1 **Evidence for Homodimerization of the c-Fos Transcription Factor in Live Cells Revealed**

2 **by Fluorescence Microscopy and Computer Modeling**

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ABSTRACT

The c-Fos and c-Jun transcription factors, members of the activator protein-1 (AP-1) complex, form heterodimers and bind to DNA via a basic leucine zipper, and regulate the cell cycle, apoptosis, differentiation, etc. Purified c-Jun leucine zipper fragments could also form stable homodimers, whereas c-Fos leucine zipper homodimers were found to be much less stable in earlier in vitro studies. The importance of c-Fos overexpression in tumors and the controversy in the literature concerning c-Fos homodimerization prompted us to investigate Fos homodimerization. FRET and molecular brightness analysis of fluorescence correlation spectroscopy data from live HeLa cells transfected with fluorescent protein-tagged c-Fos indicated that c-Fos formed homodimers. We developed a method to determine the absolute concentrations of transfected and endogenous c-Fos and c-Jun, which allowed us to determine dissociation constants of c-Fos homodimers (K_d=6.7±1.7 μM) and c-Fos–c-Jun heterodimers (on the order of 10-100 nM) from FRET titrations. Imaging fluorescence cross-correlation spectroscopy and molecular modeling simulations confirmed that c-Fos homodimers were stably associated and could bind to the chromatin. Our results establish c-Fos homodimers as a novel form of the AP-1 complex, which may be an autonomous transcription factor in c-Fos overexpressing tissues, and could contribute to tumor development.

Introduction

Activator protein 1 (AP-1) is a transcriptional regulator composed of members of the Fos, Jun and ATF families of DNA binding proteins (1, 2). c-Fos and c-Jun regulate a variety of processes including proliferation, differentiation, apoptosis and oncogenesis (3). They function as dimers binding to the promoter/enhancer regions of numerous mammalian genes (4). Their DNA binding domain is composed of a leucine zipper promoting dimerization and a basic region, which binds with high affinity to a specific 8 bp long DNA sequence (5, 6).

In addition to forming stable heterodimers with c-Fos (7-9), c-Jun can also homodimerize as revealed in vitro by electrophoretic mobility shift assay (EMSA) (8), and bind to DNA as a homodimer, although with lower affinity than the heterodimer (8, 10). In contrast, the c-Fos homodimer was found to be unstable in vitro, and thus c-Fos has been thought to interact with DNA only by forming heterodimers with c-Jun (9, 11, 12). The instability of the c-Fos dimer is thought to be due to repulsion between its negatively charged residues in the leucine zipper (6). The wild type c-Fos zipper showed no homoassociation at a concentration of 0.1 μM according to EMSA (13). O'Shea and coworkers estimated the K_d of the c-Fos leucine zipper homodimer to be 3.2 μM and 5.6 μM at 0 and 25 °C, implying that failure to detect c-Fos dimerization by others was probably due to low protein concentrations (14). It was shown by EMSA that a single amino-acid change in the leucine zipper is sufficient to allow a truncated c-Fos to homodimerize and bind to its DNA response element (15). Thermal melts on different leucine zipper dimers revealed that thermal stability increases from c-Fos–c-Fos through c-Fos–c-Jun to c-Jun–c-Jun (16).

c-Fos expression and activation can be induced by growth factors, cytokines or neurotransmitters via G-protein coupled receptors, MAPK, cAMP- or Ca^{2+}-dependent signaling pathways (17-19). c-Fos overexpression occurs in several pathological conditions, which can

65   have both proliferative and antiproliferative effects. c-Fos was overexpressed in some tamoxifen

66   resistant human breast tumors (20), and highly overexpressed in malignant oral tissues (21). It

67   could also contribute to hepatocarcinogenesis (22). In a murine skin carcinogenesis model c-Fos

68   was shown to be required for malignant tumor conversion (23). c-Fos can be up-regulated via the

69   thyroid hormone nuclear receptor α1, which is a tumor inducer in intestinal tumorigenesis (24).

70   Conversely, c-Fos overexpression inhibited cell-cycle progression and stimulated cell death in

71   hepatocytes (25). It also activated apoptosis in colorectal carcinoma cells in a p53 dependent

72   manner (26).

73   Because c-Fos but not c-Jun is overexpressed in many different types of tumors, we were

74   interested whether at higher concentrations c-Fos could form stable homodimers and bind to

75   DNA in live cells. Förster resonance energy transfer (FRET) can be used to assess distances

76   between two fluorophores in the range of 2–10 nm (27, 28), whereas fluorescence cross-

77   correlation spectroscopy (FCCS) can demonstrate the co-mobility of two molecules (29-31).

78   Using these methods previously, we demonstrated heterodimerization and chromatin binding of

79   c-Fos and c-Jun, and described the conformation of their complex in live cells (7, 32). It was

80   shown in our lab (DKFZ) by imaging FCCS that mobility and protein-protein interaction maps of

81   c-Fos and c-Jun were correlated (33).

82   Here we performed FRET measurements on fluorescent protein-tagged c-Fos molecules

83   by confocal microscopy and flow cytometry to examine whether c-Fos could form homodimers.

84   We   developed   a   method   combining   fluorescence   correlation   spectroscopy   and

85   immunofluorescence to assess the concentrations of both the fluorescently labeled and unlabeled,

86   endogenous c-Fos and c-Jun in cells. This allowed us to determine the K_d of c-Fos homodimers

87   and c-Fos–c-Jun heterodimers in live HeLa cells by FRET titrations. We found that the K_d of the

88   c-Fos homodimer is more than one order of magnitude higher than that of the heterodimer. To

89   our knowledge this is the first report on the determination of the K_d of transcription factors from

90   FRET titrations in live cells. Imaging FCCS measurements revealed co-diffusion of stable c-Fos

91   homodimers and their binding to chromatin. Our molecular dynamics simulations support that

92   Fos homodimers can form, bind to DNA and remain stable over the time span of the simulation

93   (500 ns). This novel homodimeric form of c-Fos may act as an autonomous transcriptional

94   regulator.

95

96   **MATERIALS AND METHODS**

97   **Cell culture, plasmid construction and transfection of Hela cells**

98   Cell culture, plasmid construction and transfection have been described elsewhere (34).

99   Detailed information on these procedures and plasmids is in the Supplement.

100

101   **Confocal microscopic and flow cytometric FRET analyses**

102   Confocal microscopic images were collected by using a Zeiss LSM 510 microscope. Flow

103   cytometric measurements were carried out on a Becton Dickinson FACSAria III instrument.

104   Details of data acquisition, and FRET analysis on a pixel-by-pixel or cell-by-cell basis have been

105   described earlier (34) and are detailed in the Supplement.

