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verification of small animals**

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1 **Proton radiography for inline treatment planning and positioning**
2 **verification of small animals**

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23 **Abstract**

24 **Introduction:** As proton therapy becomes increasingly well established, there is
25 a need for high-quality clinically relevant *in vivo* data to gain better insight into
26 the radiobiological effects of proton irradiation on both healthy and tumor tissue.
27 This requires the development of easily applicable setups that allow for efficient,
28 fractionated, image-guided proton irradiation of small animals, the most widely
29 used pre-clinical model.

30 **Materials & Methods:** Here, a method is proposed to perform dual-energy
31 proton radiography for inline positioning verification and treatment planning.
32 Dual-energy proton radiography exploits the differential enhancement of object
33 features in two successively measured two-dimensional (2D) dose distributions at
34 two different proton energies. The two raw images show structures that are
35 dominated by energy absorption (*absorption mode*) or scattering (*scattering*
36 *mode*) of protons in the object, respectively. Data post-processing allowed for the
37 separation of both signal contributions in the respective images. The images were
38 evaluated regarding recognizable object details and feasibility of rigid
39 registration to acquired planar X-ray scans.

40 **Results:** Robust, automated rigid registration of proton radiography and planar
41 X-ray images in scattering mode could be reliably achieved with the animal
42 bedding unit used as registration landmark. Distinguishable external and internal
43 features of the imaged mouse included the outer body contour, the skull with
44 substructures, the lung, abdominal structures, and the hind legs. Image analysis
45 based on the combined information of both imaging modes allowed image
46 enhancement and calculation of 2D water-equivalent path length (WEPL) maps
47 of the object along the beam direction.

48 **Discussion:** Fractionated irradiation of exposed target volumes (e.g.
49 subcutaneous tumor model or brain) can be realized with the suggested method
50 being used for daily positioning and range determination. Robust registration of
51 X-ray and proton radiography images allows for the irradiation of tumor entities
52 that require conventional computed tomography (CT)-based planning, such as
53 orthotopic lung or brain tumors, similar to conventional patient treatment.

54 **Keywords:** proton radiography; dual-energy; preclinical; imaging; positioning

56 Introduction

57 In recent years, technical developments and increasing numbers of proton treatment
58 facilities worldwide established this therapy for the treatment of specific, solid tumor
59 entities. However, the amount of available data regarding the effects of proton
60 irradiation on tissue *in vivo* remains rather small [1]. Preclinical *in vivo* models have
61 been studied in several publications, yet small animal irradiation is often conducted in
62 non-targeting (i.e. whole body irradiation) setups [2], for purposes not relevant for
63 clinical treatment [3] or with targeted irradiation setups that rely on the reproducibility
64 of the animal's positioning through external fixation, e.g. earpins [4] or plastic boluses
65 [5–7]. Accordingly, methods suitable for image-guided proton irradiation of normal
66 tissue and subcutaneous as well as orthotopic tumor models in small animals need to be
67 established to make preclinical *in vivo* research of proton irradiation in clinically
68 relevant settings more feasible. Positioning of small animals, especially for irradiation
69 of subcutaneous tumor models is often realized with room lasers. However, orthotopic
70 tumor models as well as immunosuppressed animals which need to be kept under
71 specific pathogen free conditions e.g. transport boxes or bedding units, require image-
72 guided positioning of the target volume at the experimental site.

73 In this manuscript, a method is suggested for proton radiography-based treatment
74 planning and positioning verification of small animals at an experimental proton
75 beamline using clinically relevant beam energies. The approach exploits the fact that the
76 passage of a proton beam through matter influences both the beam's fluence distribution
77 and residual energy distribution. The latter effect can be enhanced by the use of
78 different proton energies for the imaging. Highly sophisticated proton radiography
79 methods such as proton microscopy [8] or particle tracking [9,10] yield image

80 resolutions that can compete with conventional X-ray imaging but are technically
81 demanding. The presented imaging method, on the other hand, respects the need for
82 practicability and mobility as well as low image acquisition dose that results from
83 fractionated animal irradiation at proton beamlines without a permanently installed,
84 dedicated imaging setup. Importantly, the suggested imaging method applies the same
85 beam that is used for the treatment itself, thus providing an inherently fixed spatial
86 reference of the acquired image to the beam's isocenter. Also, water-equivalent path
87 length (WEPL) values of the imaged objects are provided, which can help reducing
88 uncertainties in beam energy selection to correctly position the proton Bragg peaks
89 inside the target volume.

