, 394–406, doi: 10.1016/j.neuroimage.2016.08.016 (2016).
611   22.   Veraart, J., Fieremans, E., Novikov, D. S. Diffusion MRI noise mapping using random
612   matrix theory. *Magnetic Resonance in Medicine*. **76** (5), 1582–1593, doi: 10.1002/mrm.26059
613   (2016).
614   23.   Braeckman, K. et al. Dynamic changes in hippocampal diffusion and kurtosis metrics
615   following experimental mTBI correlate with glial reactivity. *NeuroImage: Clinical*. **21** (August
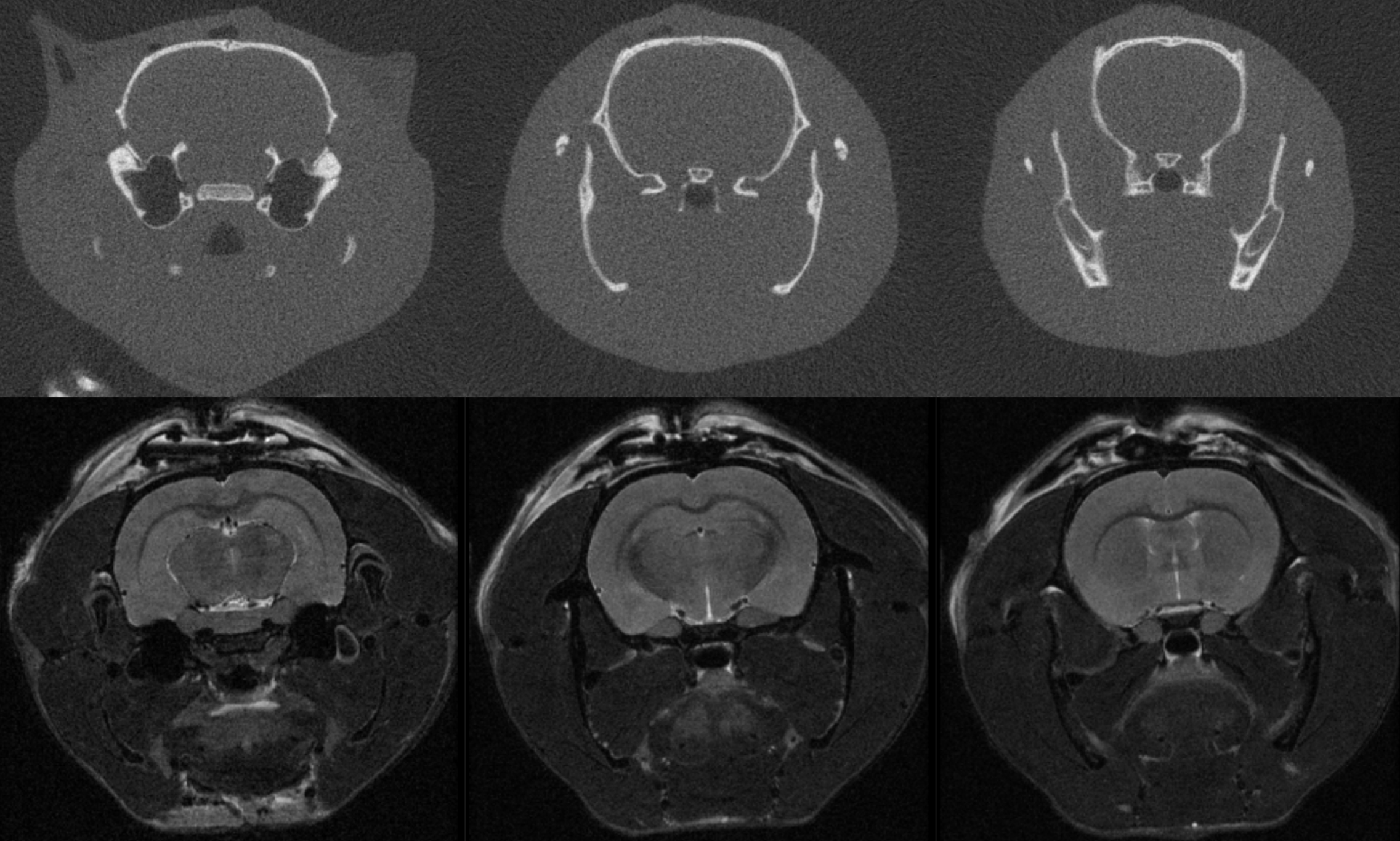616   2018), 101669, doi: 10.1016/j.nicl.2019.101669 (2019).