106

107   **Fluorescence correlation spectroscopy and calibration of fluorescence intensity as a**

108   **measure of absolute concentration**

109   In FCS (35), molecules diffuse across a subfemtoliter (<1 μm^3) detection volume defined

110   by a focused laser beam. This causes fluorescence fluctuations, which are analyzed to derive

111   dynamic parameters of the studied molecules. FCS measurements were performed on a modified

112   Olympus FluoView 1000 confocal microscope based on an inverted IX-81 stand with an

113 UPlanAPO 60× NA 1.2 water immersion objective. The FCS extension (Steinbeis Transfer Unit

114 for Biophysical Analytics, Heidelberg, Germany) equipped with two avalanche photodiodes

115 (Perkin-Elmer, Wellesley, MA) is attached to the confocal scanning unit. Fluorescence of EGFP

116 was excited by the 488 nm line of an Ar ion laser, and detected between 500–550 nm. To allow

117 FCS measurements at high EGFP concentrations (up to 25 µM), laser illumination was dimmed

118 by a neutral density filter (OD 1) to yield P=0.2 µW at the sample. FCS measurements on live

119 HeLa cells were performed in eight-well chambered coverglass plates (NUNC). Points for FCS

120 measurements were selected from confocal images. From each sample n~30 cells were measured

121 at room temperature, and 6×8 s runs per cell were recorded. Fluorescence autocorrelation

122 functions were calculated online by an ALV-5000E hardware correlator card (ALV Laser,

123 Langen, Germany). Autocorrelation curves were fitted to a two-component 3D diffusion model

124 with triplet correction by using the program QuickFit3.0 (36):

$$125 \quad G(\tau) = \frac{1 - T + T\,e^{-\tau/\tau_{tr}}}{1-T} \frac{1}{N} \left[r_1 \left(1 + \frac{\tau}{\tau_1}\right)^{-1} \left(1 + \frac{\tau}{S^2\tau_1}\right)^{-1/2} + r_2 \left(1 + \frac{\tau}{\tau_2}\right)^{-1} \left(1 + \frac{\tau}{S^2\tau_2}\right)^{-1/2} \right] \qquad (1)$$

126 where τ is the lag time, T denotes the triplet fraction, τ_{tr} is the triplet correlation time, τ_1 and τ_2 are

127 the diffusion times of the fast and slow species (average dwell times of molecules in the detection

128 volume), r_1 and $r_2 = 1 - r_1$ are the fractional amplitudes of the two components, N is the average

129 number of molecules in the detection volume and S is the aspect ratio of the ellipsoidal detection

130 volume. To assess the aggregation state of EGFP-labeled proteins, the molecular brightness or

131 fluorescence per particle, F/N, was calculated and compared with monomeric EGFP.

132 To facilitate K_d determinations, we developed a method to assess absolute concentrations from

133 fluorescence intensity, similar to that described in (37). In the first step we determined the

134 detection volume, V_{eff} of the microscope by using a 130 nM Alexa 488 solution as a standard.

135   From its autocorrelation function τ_d and S were determined by fitting, and the lateral radius ω_{xy}

136   and the axial radius ω_z were calculated with the following equations:

137     $\omega_{xy} = \sqrt{4D\tau_d}, \omega_z = \omega_{xy}S$,   (2)

138   where τ_d is the measured diffusion time of the dye, which measures mobility and is inversely

139   proportional to the diffusion coefficient and D=435 μm^2/s is its diffusion coefficient of Alexa 488

140   at 22.5 °C (38). The effective detection volume is:

141     $V_{eff} = \pi^{3/2}\omega_{xy}^2\omega_z$.   (3)

142   From autocorrelation curves with EGFP the particle numbers N were determined. From these,

143   molar concentrations c were calculated as

144     $c = N / \left(N_A V_{eff}\right)$,   (4)

145   where N_A is Avogadro's number. Before every FCS measurement the fluorescence intensity F

146   was measured with the imaging detector (photomultiplier tube) of the confocal microscope at the

147   site of FCS measurement; thus, c $vs.$ F calibration lines were generated (Fig. 4B).

148   To facilitate comparison of measurements on different days we used fluorescent beads.

149   The fluorescence intensity of 6-μm green calibration beads (bead with 1% relative intensity from

150   the InSpeck Green Microscope Image Intensity Calibration Kit, Molecular Probes, Life

151   Technologies, Inc.) was used to normalize EGFP fluorescence. Confocal sections in the

152   equatorial plane of the beads were recorded on the same day as the FCS calibration, using

153   identical instrument settings. Average fluorescence intensity per pixel in the central area of the

154   beads was obtained. Using the calibration curve in Fig. 4B, the local intensity of the bead at its

155   center corresponded to an EGFP concentration of $c_{bead\ unit}^{confocal}$ ~15.4±0.7 μM (average±SEM of five

156   experiments).

157   We could also transfer the concentration calibration to flow cytometric measurements. In

158   the microscopic calibration described above, the local fluorescence intensities at a pixel of the

159   sample and the bead are compared. In contrast, in flow cytometry the total intensity of the whole

160   cell and the bead are measured. Therefore, the ratio of the cellular and bead volumes had to be

161   taken into account. The volume of HeLa nuclei (where Fos and Jun are localized) is ~13.6-times

162   larger than that of the beads as determined by confocal microscopic 3D sectioning and using the

163   Imaris software (Bitplane AG, Zurich). In addition, the different detection efficiencies of the

164   spectra of the bead and EGFP arising from different band pass filters in the flow cytometer and

165   the confocal microscope differed by a factor of 13. Taking these factors into account, the total

166   intensity of a bead corresponded to an EGFP concentration of $c_{bead\ unit}^{flow\ cyt} \sim 1.2$ μM (distributed in a

167   HeLa nucleus) in flow cytometric experiments. If the localization of the protein is not perfectly

168   nuclear, we can correct for this as well. From confocal microscopic sectioning we determined the

169   nuclear and cytoplasmic fractions of Fos[215]-EGFP (the protein we used for FRET titrations),

170   which were 85±5% and 15±5%, independent of expression level (see Suppl. Fig. 3). With this

171   correction, a bead unit of ~1.0±0.1 μM was used for calculating the nuclear concentration of

172   Fos[215]-EGFP in flow cytometric experiments.

173

Determination of the absolute concentration of endogenously expressed Fos and Jun

175   With regular immunofluorescence assays only the relative amounts of endogenous and

176   transfected proteins can be assessed. By knowing the absolute concentration of the transfected

177   proteins, the endogenous concentration can also be determined. Therefore, we combined the

178   immunofluorescence assay with the results of FCS-based EGFP concentration calibration to

179   assess the endogenous concentrations of Fos and Jun in HeLa cells.

180   Immunofluorescence labeling was carried out as follows. After washing 3× with PBS, cells were

181   fixed with 3.7% formaldehyde (4˚C, 10 minutes), permeabilized with 0.25% Triton and 0.1%

182   TWEEN/TBS (room temperature, 30 min) and blocked with 2% BSA with 0.1% TWEEN/TBS

183   (room temperature, 30 min). Cells were then incubated with mouse anti-c-Fos (Merck,

184   Whitehouse Station, New Jersey, USA) or mouse anti-c-Jun monoclonal antibody (Millipore,

185   Billerica, Massachusetts, USA) at 20 µg/ml concentration (room temperature, 1 hour), followed

186   by incubation with NL-637-DAMIG polyclonal secondary antibody (R&D Systems,

187   Minneapolis, Minnesota, USA) at 50 µg/ml for 1 hour at room temperature in the dark. Between

188   consecutive steps cells were washed 3× with PBS. The applied antibody concentrations were

189   chosen based on titrations (Suppl. Fig. 4A-C); the used concentrations are close to saturation

190   values.

191   Flow cytometric measurements were performed on a FACSAria III flow cytometer. The

192   green EGFP signal (I^{green}) was excited at 488 nm and emission was detected between 515-545

193   nm, while the red NL637 signal (I^{red}) was exited at 633 nm and emission was detected through a

194   655 LP filter.