90

91

92 **Materials & Methods**

93 In this chapter, the key principles of the approach towards small animal proton
94 radiography are outlined, followed by the description of the experimental realization
95 and data processing. The designed setup was optimized regarding robustness and
96 efficiency.

97 *Conceptual approach*

98 The underlying idea is to acquire radiographic images of a mouse, from here on called
99 object, with an extended uniform proton field by determining the change of proton
100 fluence and proton energy caused by the object. Both kinds of changes (proton fluence
101 and energy) affect the dose distribution that is measured behind the object, which is
102 proportional to the acquired image intensity. In order to separate the sets of information,
103 two 2D dose maps are acquired (i.e., two images) that exploit different regions of the
104 proton Bragg curve by inserting slabs of plastic between object and detector for the
105 image acquisition. Hence, while the object is irradiated two times with a proton field of
106 the same energy, effectively, two different proton energies are used for the image
107 acquisition.

108

109 The image intensity I is related to the measured 2D dose distribution of the proton field.

110 For a perfectly uniform incident beam with proton fluence ϕ_0 and energy E_0 , and

111 thereby stopping power $\frac{dE}{dx_0}$, the measured signal is proportional to

$$112 \quad I \propto \frac{dE}{dx_0} \cdot \phi_0 \quad (1)$$

113 Inserting an object into the proton field leads to a change of the measured image

114 intensity

115
$$I \propto \underbrace{\left[\frac{dE}{dx_0} + \Delta \frac{dE}{dx_{\text{obj}}} + \Delta \frac{dE}{dx_{\text{Beam}}} \right]}_{\text{energy deposition}} \cdot \underbrace{[\phi_0 + \Delta\phi_{\text{obj}} + \Delta\phi_{\text{Beam}}]}_{\text{fluence inhomogeneity}} \quad (2)$$

116 which depends on the (spatial) fluence distribution and the energy deposition of the
 117 incident beam. Two major effects of the penetrated object are influencing the measured
 118 signal:

119 i) Energy deposition in the object slows down the protons which causes a change in
 120 stopping power represented by the term $\Delta \frac{dE}{dx_{\text{obj}}}$. ii) Inhomogeneous fluence is caused by
 121 multiple Coulomb scattering of the protons during the passage through the object
 122 denoted by $\Delta\phi_{\text{obj}}$. Additionally, a realistic incident proton field has an inherent energy
 123 and fluence inhomogeneity, represented by the terms $\Delta \frac{dE}{dx_{\text{Beam}}}$ and $\Delta\phi_{\text{Beam}}$. However,
 124 only the terms $\Delta \frac{dE}{dx_{\text{obj}}}$ and $\Delta\phi_{\text{obj}}$ contain the object information and need to be extracted
 125 from the measured intensity I .

126 For image acquisition at high proton energies (without plastic material between object
 127 and detector), energy deposition in the object can be assumed to be negligible ($\Delta \frac{dE}{dx_{\text{obj}}} \approx$
 128 0). The dose measurement is performed in the plateau region before the Bragg peak of
 129 the proton depth-dose curve. Hence, the intensity variation in the signal is dominated by
 130 fluence inhomogeneity caused by proton scattering within the object. In this case, the
 131 object information is encoded in $\Delta\phi_{\text{obj}}$. This imaging mode shall be referred to as
 132 *scattering mode* from here on forward.