617   24.   Jones, D. K., Knösche, T. R., Turner, R. White matter integrity, fiber count, and other
618   fallacies: The do's and don'ts of diffusion MRI. *NeuroImage*. **73**, 239–254, doi:
619   10.1016/j.neuroimage.2012.06.081 (2013).
620   25.   Matlab code DKI and WMTI model. at <https://github.com/NYU-DiffusionMRI/Diffusion-
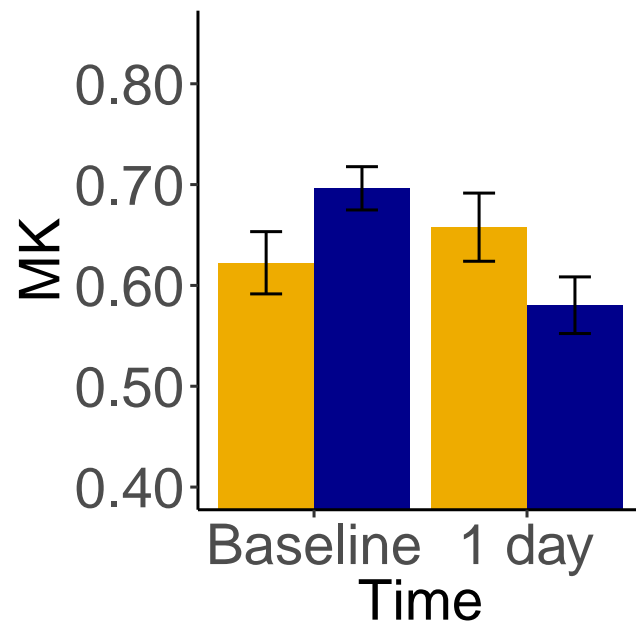621   Kurtosis-Imaging>.
622
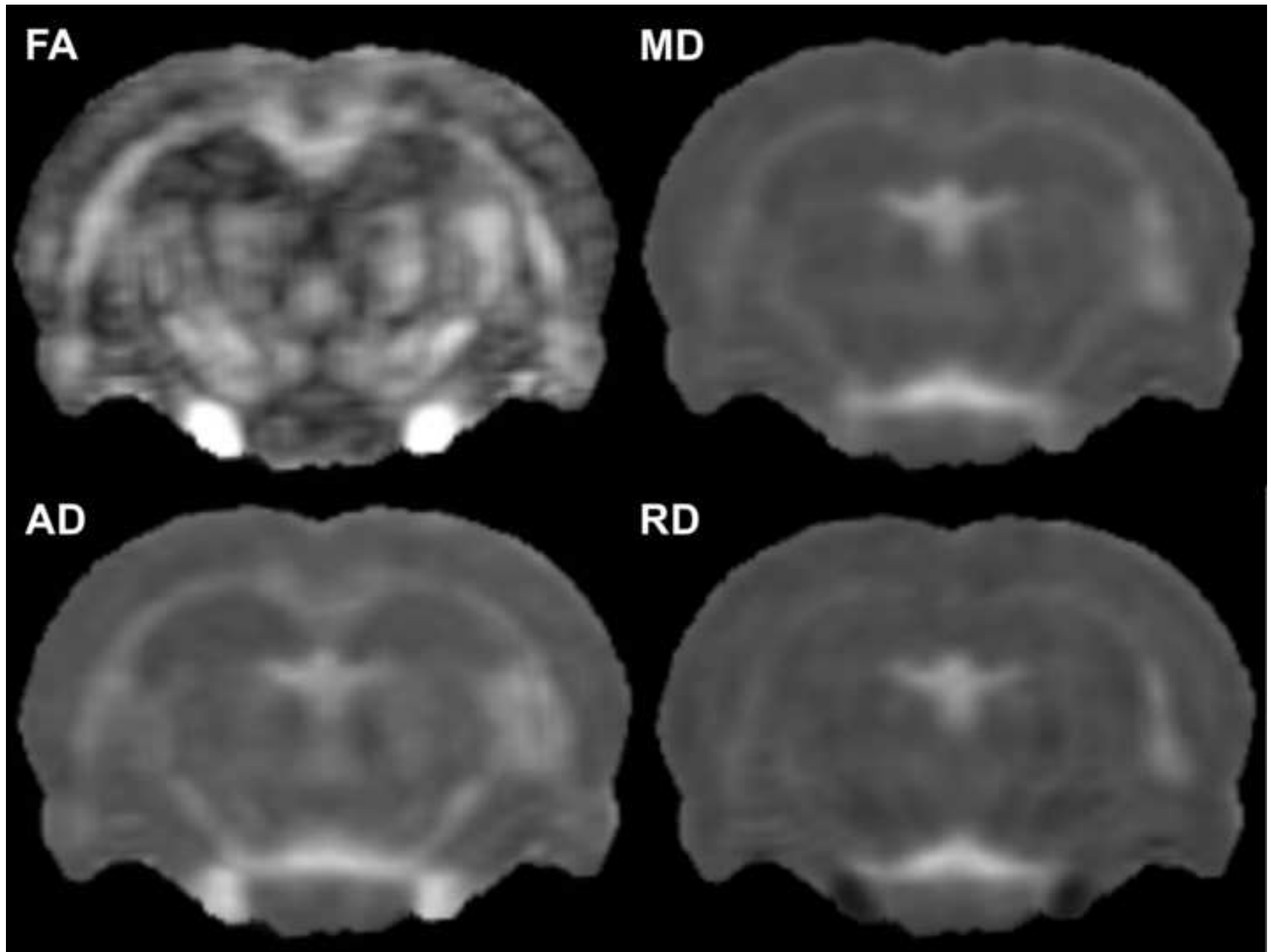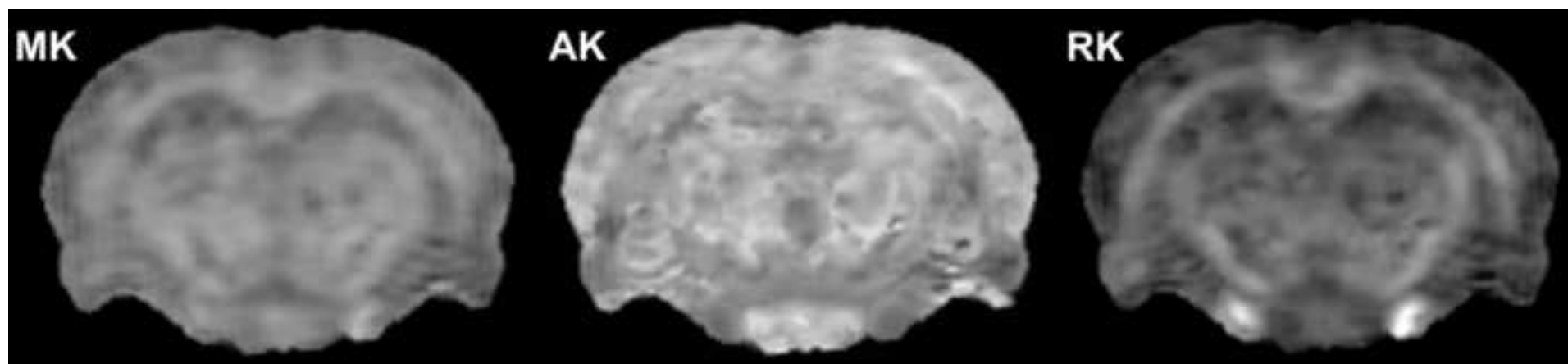