195   The amount of Fos-EGFP was determined by comparing its I^{green} fluorescence signal to

196   that of the green bead used for concentration calibration. The red signal I^{red} of the NL637-

197   DAMIG antibody used for immunofluorescence labeling is proportional to the total amount of

198   Fos: the endogenous Fos in the non-transfected sample, and the endogenous Fos + Fos-EGFP in

199   the transfected one. The concentrations of endogenous Fos ($c_{Fos\text{-}endogen}$) and Fos-EGFP ($c_{Fos\text{-}EGFP}$)

200   were calculated from the green and red signals using transfected and non-transfected cells as:

201   $$c_{Fos-EGFP} = \frac{I^{green}_{transfected}}{I^{green}_{bead}} \times c^{flow\,cyt}_{bead\,unit} \tag{5}$$

202
$$\frac{c_{Fos-EGFP} + c_{Fos-endogenous}}{c_{Fos-endogenous}} = \frac{I_{transfected}^{red}}{I_{non-transfected}^{red}}$$
(6)

203
$$c_{Fos-endogenous} = \frac{c_{Fos-EGFP}}{\left(I_{transfected}^{red} / I_{non-transfected}^{red}\right)-1} = \frac{\left(I_{transfected}^{green} / I_{bead}^{green}\right) \times c_{bead\,unit}}{\left(I_{transfected}^{red} / I_{non-transfected}^{red}\right)-1}$$
(7)

204 with $c_{bead\,unit}^{flow\,cyt} \sim 1.2$ µM in our measurements. I^{red} signals were collected from fixed cells (required

205 by the immunofluorescence labeling protocol), whereas I^{green} signals were measured in non-fixed

206 live cells (from the same transfected population) to avoid deterioration of EGFP fluorescence due

207 to fixation. The concentration of endogenous and EGFP-tagged Jun was determined using the

208 same principle.

209 **Calculation of dissociation equilibria from FRET data**

210 To assess the K_d of Fos homodimers and Fos-Jun heterodimers we carried out FRET

211 titration experiments. In the derivation of dissociation equilibria we assumed that the heterodimer

212 was more stable than the Fos homodimer. Therefore, in the case of Fos-Jun association we

213 neglected the presence of Fos homodimers (assuming they were not present at lower

214 concentrations). The law of mass action for heterodimer formation is:

215 $[F][J]/[FJ] = K_d^{FJ}$
(8)

216 where square brackets denote concentrations of free monomers and heterodimers, and K_d^{FJ} is the

217 dissociation constant of the heterodimeric complex. The total concentration of Fos or Jun can be

218 written as the sum of the concentrations of free monomers F and J and heterodimers FJ:

219 $[F]_t = [F] + [FJ]; \quad [J]_t = [J] + [FJ]$
(9)

220 The concentration of the heterodimer is:

221
$$[FJ] = \frac{1}{2}\left([F]_t + [J]_t + K_d^{FJ} - \sqrt{[F]_t^2 - 2[F]_t \times \left([J]_t - K_d^{FJ}\right) + \left([J]_t + K_d^{FJ}\right)^2}\right)$$
(10)

222   The measured FRET efficiency between EGFP- and mRFP1-labeled proteins is an average value

223   stemming from FRET-producing and non-FRET-producing donor molecules. Free donors or

224   donors associated with endogenous unlabeled protein give zero FRET. Only donors forming a

225   complex with an acceptor have a positive contribution to FRET (Fig. 5). Thus, we need to

226   calculate the concentration of Fos-Jun dimers labeled with both donor and acceptor. The total

227   concentrations of Fos and Jun are:

228    $\left[F\right]_t=\left[F_D\right]_t+\left[F_e\right]_t$ and $\left[J\right]_t=\left[J_A\right]_t+\left[J_e\right]_t,$ (11)

229   where the indexes D, A and e refer to donor-tagged, acceptor-tagged and endogenous molecules.

230   The fractions of donor-tagged Fos (p_D) and acceptor-tagged Jun (p_A) are:

231    $p_D=\dfrac{\left[F_D\right]_t}{\left[F_D\right]_t+\left[F_e\right]_t}$ and $p_A=\dfrac{\left[J_A\right]_t}{\left[J_A\right]_t+\left[J_e\right]_t}$ (12)

232   The concentration of doubly labeled Fos$_D$-Jun$_A$ dimers is:

233    $\left[F_DJ_A\right]=\left[FJ\right]\times p_D\times p_A=\left[FJ\right]\times\dfrac{\left[F_D\right]_t}{\left[F_D\right]_t+\left[F_e\right]_t}\times\dfrac{\left[J_A\right]_t}{\left[J_A\right]_t+\left[J_e\right]_t}$ (13)

234   We denote the FRET efficiency in the complex of a single donor-tagged Fos and an acceptor-

235   tagged Jun by E_0. The measured apparent FRET efficiency E_{meas} can be written as:

236    $E_{meas}=\dfrac{\left[F_DJ_A\right]}{\left[F_D\right]_t}\times E_0+\dfrac{\left[F_D\right]_t-\left[F_DJ_A\right]}{\left[F_D\right]_t}\times 0=\dfrac{\left[F_DJ_A\right]}{\left[F_D\right]_t}\times E_0$ (14)

237   where $\left[F_DJ_A\right]$ is the concentration of complexes of donor-tagged Fos with acceptor-tagged Jun,

238   and $\left[F_D\right]_t$ is the total concentration of donor-tagged Fos (without respect to being monomeric or

239 in a Fos-Jun complex). $[F_D]_t - [F_D J_A]$ is the concentration of donor-tagged Fos not complexed

240 with acceptor-tagged Jun (Fos-EGFP in monomeric form or complexed with endogenous Jun),

241 contributing zero FRET efficiency. By introducing the acceptor-to-donor expression ratio

242 $N_A/N_D = [J_A]/[F_D]$ (Suppl. eq. S8) and combining equations 10, 13 and 14, the measured FRET

243 efficiency can be expressed as:

244 $$E_{meas} = \frac{[F]_t + [J]_t + K_d^{FJ} - \sqrt{[F]_t^2 - 2[F]_t \left([J]_t - K_d^{FJ}\right) + \left([J]_t + K_d^{FJ}\right)^2}}{2[F]_t [J]_t} \times [F_D]_t \frac{N_A}{N_D} E_0, \qquad (15)$$

245 By substituting Eq. 11 and the expression for the N_A/N_D ratio into Eq. 15 we get the formula used

246 in the nonlinear fit (see Eq. S12 in the Supplement) with variables $[F_D]_t$ and N_A/N_D.