133 However, if material (e.g. plastic (polycarbonate) slabs) is inserted between the object
 134 and the detector, the dose acquisition is carried out in the part of the proton depth-dose
 135 curve with a steep dose gradient. Then, even small changes in the water-equivalent path
 136 length of the object are translated into measurable dose changes in the detector.
 137 Consequently, the acquired signal is an overlay of inhomogeneous fluence caused by

138 scattering combined with energy absorption of the protons upon their passage through
139 the material (object + slabs of plastic). This imaging mode shall be referred to as
140 *absorption mode* from here on forward.

141 The setup of the dual-energy proton radiography concept is schematically shown in
142 Figure 1. A laterally uniform incident proton field passes through the object to be
143 imaged. A 2D flat panel scintillation detector, placed at a distance d from the object,
144 measures the 2D inhomogeneous dose distribution for two different imaging modes
145 (i.e., two images): either without (scattering mode) or with (absorption mode) slabs of
146 plastic inserted in between object and detector with no additional changes to the setup.

147 ***Experimental setup***

148 A recently developed mobile double-scattering setup [11] was used to produce an
149 extended proton field with the size of $10 \times 10 \text{ cm}^2$. The scattering setup was optimized
150 for a fixed initial proton energy of 150 MeV and provided a homogeneous field with an
151 effective energy of $E = 125 \text{ MeV}$. The 2D dose distributions were acquired with a
152 ‘Lynx’ flat panel scintillation detector (IBA dosimetry, Schwarzenbruck, Germany)
153 which has a pixel pitch of $0.5 \times 0.5 \text{ mm}^2$. The scintillation screen’s signal is measured
154 by a CCD sensor (*charge-coupled device*, light-sensitive electronic detector element).
155 Additional dosimetric measurements were conducted with an advanced Markus
156 ionization chamber (PTW, Freiburg, Germany) to evaluate the applied doses during the
157 imaging process. For each image, the monitor units (MU) measured by a monitor
158 ionization chamber at the proton beam exit were recorded as a relative measure of the
159 total dose. The dose is given as equivalent dose under the assumption of a relative
160 biological effectiveness of 1.1.

161 Planar X-ray scans of objects were acquired with a small animal imaging platform [12]
162 to evaluate the feasibility of automated, rigid registration of proton radiography images

163 and planar X-ray scans as well as to facilitate proton radiography image interpretation.
164 The X-ray image was used to identify the lung, the skull, abdominal organs and the
165 backbone of the object. Segmentation of structures was performed by two experienced
166 observers (R. Bütof and A. Dietrich) and the segmented contours were then projected
167 onto the proton radiography image for illustration and verification. This enabled the
168 evaluation of the method's potential for usage as a stand-alone tool or in combination
169 with other imaging modalities such as planar X-ray.
170 The test objects (deceased laboratory mice) were placed within an in-house developed,
171 opaque and fully physically closed bedding unit. This setup is designed to prevent
172 contact of immunosuppressed mice with an uncontrolled environment allowing for
173 future research with living animals (e.g. low hygiene and non-pathogen-free
174 environment, respectively).

175

176 *Image acquisition*

177 Proton radiography images of the object were acquired in scattering mode and
178 absorption mode. Additionally, images of the proton field without the object were
179 acquired in both modes, representing the background signal of the beam. The images
180 are referred to as A_{obj} and A_{Beam} (absorption mode images) and S_{obj} and S_{Beam} (scattering
181 mode images), respectively.

182 Each of the eight inserted plastic slabs had a WEPL thickness of
183 $\text{WEPL}_{\text{polycarbonate}} = 8.85$ mm. Additionally, a calibration curve for the conversion of
184 measured image intensity to WEPL was obtained in absorption mode. Slabs of plastic
185 with known WEPL were used as objects to relate their thickness with the measured
186 intensity to achieve energy absorption to thickness conversion. The total number of
187 eight absorber slabs was found to be a good compromise for the imaging of the intended

188 mouse irradiation setup. In general, the summed thickness of absorber slabs and object
189 to be imaged have to ensure a positioning of the detector screen in the part of the proton
190 depth-dose curve with monotonously increasing dose, i.e., proximal to the Bragg peak.
191 This allows for a monotonous conversion from pixel value to WEPL. On the other hand,
192 a high resolution of the WEPL of the object can be obtained when the detector is placed
193 close to the Bragg peak, where the dose gradient is high. Then, small changes in object
194 thickness translate into large dose differences.