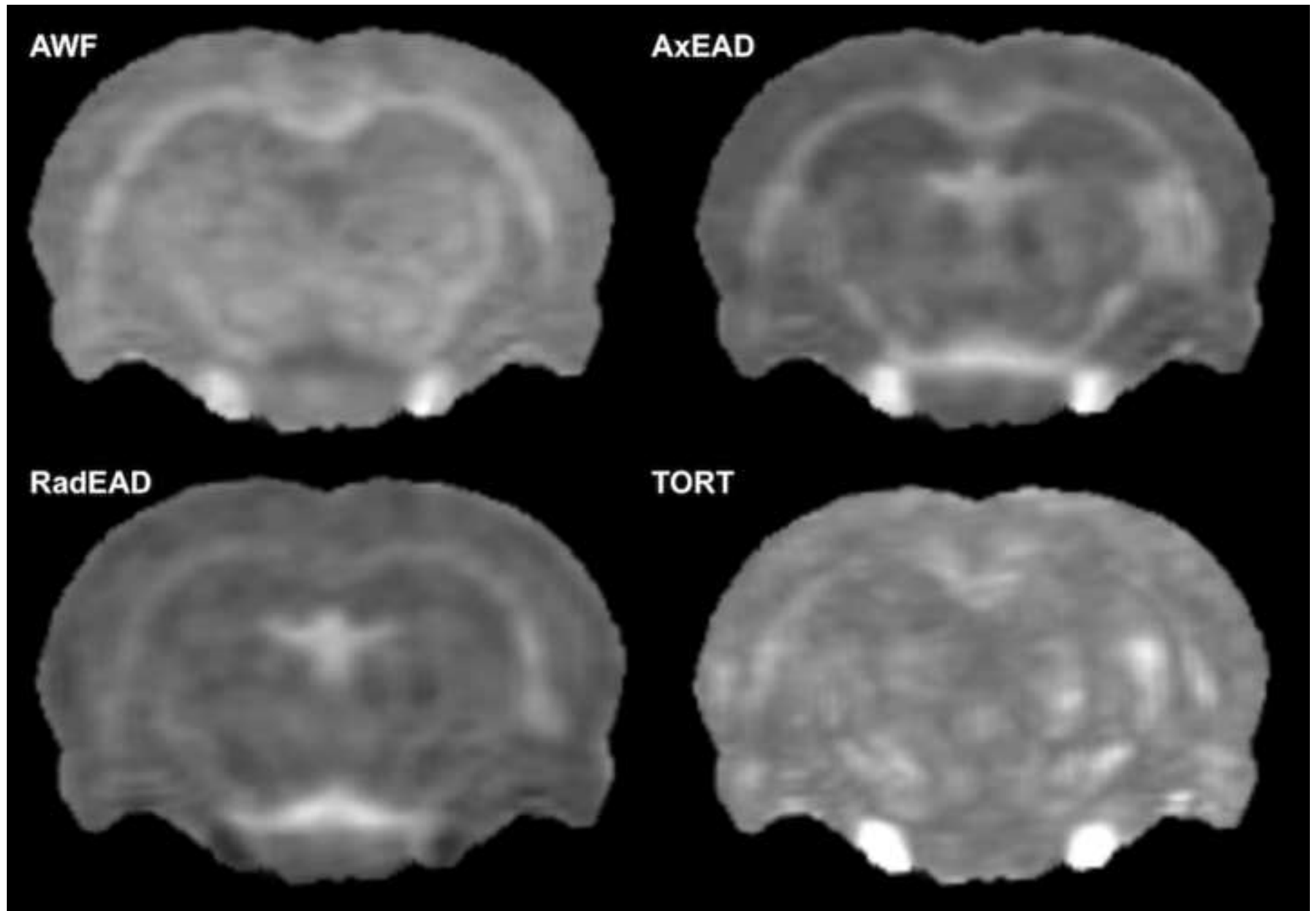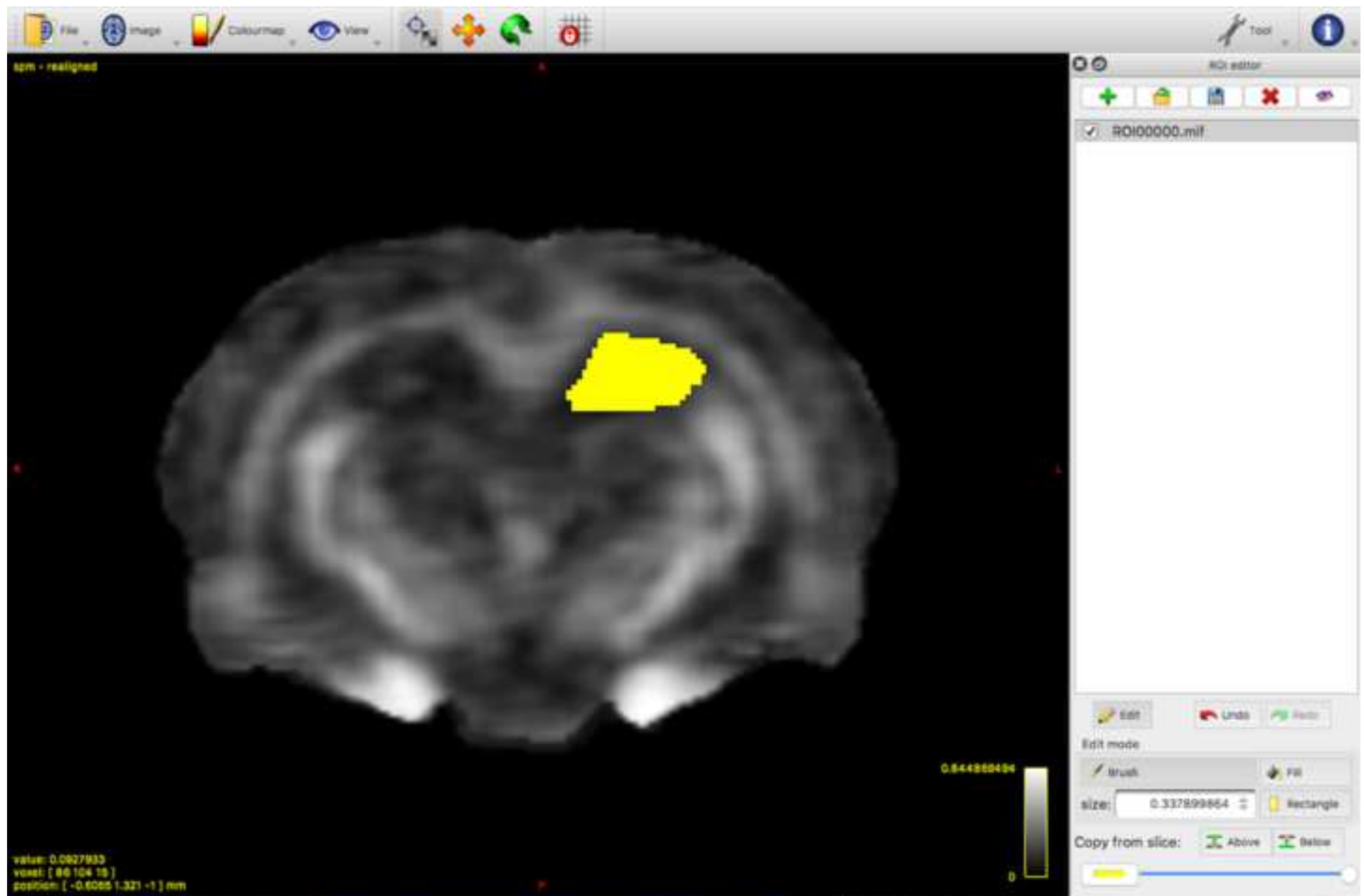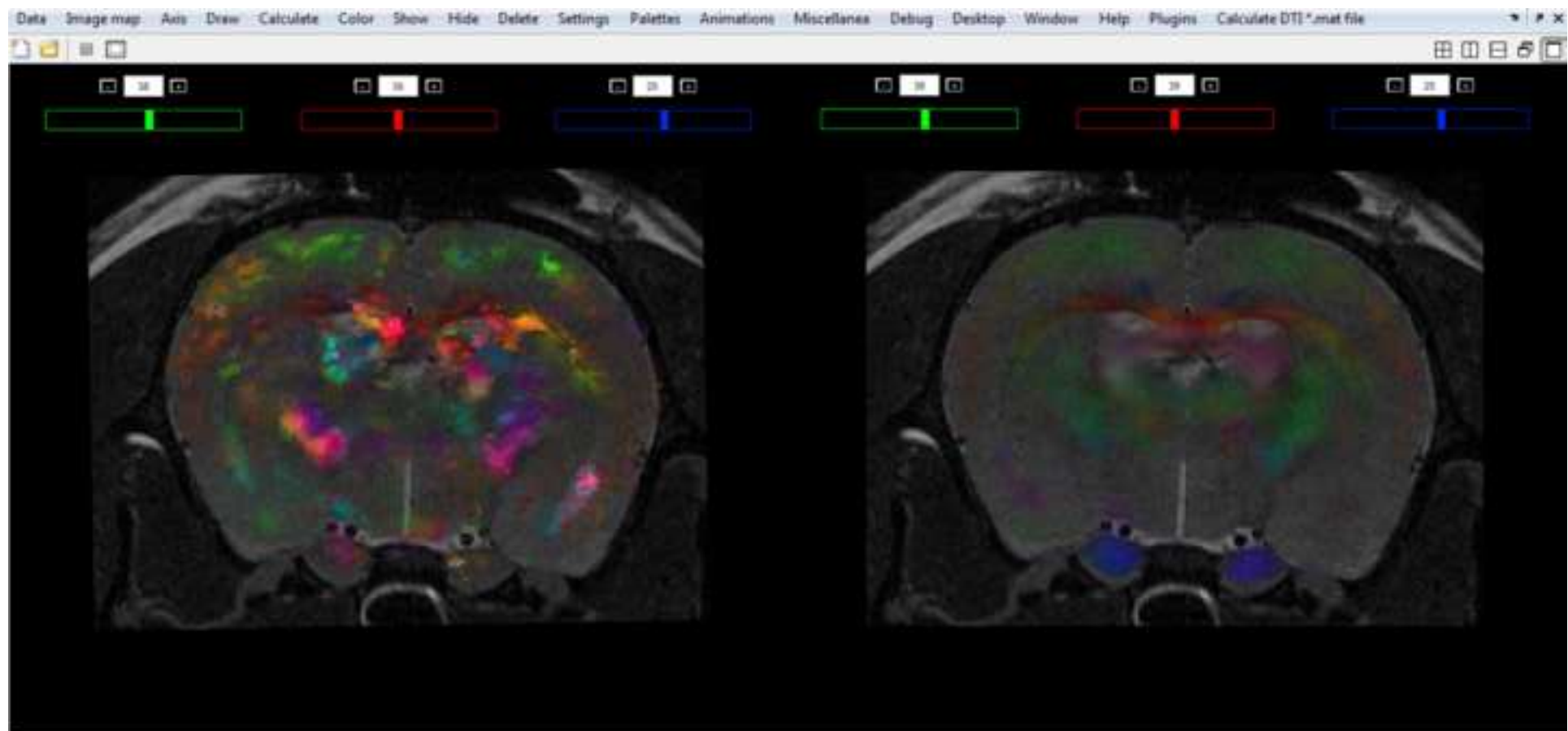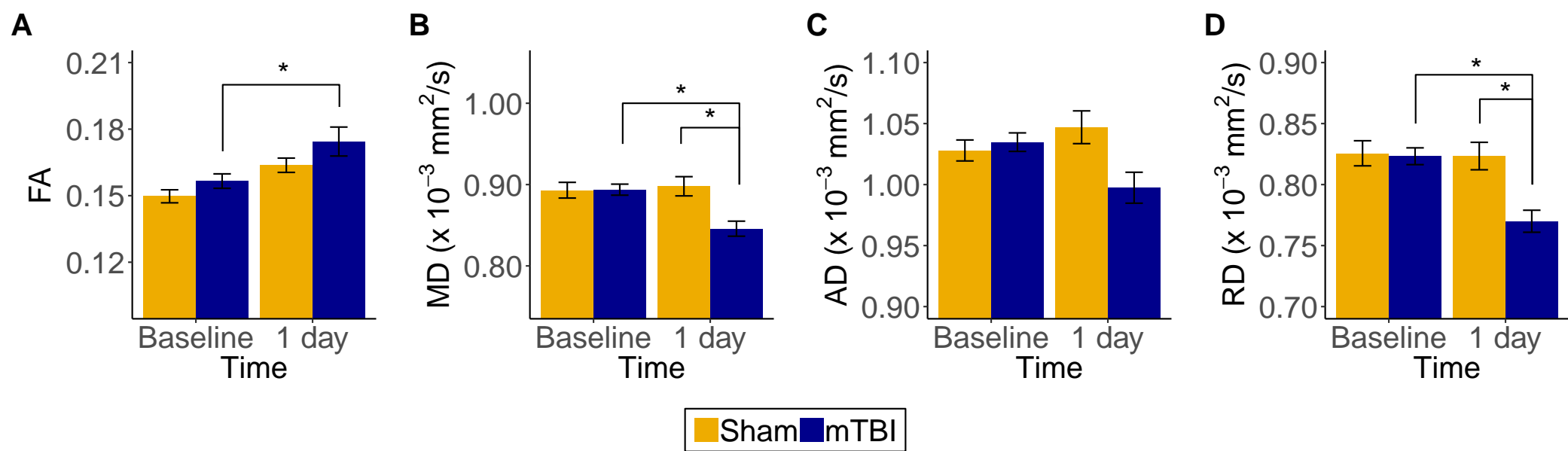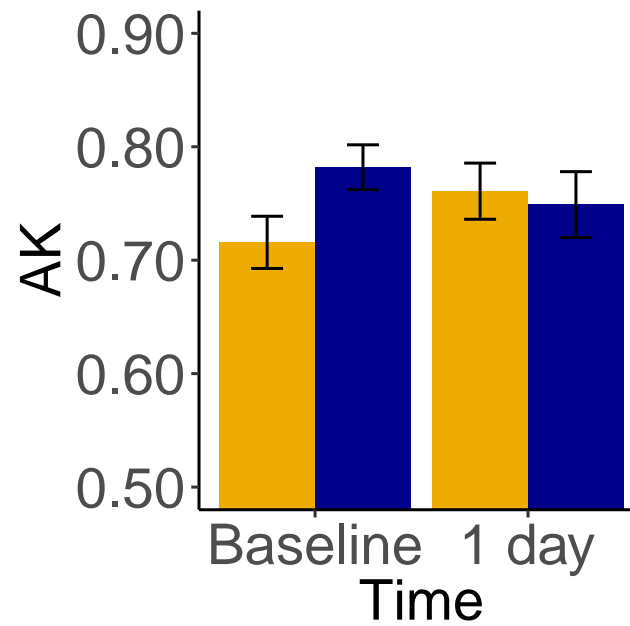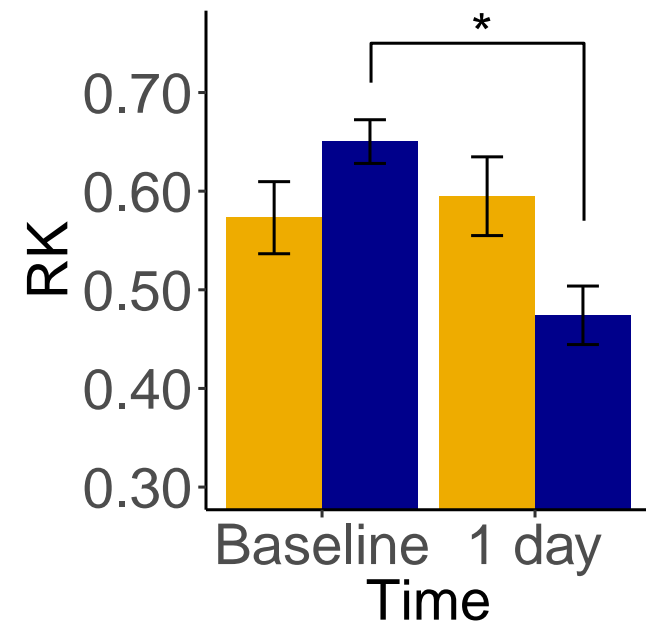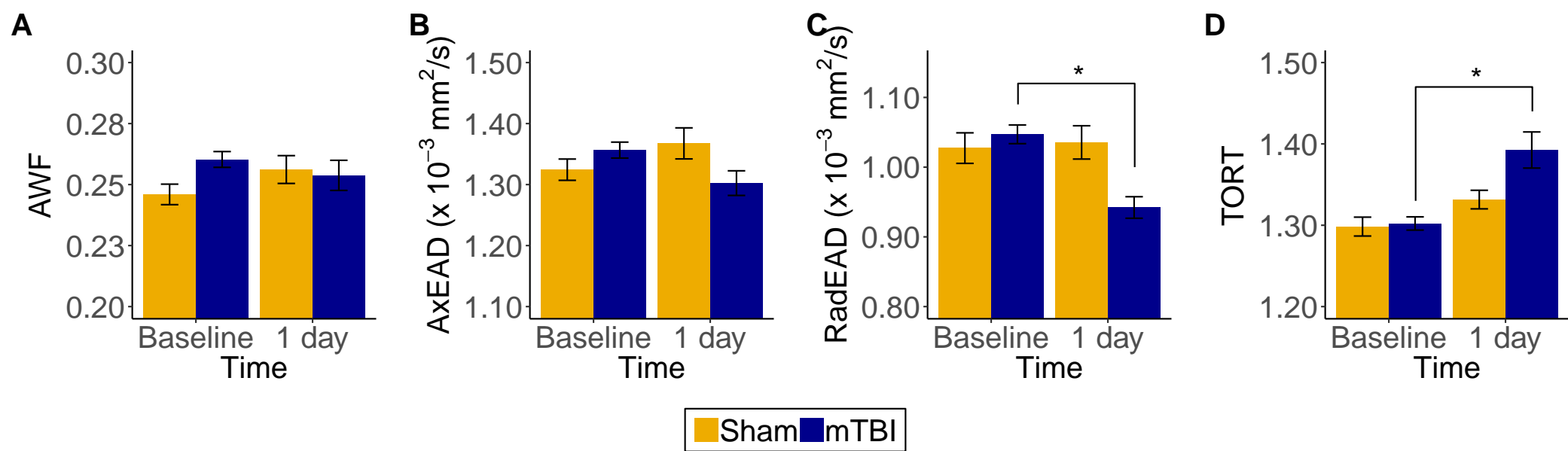
Name of Material/Equipment	Company	Catalog Number	Comments/Description
Induction of trauma			
0.9% NaCl physiologic solution	B Braun	394496	
brass weight 450g	custom made	custom made	diamter 18mm and 210 mm height
catheter	Terumo	Versatus-W	26G