247 For calculating Fos-Fos equilibria, we have to take into account Fos-Jun formation as

248 well. Since the heterodimer is more stable, we make the simplifying assumption that all Jun

249 molecules present are in complex with Fos at the high Fos concentrations where Fos

250 homodimerization takes place, leaving no free Jun. We can write the following equilibrium

251 equation:

252 $$[F][F]/[FF] = K_d^{FF},$$
$$[J]_t = [FJ], \quad [F]_t = [F] + [FJ] + 2[FF] \qquad (16)$$

253 where K_d^{FF} is the dissociation constant of the Fos homodimer, FF and FJ denote the homo- and

254 the heterodimer. The amount of Fos homodimer can be expressed as:

255 $$[FF] = \frac{1}{8}\left(4[F]_t - 4[J]_t + K_d^{FF} - \sqrt{8K_d^{FF}[F]_t - 8K_d^{FF}[J]_t + \left(K_d^{FF}\right)^2}\right) \qquad (17)$$

256 Fos molecules can be labeled with donor, acceptor or can be unlabeled, and only homodimers

257 containing both a donor and an acceptor will produce FRET (Fig. 5). The fraction of such double-

258 labeled pairs follows a multinomial distribution, and equals

259 $$p_{D,A} = 2p_D p_A \qquad (18)$$

260 where $p_D = [F_D] \big/ ([F_D]_t + [F_A]_t + [F_e]_t)$ and $p_A = [F_A] \big/ ([F_D]_t + [F_A]_t + [F_e]_t)$ are the donor and

261 acceptor-tagged fractions of Fos. The measured FRET efficiency is:

262 $$E_{meas} = \frac{[F_D F_A]}{[F_D]_t} \times E_0 = \frac{[FF] p_{D,A}}{[F_D]_t} \times E_0 \qquad (19)$$

263 Combining equations 17 and 19 (see also Eq. S14 in the Supplement) yields

264 $$E_{meas} = \frac{1}{4}\left(4[F]_t - 4[J_e]_t + K_d^{FF} - \sqrt{K_d^{FF}}\sqrt{8[F]_t - 8[J_e]_t + K_d^{FF}}\right) \frac{[F_D]_t \frac{N_A}{N_D} E_0}{[F]_t^2} \qquad (20)$$

265 with

266 $$[F]_t = [F_D]_t + [F_A]_t + [F_e]_t = [F_D]_t\left(1 + \frac{N_A}{N_D}\right) + [F_e]_t \qquad (21)$$

267 where $[F]_t$ is the total Fos concentration (including donor-tagged, acceptor-tagged and

268 endogenous Fos, without respect to monomeric or dimeric state) and E_0 is the FRET efficiency

269 between a donor-acceptor pair (this may be different from the E_0 of the heterodimer). These

270 equations were used to determine the K_d of dimers from flow cytometric FRET experiments by

271 nonlinear regression.

272 In the analysis we also considered the presence of dark acceptor species due to imperfect

273 maturation, and a cytoplasmic fraction of Fos (see Supplemental Material). These factors

274 influence E_0, but not the value of K_d.

275

276 **Single Plane Illumination Microscopy – fluorescence cross-correlation spectroscopy (SPIM-**

277 **FCCS)**

278   The SPIM-FCCS measurements were performed on an in-house built selective plane

279   illumination microscope setup based on the design described in (39, 40). Data were analyzed

280   using the software QuickFit 3.0. Details of the experimental setup and analysis are summarized in

281   the Supplement.

282

Molecular dynamics simulation of Fos-Jun and Fos-Fos complexes

284   Two systems were submitted to molecular dynamics simulation. The first one is

285   constructed from the Fos (139-198)_Jun (257-313) protein fragments associated to the DNA

286   fragment as was deposited in the protein data bank (41). It was completed by adding the missing

287   hydrogen atoms and closing the N- and C-terminal residues by the acetyl and N-methyl groups,

288   respectively. The second system, a Fos (139-198)_Fos (139-198) was obtained from the first one

289   using the Jun fragment as a template in a proper position for homology modeling of the Fos

290   protein. Each of these systems was put in a dodecahedral box, solvated by the TIP3P explicit

291   water model, neutralized by Na^+ ions, and further Na^+ and Cl^- ions were added to set the ionic

292   strength to 0.15 M. They were then minimized, slowly heated to 310 K and after an 80 ns

293   equilibration period they were submitted to a 500 ns constant particle number (123888 and

294   123870 for the Fos-Jun and Fos-Fos systems, respectively), constant pressure ($P=10^5$ Pa),

295   constant temperature (T=310 K) production dynamics. For the simulations the AMBER99SB

296   force field (42) and periodic boundary condition were used. Short range electrostatic and van der

297   Waals interactions were calculated explicitly within a 1 nm cut-off. For the long range

298   electrostatics the particle mesh Ewald method (43) was applied. A Berendsen barostat and

299   thermostat (44) was used during this simulation. For the simulations the GROMACS packages

300   were used (45, 46).

301　　　　For control purpose molecular dynamics simulations on the Leu zipper region only of the

302　c-Fos:c-Jun and c-Fos:c-Fos dimeric structures were also carried out using the same set-up

303　protocol which is detailed above. The Leu zipper region we considered consisted of the 275-313

304　and 160-198 amino acid residues for the c-Jun and c-Fos fragments, respectively. Simulations

305　were completed for both the wild type Leu zipper regions and the corresponding structures

306　applying Leu280Asp, Leu294Asp virtual mutations in c-Jun- and Leu165Asp, Leu179Asp

307　mutations in c-Fos protein fragments.

308

309　**RESULTS**

310　**FRET microscopy implies Fos homodimerization**

311　　　　FRET is the radiationless transfer of energy from a donor fluorophore to a nearby

312　acceptor (27, 28), which is often used to assess molecular distances. To measure the association

313　of c-Fos molecules (referred to as Fos in the following sections), we used Fos and its C-terminal

314　truncation mutant Fos[215] tagged with ECFP (donor) or EYFP (acceptor) in confocal microscopic

315　FRET experiments. Fos[215] was prepared (32) to bring the FP-labeled C-termini of Fos and Jun to

316　a similar distance from the leucine zipper to enhance FRET (Fig. 1). Images of donor, transfer

317　and acceptor signals were recorded, and FRET efficiencies E between labeled proteins, as well as

318　acceptor-to-donor molecular ratios N_A/N_D were calculated on a pixel-by-pixel or cell-by-cell

319　basis.

320　　　　Pixel-by-pixel FRET efficiency maps and histograms of representative cells are shown in

321　Fig. 2. Cells cotransfected with full length Fos-ECFP+Fos-EYFP (top row) yielded a mean FRET

322　efficiency of E=5.0±0.5% (n~30 cells, ±SEM); for Fos[215]-ECFP+Fos[215]-EYFP (2nd row, n~30) it

323　was 10.0±0.5%. The higher E between the truncated Fos[215] molecules is probably due to the

324　reduced distance between the shorter C-terminal regions (Fig. 1). We also measured the FRET

325   efficiency for the Fos-ECFP+Jun-EYFP and Fos[215]-ECFP+Jun-EYFP samples (3rd and 4th rows,

326   n~30), which was 7.9±0.4% and 15.0±1.1%. For these pairs we have shown heterodimer

327   formation earlier by FCCS and FRET (7, 32, 34). Both Fos and Jun molecules showed strong

328   nuclear enrichment. The negative control (ECFP coexpressed with EYFP as separate proteins)

329   and the positive control (ECFP-EYFP fusion protein) had mean E values of 2.8±0.4% and

330   48.6±0.8% (5th and 6th rows, n~20). These proteins had a diffuse distribution in the whole cell.

331   The FRET efficiency of the Fos-Fos (or Fos[215]-Fos[215]) samples was lower than that of the Fos-

332   Jun or Fos[215]-Jun heterodimers, but significantly higher than for the negative control, indicating

333   that Fos formed homodimers in these cells.