195 *Data processing*

196 The intensity of each image was divided by the number of applied MUs to achieve
197 normalization. The insertion of an object into the proton field imposes a change on the
198 measured signal, which originates from energy deposition in the object and fluence
199 inhomogeneity (see above). These contributions can be separated mathematically to a
200 large extent: in scattering mode, the background-corrected image S is obtained by
201 subtracting S_{Beam} from S_{obj} . According to Eq. (2), S is mainly dominated by the variation
202 of $\Delta\phi_{\text{obj}}$ under the assumption that energy absorption is negligible in this setup
203 ($\Delta\frac{dE}{dx_{\text{obj}}} \approx 0$). The absorption mode image A has to be cleared of the fluence
204 inhomogeneity contribution which is achieved by the following operation:

$$205 \quad A = \frac{A_{\text{obj}}}{S_{\text{obj}}} - \frac{A_{\text{Beam}}}{S_{\text{Beam}}} \quad (3)$$

206 According to Eq. (2), A is mainly dominated by the energy loss $\Delta\frac{dE}{dx_{\text{obj}}}$ and therefore
207 the radiologic thickness of the object. The measured WEPL calibration curve could be
208 applied to the absorption image A to obtain a 2D map encoding the WEPL of the object
209 along the beam direction.

210 The generated images were further processed by using 2D histograms of the pixel
211 values from data of the images A and S. This visualization allowed for the identification
212 of characteristic regions in the histogram that could be assigned to specific image
213 features, e.g. the bedding unit, the outer contour or internal structures of the object.
214 Lastly, an unsharp masking algorithm was applied to the images to enhance image
215 contrast for better visibility. All processing steps were implemented as Python 2.7
216 scripts.
217

218 **Results**

219 The presented imaging method for preclinical proton irradiation experiments was
220 evaluated regarding its feasibility as a stand-alone tool (dual-energy) or in combination
221 with a planar X-ray scan, thus requiring image co-registration. An example of a raw,
222 unprocessed scattering mode image of a deceased laboratory mouse is shown in Figure
223 2 to depict the following processing steps more comprehensively. Faint contours of the
224 object can be seen within the bedding unit, framed by the collimated, rectangular proton
225 field.

226 *Image registration*

227 The implemented rigid image registration method yielded highly robust and
228 reproducible results. An example of a co-registered planar X-ray scan and the respective
229 scattering mode proton radiography image, taken with a dose of 189.8 mGy, are shown
230 in Figure 3. Both images were automatically cropped with the bedding unit's edges
231 being used as landmark. Automated detection of these edges was robust and
232 reproducible with doses as low as 6.1 mGy.

233 Furthermore, the outline of the object can be clearly distinguished from the background.
234 The skull of the object with substructures can be identified whereas the front of the skull
235 is superimposed by the signal of the fixation mask. Abdominal and intrathoracic
236 structures are visible in the scattering mode proton radiography image such as the
237 stomach and the lung.

238 The image quality and dose dependence of the performed image co-registration was
239 assessed by the reproducibility of the width of the bedding unit (number of pixels) in the
240 planar X-ray image and in the proton radiography image, respectively. Varying the
241 proton imaging dose (between 274 mGy and 6.5 mGy), lead to a stable pixel spacing

242 ratio k with $k = 5.6 \pm 0.2$ ($n = 11$, number of co-registered pairs of images), whereas
243 imaging doses of less than 22 mGy prevented visual identification of the mouse's body
244 contour.