ethilon II	Ethicon	EH7824	FS-3, 4-0, 3/8, 16mm
Matrass	Foam to Size	Type E	
Plexiglas tube	ISPA Plastics	416564	M1 PMMA XT GOO tube 25x19 mm (inner diamter 19 mm, minimal length of 1.50 m)
Preclinical CT scanner	Molecubes	X-cube	
Steel helmet	custom made	custom made	diameter 10 mm and 3 mm thickness
Vetbond Tissue Adhesive	3M	1469SB	
Vetergesic (buprenorphin)	EcuPhar	VETERG20	0.05 mk/kg
Xylocaine 2% gel	AstraZeneca	Xylocaine 2%	gel
Xylocaine (lidocain 2%)	Aspen/AstraZeneca	Xylocaine 2% gel	100 µl injection
Diffusion MRI			
Preclinical MRI acquisition software	Bruker Biospin MRI GmbH	Z400_PV51_CENTOS55	ParaVision 5.1 MRI software
Preclinical MRI scanner	Bruker Biospin MRI GmbH	PharmaScan 70/16	7T MRI scanner
Quadrature volume coil	Bruker Biospin MRI GmbH	RF RES 300 1H 075/040 QSN TR	Model No: 1P T13161C3
Small animal physiological monitoring unit	Rapid Biomedical	EKGHR02-0571-043C01	Unit for respiratory monitoring
Water-based heating unit	Thermo Fisher Scientific	Haake S 5P	Model No: 1523051