334   Dimer formation depends on the concentrations of the interacting partners; therefore, we

335   analyzed FRET in cells expressing various amounts of the proteins. We calculated average

336   intensities in the whole nucleus for Fos or Jun, and in the whole cell for the positive and negative

337   controls in single cells, and determined FRET on a cell-by-cell basis. This method allowed a

338   rapid analysis of several hundred cells. Fig. 3A and B show the mean E value as a function of the

339   acceptor-to-donor molecular ratio N_A/N_D for the Fos[215]-ECFP+Fos[215]-EYFP and Fos-ECFP+Fos-

340   EYFP samples. Data were grouped into three subsets according to donor concentration based on

341   the fluorescence intensity of the donor (low, medium, high). For both protein pairs, E increases

342   from low to high donor concentrations, in accordance with the higher probability of complex

343   formation. E also increases with increasing N_A/N_D because more acceptor-tagged Fos molecules

344   are available for donor-tagged ones to form a complex. The plateau or in its absence the average

345   of the highest E values are presented in Fig. 3C, which shows that at higher Fos concentrations

346   the extent of homodimerization increased as expected. Similar to the pixel-by-pixel analysis, the

347   mean FRET efficiencies of the Fos-Fos and Fos[215]-Fos[215] dimers are between those of the

348   negative control and the respective Fos-Jun or Fos[215]-Jun dimers.

349   The measured E values depend on the FRET efficiency in a single donor-acceptor

350   complex determined by the dye-to-dye distance and orientation, and on the fraction of donors

351   forming dimers with an acceptor. The length of the Fos[215] molecule downstream of the

352   dimerization domain is similar to that of Jun, thus, the dye-to-dye distances in the Fos[215]-Fos[215]

353   and Fos[215]-Jun complexes should be similar. However, in the case of Fos homodimers, only

354   complexes of donor- and acceptor-tagged proteins yield FRET. The measured mean E value is a

355   weighted average of non-FRET-ting and FRET-ting dimers. Thus, the mean FRET efficiency of

356   the homodimer is expected to be lower than that of the heterodimer. This was taken into account

357   in our subsequent analyses.

358

359   **Calibration of fluorescence intensity to measure absolute EGFP concentration**

360   The above FRET titrations curves demonstrated that FRET efficiency can be used to

361   monitor the extent of homo- and heteroassociations quantitatively in our system. The stability of

362   a complex is characterized by its dissociation constant, K_d. In the Materials and Methods section

363   we outlined a method to determine the K_d of interacting proteins in live cells from FRET titration

364   curves. This requires knowing the absolute concentrations of all interacting molecules: the

365   transfected fluorescent, and the endogenous non-fluorescent ones. In subsequent measurements

366   we used the EGFP-mRFP1 dye pair because of the higher photostability of EGFP compared to

367   ECFP. First, we developed a calibration method to determine the concentration of fluorescent

368   proteins. Confocal images of cells expressing free EGFP were taken, and autocorrelation curves

369   (ACFs) were recorded at selected points of the images (Fig. 4A). From ACFs local dye

370   concentrations were determined by nonlinear fitting yielding a calibration curve of EGFP

371   concentration vs. fluorescence intensity per pixel, $c(F)$ (Fig. 4B) (37). To make the concentration

372   calibration portable and facilitate comparison of measurements on different days, we normalized

373 EGFP fluorescence by using a fluorescent bead as an intensity standard. The EGFP

374 concentration corresponding to one bead unit was ~15.4±0.7 µM for our confocal microscope and

375 1.0±0.1 µM for the flow cytometer. For the latter calculation we took the nucleus-to-bead volume

376 ratio (13:1) and the nuclear fraction of Fos[215]-EGFP (85%) into account. This way, the molar

377 concentration of EGFP-tagged protein could be assessed by simply comparing its intensity to that

378 of the bead measured on the same day in the microscopic or flow cytometric setup without having

379 to repeat the FCS calibration.

380

381 **Determining the absolute concentrations of endogenous and transfected Fos and Jun**

382 For calculating the K_d of dimers we also need to know the amount of endogenous Fos and Jun,

383 since they can also form dimers with each other or with their fluorescent counterparts. First, we

384 detected the green fluorescence signal of Fos-EGFP in transfected cells, and compared it to that

385 of the calibration bead to determine the absolute concentration of transfected protein (Eq. 5 in the

386 Materials, Suppl. Fig. S4 D-G). Then we used immunofluorescence labeling, paired with far red

387 channel flow cytometry, to detect the total Fos pool in non-transfected and in Fos-EGFP

388 transfected samples. The immunofluorescent signal of the non-transfected sample is proportional

389 to the endogenous Fos concentration, while that of the transfected one corresponds to the sum of

390 the endogenous and transfected amounts. Thus, using the known concentration of Fos-EGFP, we

391 deduced the average concentration of endogenous Fos (113±11 nM) and Jun (94±10 nM) in HeLa

392 cells (see Eq. 7 in the Materials). Our procedure combining immunofluorescence and EGFP-

393 tagged protein expression can be generally used to assess the absolute concentration of any

394 endogenously expressed non-fluorescent protein.

395

Determination of the dissociation constant of Fos-Jun heterodimers and Fos homodimers in live cells using flow cytometric FRET data

We wanted to determine the dissociation constants of homo- and heterodimers from FRET titrations. Therefore, we derived the expressions of FRET efficiency in terms of the concentrations of donor-tagged, acceptor-tagged and unlabeled, endogenous proteins of interest and the K_d's (eqs. 15, 20 and supplementary equations S12, S14). Flow cytometric FRET experiments were carried out on large cell populations expressing EGFP- and mRFP1-tagged proteins at various concentrations. Cell-by-cell values of FRET efficiency (E), donor concentration $\left[F_D\right]_t$ (derived from the FRET-corrected I_D donor intensity by comparison to beads), and acceptor-to-donor molecular ratio (N_A/N_D) data were determined. To characterize heterodimers, cells were cotransfected with Fos[215]-EGFP+Jun-mRFP1 or Jun-EGFP+Fos[215]-mRFP1 pairs. The extent of association depends on the concentrations of both molecules. To create functions with a single variable, cells were grouped into classes with approximately constant N_A/N_D ratios. E values of selected N_A/N_D groups were plotted as a function of the donor concentration (Fig. 6A,C). The FRET efficiency increases with increasing N_A/N_D ratio as expected. Figures 6B,D display the theoretical values of the maximal FRET efficiencies at various N_A/N_D ratios (Eq. S13 in the Supplement), which could be measured if all possible donor-acceptor complexes were formed (association were complete). The function increases linearly up to $N_A/N_D=1$ where it reaches E_0, the FRET efficiency between a single donor-acceptor pair, and remains constant at higher N_A/N_D ratios. E vs. N_A/N_D data were fitted to the model function describing the heterodimerization process (Eq. S12 in the supplement), taking the presence of FP-tagged and unlabeled endogenous Fos and Jun into account. The apparent K_d values derived from the fits varied with varying N_A/N_D for both pairs between 10 and 370 nM.

419   We received smaller apparent K_d-s where Fos was present in excess (larger Fos:Jun ratios) (Fig.

420   6E).