245 *Dual-energy approach*

246 As described above, the method can also be used as a stand-alone tool to circumvent
247 prior X-ray imaging. Figure 4 (A) shows the raw data of an absorption mode proton
248 radiography image of the object with (B) showing the result after image correction and
249 conversion from pixel value/MU to WEPL. The applied correction removed the
250 contribution to the signal originating from proton fluence inhomogeneity (bright corona
251 around mouse in raw image) to a large extent. The measured maximal radiologic
252 thickness of the object in the bedding unit, $WEPL_{\text{mouse+bedding}} \approx 30$ mm, corresponds well
253 with the actual thickness of the object under the assumption that the tissue composition
254 of the object in the abdominal region is equivalent to water. Furthermore, the WEPL
255 measurement accuracy can be estimated by the WEPL values of the image background
256 outside the bedding unit. In the case of the absorption mode image (Figure 4) the WEPL
257 of the background was $WEPL_{\text{Background}} = 0.0 \pm 1.0$ mm. The WEPL measurement of a
258 plastic plate ($WEPL_{\text{plate}} = 15.0$ mm) yielded a value of 15.6 ± 1.0 mm. Furthermore,
259 geometric proportions of objects in the image (e.g. radius of the bedding unit,
260 $R = 16.5$ mm) could be measured on the absorption mode image. The latter's radius was
261 determined through a fit and was found to be $R_{\text{meas}} = 17.6$ mm with an R^2 -value of 0.95
262 (Figure 6, supplementary materials).

263 Subsequently, the corrected absorption-based proton radiography image allowed
264 evaluation of the scatter-based image data in the context of the radiologic thickness as
265 demonstrated in Figure 5 by means of a 2D histogram of both images (B). Within the
266 2D histogram (Figure 5 (B)), distinct areas can be attributed to certain regions of

267 interest in the image. Pixels containing the background of the image (mostly air) were
268 found to be clustered in a 2D histogram region of low scattering and low-energy
269 absorption, i.e., small WEPL. This region is marked accordingly with (a) in the 2D
270 histogram. The rims of the bed are characterized by a strong scattering signal due to its
271 distinct edges (air-material gradient) and an absorption signal slightly above
272 background (b). Lastly, the body of the mouse, which features a range of WEPL values
273 greater than ~ 5 mm and broadly ranging scattering signal due to internal structures, is
274 identified as region (c) in the 2D histogram.

275 In region (c), the WEPL values correlated positively with the scatter signal, as can be
276 seen from the 2D histogram. This characteristic originates from the energy absorption in
277 scattering mode which is small but highly correlated with the WEPL of the object. It
278 could be corrected in first-order by minimizing its correlation with WEPL. Subsequent
279 application of image sharpening (unsharp masking algorithm) and suitable windowing
280 resulted in the 2D histogram in Figure 5 (D).

281 The performed operations were found to produce scattering mode images which
282 preserve the outer features (e.g. bedding unit, body contour and skull) as well as
283 enhance contrast and detectability of internal structures such as abdominal internal
284 structures, the lung or substructures of the skull.

285 **Discussion**

286 Experiments with deceased mice were conducted to evaluate the feasibility of proton
287 radiography both in combination with other imaging modalities e.g. planar X-ray scans
288 or as a stand-alone tool for positioning verification and treatment planning for proton
289 irradiation of small animals.

290 A high level of image detail could be obtained from the performed scattering mode
291 imaging experiments in combination with planar X-ray imaging. Robust detection of
292 landmarks, e.g. the bedding unit, could be achieved, allowing the method to be used as
293 positioning tool in combination with additional external X-ray/CT imaging. Rigid
294 registration could even be reproducibly achieved for a high noise level (doses as low as
295 22 mGy). The co-registration of scattering mode images and X-ray images can be
296 reliably performed using the bedding unit as a rigid reference frame. The positioning
297 accuracy in this setup is inherently limited by the uncertainty of the co-registration
298 (given by the pixel spacing ratio k , $\Delta k = \pm 0.2$), the spatial resolution of the used X-ray
299 device (± 0.2 mm) [12] and the Lynx detector's pixel pitch (± 0.5 mm). The squared sum
300 of these uncertainties give an estimate of the theoretically achievable positioning
301 accuracy with the proposed setup. The suggested estimate yields a positioning
302 uncertainty of ± 0.6 mm.