Anaesthesia

Anaesthesia movable unit isoflurane: Isoflo	Veterenary technics Zoetis	B506	BDO - Medipass, Ijmuiden
Oxygen generator	Veterenary technics	7F-3	BDO - Medipass, Ijmuiden

Diffusion image processing

Amide	http://amide.source forge.net	Version 1.0.5.	Medical Imaging Data Examiner Toolbox (Loening AM, Gambhir SS, " AMIDE: A Free Software Tool for Multimodality Medical Image Analysis", Molecular Imaging, 2(3):131-137, 2003)
ExploreDTI	http://www.explore dti.com	Version 4.8.6	Toolbox for (pre-)processing and analysis of diffusion weighted MR images (Leemans A, Jeurissen B, Sijbers J, and Jones DK. ExploreDTI: a graphical toolbox for processing, analyzing, and visualizing diffusion MR data. In: 17th Annual Meeting of Intl Soc Mag Reson Med, p. 3537, Hawaii, USA, 2009)

| MRtrix3 | http://www.mrtrix.org | Version 3.0_RC3-86-g4b523b41 | Toolbox for (pre-)processing and analysis of diffusion weighted MR images |

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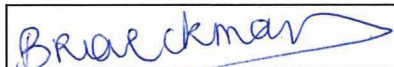Department: Infinity Lab - Medical Imaging and Processing Group

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Review Editor
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Kim Braeckman
PhD student

kim.braeckman@ugent.be
InfinityLab, IBiTech-Medisip
Faculty of Engineering Sciences and Architecture
Ghent University
Blok B-5 (Ingang 36),
Corneel Heymanslaan 10,
9000 Gent
BELGIUM

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