421   Fos homodimerization was studied by measuring the FRET efficiency between

422   Fos[215]-EGFP and Fos[215]-mRFP1 (Fig. 6F). Here, homodimers can contain donor-acceptor, donor-

423   donor, and acceptor-acceptor tagged protein pairs (plus dimers containing endogenous Fos); thus,

424   the dependence of E on the N_A/N_D ratio is different than for heterodimerization (Fig. 6G). The

425   theoretical E_{max} value approximates the value of E_0 asymptotically as N_A/N_D tends to infinity (Eq.

426   S15 in the Supplement). Curves were fitted taking into account both the homoassociation of Fos

427   (in all combinations of donor-tagged, acceptor-tagged and endogenous molecules) and its

428   heteroassociation with endogenous Jun (Eq. S14 in the supplement). In the fits the K_d and E_0

429   values were linked for data sets with different N_A/N_D values, yielding K_d=6.7±1.7µM and E_0=9.5

430   ± 0.8% for the homodimerization process. When different N_A/N_D groups were fitted

431   independently, K_d and E_0 ranged between 5.4-9.7 µM and 9.1-11.9%. As expected, the K_d of the

432   Jun-Fos[215] heterodimer is much smaller than that of the Fos homodimer, which means that Fos

433   homodimerization will be significant only in case of its selective overexpression. At equal Fos

434   and Jun concentrations the formation of Fos-Jun heterodimers is more probable than the

435   formation of Fos homodimers.

436

437   **Fluorescence brightness and slow diffusion indicate Fos homoassociation and DNA binding**

438   FCS was not only used for concentration calibration, but also as an additional tool to

439   probe Fos homoassociation. Diffusing particle concentration vs. fluorescence intensity curves

440   were generated for EGFP, Fos[ΔΔ]-EGFP (a mutant form lacking the DNA-binding and

441   dimerization domains, see Fig. 1), full length Fos-EGFP and Fos-EGFP+Jun-mRFP1 samples

442   (Fig. 4B). The slopes of the fitted straight lines for EGFP and Fos[ΔΔ]-EGFP were 30.0 and 30.3

443   (nM/intensity unit). The similar slopes indicate that equal intensities of EGFP or Fos$^{\Delta\Delta}$-EGFP

444   correspond to equal particle number, suggesting that this mutant contains one fluorophore per

445   particle, i.e., it is monomeric. In contrast, the full length Fos-EGFP protein yielded a slope of

446   11.5, which is less than half of the previous values. Thus, an equal intensity of Fos-EGFP

447   corresponds to a little less than $1/2\times$ the particle concentration of the monomeric proteins,

448   implying the formation of Fos homodimers. In fact, this curve is not expected to be linear in the

449   low concentration regime because of the monomer-dimer transition. The slope of the Fos-

450   EGFP+Jun-mRFP1 sample (with an average Jun:Fos ratio of 0.7) is between the monomeric and

451   dimeric slopes. Therefore, Fos-EGFP is partially complexed with Jun-mRFP1, where the

452   brightness of EGFP is similar to that of monomers, whereas the rest of Fos-EGFP may form

453   homodimers. We also analyzed the specific particle brightness F/N, defined as the ratio of the

454   fluorescence intensity F to the number of particles N. This parameter characterizes the association

455   state of a labeled protein, and is proportional to the number of fluorophores in a jointly diffusing

456   complex. F/N vs. EGFP concentration values are shown in Fig. 4C. Fos-EGFP is brighter than

457   EGFP, Fos$^{\Delta\Delta}$-EGFP or Fos-EGFP+Jun-mRFP1, corroborating the conclusion that Fos-EGFP is

458   homodimerized when there is not enough Jun present. The brightness of Fos-EGFP increases

459   with concentration indicating that dimerization is enhanced at higher concentrations, whereas the

460   brightness of EGFP or Fos$^{\Delta\Delta}$-EGFP does not vary with concentration, just as expected for

461   monomers.

462   From the autocorrelation functions (ACFs) we also determined molecular diffusion

463   properties (Fig. 4D). ACFs from the EGFP, Fos-EGFP, Fos$^{\Delta\Delta}$-EGFP and Fos-EGFP+Jun-mRFP1

464   samples were fitted to a model assuming a fast, freely diffusing and a slowly moving component

465   (7). Diffusion coefficients and the fractions of the species are shown in (Fig. 4E). The average

466   fraction of the slow components was 0.35±0.14 for Fos-EGFP expressed alone and 0.38±0.10 for

467   Fos-EGFP when co-expressed with Jun-mRFP1, whereas it was only 0.19±0.12 for the non-

468   binding Fos[ΔΔ]-EGFP mutant, and 0.07±0.05 for EGFP. The similarly increased slow fractions of

469   Fos-EGFP expressed alone or together with Jun-mRFP1 suggest that Fos can bind to chromatin

470   not only as a heterodimer, but also as a homodimer.

471

472   **SPIM-FCCS confirms stable homoassociation and chromatin binding of Fos proteins**

473   We used fluorescence cross-correlation spectroscopy (FCCS), the two-color version of

474   FCS to characterize the co-mobility of dimer-forming Fos molecules. In FCCS, the auto- and the

475   cross-correlation functions (CCF) from two molecular species tagged with different colors are

476   determined. A non-zero CCF amplitude indicates that the molecules are moving together. The

477   ratio of the CCF and ACF amplitudes from a double-labeled sample is proportional to the fraction

478   of molecules forming a complex. FCCS measurements were performed on a single plane

479   illumination microscope (SPIM) using an EM-CCD camera as sensor, which allows simultaneous

480   measurements at many pixels in a cell. This improves the statistics and provides 2D interaction

481   and mobility maps (40).

482   Measurements were carried out on cells co-transfected with the following protein

483   combinations: Fos[215]-EGFP+Fos[215]-mRFP1, Fos[215]-EGFP+Jun-mRFP1, Fos[ΔΔ]-EGFP+Fos[215]-

484   mRFP1 (negative control) and EGFP-P30-mRFP1 (positive control, EGFP and mRFP1 connected

485   by a 30-residue long polyproline linker). Cells expressing about equal amounts of green and red

486   fluorophores were selected from the concentration range used in FRET experiments (0.3 – 10

487   μM). Fig. 7A shows typical correlation curves obtained from the four samples. Amplitudes of the

488   curves were low due to the high concentration required to see Fos homodimerization, but our data

489   showed that quantitative measurements were possible even in this concentration regime. At each

490   pixel we performed a global FCCS fit to the green and red ACFs and the CCF (see (40) and Eqs.

491   S16-S18 in the Supplement). The fit functions were parameterized by the concentrations of three

492   diffusing species (green-only, red-only, green/red-dimers), which were linked over all three

493   curves. We assumed a two-component diffusion model for the ACFs and a one-component model

494   for the CCF. Diffusion coefficients were not linked. Fig. 7B shows exemplary maps and

495   histograms of the relative GR-dimer concentration $c_{GR}/\left(c_{G-only}+c_{R-only}+c_{GR}\right)$ obtained from

496   these fits. Fig. 7C shows the statistics from n>10 cells as average ± standard deviation of the

497   medians extracted from the pixel distributions in each cell. As expected, the negative control had

498   the lowest and the positive control the highest apparent dimer fraction, defining the dynamic

499   range of the measurements (0.06 − 0.32). The upper limit is less than one because of the

500   imperfect overlap of the green and red detection volumes, partial photobleaching of the dyes and

501   imperfect dye maturation/folding producing green-only and red-only species besides doubly

502   labeled molecules. The Fos-Jun heterodimer showed a high apparent dimer fraction (0.22±0.07),

503   whereas the value from the Fos[215] homodimer was slightly smaller (0.16±0.05), but still

504   significantly larger than that of the negative control.