303 The presented approach is also promising as a stand-alone method for image-guided
304 irradiation of small animals. The hind leg and the tail of the mouse can clearly be
305 identified in the scattering mode image which allows sparing of healthy surrounding
306 tissue (e.g. abdomen) by conformal proton irradiation and adequate positioning of the
307 animal. This is of key importance to prevent undesired harmful normal tissue reactions,
308 especially if visual positioning of the animal is not possible due to an opaque animal
309 setup. Furthermore, the skull and internal substructures (e.g. ear canal, Figure 3 (A));

310 eyehole, Figure 5(C)) as well as the lung can be identified in the scattering mode
311 images.

312 In absorption mode, information about the internal structures of the mouse is lost since
313 protons undergo multiple Coulomb scattering processes upon passing through the
314 plastic slabs behind the object. The distribution of the proton scattering angle caused by
315 multiple Coulomb scattering in the plates is (approximately) Gaussian [13]. Therefore,
316 the slabs effectively induce a Gaussian blurring of the absorption mode image. Yet, the
317 WEPL of the object could be determined from the 2D WEPL maps (see Figure 4(B)).
318 This information can then be used – e.g., together with an X-ray CT – to determine the
319 proton energy to achieve a correct spread-out Bragg peak positioning in the tissue.
320 Thus, the combination of absorption and scattering mode proton radiography imaging
321 can provide a potential tool for an on-site treatment planning and positioning
322 verification, as both, information about the internal structure of the animal as well as on
323 the radiologic thickness of the object, is provided.

324 The identification of the animal's skull including substructures and abdominal as well
325 as intrathoracic structures can potentially allow for image-guided, targeted irradiation of
326 these structures. This is of particular interest for studying normal tissue reactions in
327 organs like brain or lung. In addition, orthotopic tumor models (e.g. glioblastoma, lung
328 tumors) have been found to become of increasing importance in the context of
329 preclinical research, as they can provide clinically relevant insights into tumor-related
330 biological characteristics [14] which cannot be addressed through clinical trials [15].

331 The proposed imaging modes (dual-energy or combination with external imaging) need
332 to be chosen with regard to the respective scenario. The (fractionated) irradiation of
333 spatially exposed structures such as subcutaneous tumors or full and partial brain
334 irradiation, respectively, can be achieved with dual-energy proton radiography as a

335 method for daily positioning and range determination. Target volumes that require
336 external imaging (planar X-ray, X-ray CT) for treatment planning (e.g. sub-volumes of
337 healthy lung tissue, orthotopic lung tumors, orthotopic brain tumors) can be positioned
338 for daily treatment using single-energy proton radiography. In both scenarios,
339 absorption mode proton radiography can provide the necessary data for the
340 determination of the proton beam's range in the animal. This is of particular importance
341 for the conduct of clinically relevant studies since the biological effect of proton
342 irradiation is known to depend on the residual proton energy at the target location [1].
343 Due to the fact that the object is irradiated with energies in the plateau region of the
344 Bragg curve in both radiography modes, the applied doses can be kept small in
345 comparison to the applied treatment doses.

346 The presented method is conceptually very similar to X-ray single-source, dual-layer
347 computed tomography [16] which is realized by implementing the necessary energy
348 selection for dual-energy imaging as layered detectors on the posterior site of the object.
349 The conducted experiments showed that the implementation of a single-source dual-
350 energy approach in the context of proton radiography provides additional information
351 compared to single-energy proton radiography.