505   We also analyzed the protein mobility of the Green/Red dimers that could be extracted

506   from the fits of the CCFs. We used a single component fit; a second component could not be

507   fitted to the CCFs of the Fos-EGFP + Jun-mRFP1, Fos[215]-EGFP + Fos[215]-mRFP1 samples

508   suggesting the presence of only a single slow species. The CCFs of the negative control could not

509   be fitted reliably due to their very low amplitude. The diffusion coefficient of the EGFP-P30-

510   mRFP1 fusion protein was D_{cross}~4.3 µm²/s (a mean of two components present for this protein),

511   whereas the average diffusion coefficients of the Fos[215]-Fos[215] and Fos-Jun dimers were much

512   lower, ~0.3 and 0.4 µm²/s (Fig. 7D). The presence of a single, slow component for Fos

513   homodimers and Fos-Jun heterodimers indicated that these complexes could bind to slowly

514   moving nuclear components, supposedly the chromatin.

515

516   **Fos-Jun and Fos-Fos complexes form stable dimers as revealed by MD modeling**

517   Based on the distance-related data from FRET measurements, we performed MD

518   modeling to testify the stable formation of Fos homodimers. In Fig. 8 the first frames from the

519   production dynamics trajectories are presented. During the 500 ns simulation interval

520   (Supplemental movies 5 and 6) not only the Fos-Jun heterodimer but also the Fos-Fos

521   homodimer remain bound to DNA, and the dimeric structures (coiled coil motifs) stay strongly

522   associated by their leucine zipper regions. It is noteworthy that the Fos-Fos homodimer exists

523   despite the net negative charge of the leucine zipper regions. This indicates that in the dimeric

524   form hydrophobic interactions play a crucial role, and electrostatic forces are largely shielded by

525   counterions. While H-bonds and even salt bridges can contribute to the stabilization of the

526   dimeric structure as well, the H-bond networks between protein chains have variability in their

527   connection patterns, which further supports the importance of hydrophobic interactions in the

528   dimeric structures.

529   Simulations carried out solely on the leucine zipper region of c-Jun:c-Fos and c-Fos:c-Fos

530   dimers indicated stable structures with coiled coil motif (Figure 8C,D). These results are both in

531   good accordance with former simulations carried out on the c-Jun:c-Fos leucine zipper region

532   (47)and underline again the role of hydrophobic forces even in the stability of the c-Fos:c-Fos

533   homodimer. Whereas, introducing Leu-Asp virtual mutations into these dimeric structures (as a

534   negative control) the contact between the corresponding regions of helices was either weakened

535   (c-Jun:c-Fos) or even destroyed (c-Fos:c-Fos) as demonstrated in Figure 8E,F.

536

DISCUSSION

Homodimer formation of short fragments, mainly the leucine zippers, of Fos proteins has been studied earlier. However, in vitro studies reported low stabilities of the homodimer, and it was assumed that it could not be present in live cells. By combining FRET, FCS and imaging FCCS we demonstrated that Fos proteins formed homodimers in live cells, and presented a method for calculating their dissociation constant. The K_d of Fos homodimers in HeLa cells was 6.7±1.7 µM, which is the same order of magnitude as the value 5.6 µM determined for its isolated leucine zippers in vitro by circular dichroism (14). Values reported for the heterodimers of the isolated leucine zippers (10, 48, 49) or longer polypeptides (50) in vitro varied between 1 and 140 nM. For the Fos-Jun heterodimer we found a K_d range of 10-370 nM in live cells, which depended on the Fos:Jun ratio, and on putting the donor and acceptor tags on one or the other protein. The variation of the K_d with different Fos:Jun ratios may be caused by the formation of Jun homodimers, which could interfere with the heterodimerization process. At lower Fos:Jun ratios, when there is excess Jun present, the relative amount of Jun homodimers is expected to be higher; thus, the amount of available free Jun is less and the heterodimerization process could shift toward higher concentrations (Fig. 6E), resulting in a higher apparent K_d. At higher Fos:Jun ratios, where the Jun homodimer is expected to be less abundant, we got K_d <100 nM for the heterodimer, in agreement with earlier in vitro results. The shift between the Fos-Jun and Jun-Fos curves in Fig. 6E might be due to dark states (51) and incomplete maturation of mRFP1, resulting an error in the acceptor-to-donor ratios. Furthermore, the autofluorescence intensity of HeLa cells in the green channel corresponds to the specific intensity of ~50 nM EGFP; therefore, the signal-to-noise ratio in the concentration range of the K_d is lower than in the case of the homodimer, making the K_d for the heterodimers less accurate.

560   Several groups used FRET to determine the K_d of isolated proteins (50, 52). Other groups

561   used microscopic FRET to determine K_d in cells, utilizing prior estimates of protein copy number

562   per cell (53), or applying in vitro concentration calibration with purified proteins (54). Here we

563   presented a method to calculate K_d values based on FRET titrations after concentration

564   calibration by FCS, where the whole procedure was carried out in live cells. None of the earlier

565   studies took into account the presence of endogenous, unlabeled proteins. With our method, the

566   absolute concentrations of both overexpressed fluorescent and endogenous non-fluorescent

567   proteins were determined and included in dissociation equilibria. Our concentration calibration

568   method is transferable to measurements performed on different instruments or days by utilizing

569   fluorescent beads as a standard. The procedure can be generally used to determine K_d-s and

570   absolute concentrations of proteins in live cells.

571   FRET reveals that a certain fraction of molecules are colocalized within Förster distance.

572   We used FCS to assess the co-diffusion of molecules, which is a direct indication of stable

573   interaction. Our molecular brightness analysis of FCS data indicated that Fos-EGFP, when

574   expressed alone, had a higher ($\geq$2×) molecular brightness than its dimerization- and DNA-

575   binding-deficient Fos$^{\Delta\Delta}$ mutant or the free EGFP dye. This corroborated that at a few micromolar

576   concentration Fos was present mainly as a homodimer, which was stable at least for a few tens of

577   milliseconds (the mean dwell time of particles in the focal volume setting the upper limit of

578   observed timescales in our FCS experiments). When fitted with a slow and a fast diffusion

579   component, the slow fraction of Fos was about the same whether expressed alone or together with

580   Jun; in contrast, the slow fraction of the Fos$^{\Delta\Delta}$ mutant was significantly lower, hinting at DNA

581   binding of the wild type Fos either as a homo- or as a heterodimer. The presence of a very small

582   apparent slow fraction in the case of lone EGFP and Fos$^{\Delta\Delta}$ is probably due to molecular crowding

583   in the nucleus leading to anomalous subdiffusion (37). This makes the autocorrelation curves less

584 steep than for free diffusion, mimicking the presence of a second, slowly moving component with

585 a longer diffusion time.