352 Furthermore, the experimental setup was easy to install and dismantle at a (multi-
353 purpose) experimental site while delivering results of reproducible and reliable image
354 quality. This is of key importance for the conduct of high precision irradiation of small
355 animals at multi-purpose experimental facilities which may not provide a dedicated
356 setup for image-guided irradiation as it was presented by Ford et al. [17].

357 In summary, the presented method for proton radiography is feasible for performing
358 inline position verification and planning of image-guided treatments of small animals in
359 a stand-alone or combined, multi-modal imaging approach while delivering images of

360 lower spatial resolution than more sophisticated approaches [8,9]. A strength of the
361 presented setup is the fact that the proton radiography imaging and the subsequent
362 treatment are performed in the same setup and with the same radiation source. It allows
363 for a direct registration of the radiography image relative to the treatment field both
364 measured with the same detector. Thereby, the detector provides a fixed relative
365 reference frame between the object to be irradiated and the proton beam. Based on rigid
366 co-registration (as demonstrated), this spatial reference can be extended towards a
367 planar X-ray image. Current efforts concentrate on the application of the method for the
368 first fractionated irradiation experiments of sub-cutaneous tumor models in mice and
369 research on radiation-induced normal tissue complication after targeted organ
370 irradiation. The current implementation of the approach and therefore the achievable
371 image quality is expected to improve after the availability of proton fields with higher
372 energies (about 230 MeV).

373

374

375

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377

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382

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459 Figure 1: Schematic figure of the used setup for dual-energy proton radiography
460 imaging in both imaging modes. The blue arrow on the left indicates the incident proton
461 field. In *absorption mode*, the measured dose distribution encodes the residual energy of
462 the beam after passing the object and hence the combined thickness of the plates and the
463 object. In *scattering mode*, the fluence of the field is disturbed by the passage of the
464 beam through regions of different density (here depicted as different shades of grey).
465 The distance d between object and detector remains unchanged

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469 Figure 2: Raw scattering mode proton radiography image of a mouse (**a**) within the
470 bedding unit (**b**). The edge of the brass collimator which shapes the extended proton
471 field (**c**) can be visually identified by the bright corona (**d**) at its edges.

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474 Figure 3: **(A)** Proton radiography image acquired in scattering mode. **(B)** Planar X-ray
475 scan of the same mouse prior to proton radiography acquisition. Both images were
476 cropped with the bedding unit used as landmark. The markers indicate the contours of
477 internal and external structures of the mouse that could be identified in the planar X-ray
478 image. Structures that could be distinguished were **(a)** abdominal intestines, **(b)** the
479 stomach, **(c)** the lung, **(d)** the vertebral spine and **(e)** the body contour of the mouse.
480 These contours are also shown in the proton radiography image **(A)** for illustration.

481

482 Figure 4: Absorption mode proton radiography. **(A)** Raw image and **(B)** background-
483 corrected image with conversion from pixel value/MU to WEPL applied. **(C)** Post-
484 processed image using an unsharp masking filter for the enhancement of internal
485 structures.

486 Figure 5: Overview of applied data processing and post-processing operations. **(A)**
487 Background-corrected scattering mode image S. **(B)** Native 2D histogram of pixel
488 values from background-corrected images in scattering and absorption mode along the x
489 and y axis, respectively. The highlighted regions could be assigned to specific image
490 features such as **(a)** the body of the mouse, **(b)** the bedding unit and **(c)** the air-filled
491 image background. **(C)** Processed scattering mode image and **(D)** corresponding
492 modified 2D histogram after the applied operations (for details see materials &
493 methods).

494

495 Figure 6: Mean WEPL cross-section of the massive plastic plate at the bedding unit's
496 top end (Figure 4B) and fitted WEPL curve corresponding to the ideal, expected WEPL
497 cross-section of the measured object. The fitted parameters are the center of the bedding
498 unit in the image (x_0), the bedding unit's radius (R) and the relative stopping power of
499 the material (k). The fit yielded values of $x_0 = -0.3$ mm, $R = 17.4$ mm and $k = 0.52$.