586   SPIM-FCCS allowed us to confirm the presence, visualize the distribution and

587 characterize the intranuclear mobility of Fos homodimers. These were stable for at least a few

588 hundred milliseconds, the time window defined by the cross-correlation diffusion time. Their

589 diffusion coefficient derived from the cross-correlation curve was ~0.3 $\mu m^2/s$, similar to that of

590 Fos-Jun heterodimers (0.4 $\mu m^2/s$). The measured diffusion coefficients are similar to those

591 determined by confocal FCCS for the same proteins (7), and to values observed for other

592 chromatin-binding proteins, e.g. nuclear receptors (55, 56) or HP1α (57).

593   Our molecular dynamic modeling simulations also supported the possibility of

594 homodimerization showing that homodimers stayed together for the duration of the simulation.

595   The existence of stable Fos homodimers capable of chromatin binding brings up the

596 possibility that they may act as transcriptional regulators, and may explain the importance of Fos

597 overexpression in oncogenesis. Various complexes of different Fos and Jun variants occur in

598 different cell types contributing to cell proliferation or apoptosis (58-60). It is not clear yet

599 whether the Fos homodimer could function as an autonomous transcription factor, or

600 alternatively, it could occupy the binding sites of AP-1 heterodimers preventing their normal

601 function and interfering with their proliferative or antiproliferative effects.

602

616

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781   **FIGURE LEGENDS**

782   **Figure 1**

783   **Schematic drawing of c-Fos, its mutants and c-Jun**

784   From top: full length Fos with fluorescent protein tag at the C terminus; C-terminally truncated

785   Fos^{215}, Fos$^{\Delta\Delta}$ where the DNA binding and dimerization domains were deleted; and Jun. Pink

786   color denotes the DNA-binding domain, yellow the leucine-zipper, and the dotted line the linker

787   between Fos/Jun and the fluorescent protein tag (ECFP, EYFP, EGFP or mRFP1).

788

789   **Figure 2**

790   **Subcellular, pixel-by-pixel analysis of dimerization by confocal microscopic FRET on HeLa**

791   **cells**

792   ECFP (donor channel) was excited at 458 nm and detected between 475-525 nm; in the transfer

793   channel the excitation was at 458 nm and detection between 530-600 nm; EYFP (acceptor

794   channel) was excited at 514 nm and detected between 530-600 nm. Full length Fos-ECFP+Fos-

795   EYFP (top row), Fos^{215}-ECFP+Fos^{215}-EYFP (2nd row), Fos-ECFP+Jun-EYFP (3rd row) and

796   Fos^{215}-ECFP+Jun-EYFP (4th row) showed nuclear localization; the negative control, ECFP and

797   EYFP expressed independently, and the positive control, the ECFP-EYFP fusion protein (5th and

798   6th rows) were evenly distributed in the whole cell. FRET efficiency E was calculated in each

799   pixel. Histograms show the statistics of the subcellular distribution of E.

800

801   **Figure 3**

802   **Cell-by-cell analysis of dimerization by confocal microscopic FRET**

803   A and B) FRET efficiencies between donor- (ECFP) and acceptor- (EYFP) tagged Fos^{215} or full

804   length Fos molecules as a function of the acceptor-to-donor molecular ratio (N_A/N_D). Data of 300

805 cells were grouped into three subsets as a function of donor intensity (low: <800, medium: 800-

806 1200 and high: >1200, a.u.). Cellular data were binned in 0.25 wide intervals of the N_A/N_D values

807 to reduce data scatter. FRET efficiencies increased with increasing N_A/N_D. C) Saturation values of

808 FRET efficiencies at high acceptor-to-donor ratios ($N_A/N_D>0.95$). ECFP-EYFP fusion protein

809 served as a positive, and independently expressed ECFP and EYFP as a negative control. FRET

810 data of the Fos-Jun and Fos[215]-Jun pairs were published in (34).

811

812 **Figure 4**

813 **FCS-based concentration calibration and brightness analysis**

814 A) EGFP concentration in HeLa cells was determined from the amplitude of the autocorrelation

815 function. The curve was fitted to a two-component free diffusion model with triplet correction. B)

816 Diffusing particle concentration ($1/G_0$) as a function of the fluorescence intensity per pixel of

817 EGFP, Fos$^{\Delta\Delta}$-EGFP, Fos-EGFP and Fos-EGFP coexpressed with Jun-mRFP1. Data were fitted

818 with straight lines using Deming regression. C) Fluorescence per particle or molecular brightness

819 values characterizing the aggregation state, plotted as a function of the concentration of the EGFP

820 tag. Symbols are the same as in panel B. D) Normalized autocorrelation functions fitted to a two-

821 component free diffusion model. E) Diffusion constants and fractions of the second, slow

822 component derived from the fits (n: number of cells).

823

824 **Figure 5**

825 **Possible combinations of fluorescently tagged and endogenous Fos and Jun**

826 A) In the monomer-heterodimer equilibrium fluorescently tagged and endogenous, unlabeled Fos

827 and Jun molecules participate. The three species containing a donor tag contribute to the

828 measured value of the FRET efficiency E_{meas}: the doubly labeled heterodimer having a FRET

829   efficiency E_0, and the donor-labeled Fos in complex with endogenous Jun or present as a

830   monomer; the latter two species are characterized by zero FRET efficiency. The fraction of the

831   different heterodimers follows a multinomial distribution. E_{meas} is a weighted average of the

832   species-specific E values (given by Eq. S12 in the Supplement). B) In the monomer-homodimer

833   equilibrium donor-tagged, acceptor-tagged and endogenous Fos and endogenous Jun participate.

834   Four heterodimeric species and the donor-tagged monomer contribute to E_{meas} (derived in Eq.

835   S14 in the Supplement).

836

837   **Figure 6**

838   **Determination of the dissociation coefficients of Fos-Jun heterodimers and Fos-Fos**

839   **homodimers from flow cytometric FRET titrations**

840   A) and C) FRET efficiency measured in cells co-transfected with Fos[215]-EGFP+Jun-mRFP1 and

841   Jun-EGFP+Fos[215]-mRFP1, plotted as a function of donor-tagged Fos[215] or Jun concentration.

842   Data were grouped according to acceptor-to-donor molecular ratios (N_A/N_D) and fitted as

843   described (Suppl. Eq. S12, solid lines), yielding the K_d value of the heterodimers and the E_0

844   FRET efficiency of individual donor-acceptor pairs. Endogenous Fos and Jun were also taken

845   into account. B) and D) The solid lines represent the maximal theoretically attainable E values at

846   different N_A/N_D ratios (assuming E_0=15% and 14.1% based on the fits) when all available Jun-

847   mRFP1 molecules are engaged in heterodimers with Fos; the marked points correspond to the

848   experimental N_A/N_D values (Suppl. Eqs. S13, S15). E) Dependence of the K_d values from the fits

849   on the Fos:Jun ratio. F) FRET efficiency of Fos[215]-EGFP+Fos[215]-mRFP1 homodimers as a

850   function of donor-tagged Fos[215] concentration with K_d and E_0 yielded from a linked fit (see suppl.

851   Eq. S14). G) Maximal attainable FRET efficiencies at different N_A/N_D ratios (assuming E_0=9.47%

852   based on the fit), when all Fos molecules form homodimers.

853 **Figure 7**

854 **SPIM-FCCS data analysis show co-diffusion and DNA-binding of Fos homodimers**

855 A) Autocorrelation (ACF) and cross-correlation (CCF) functions from SPIM-FCCS

856 measurements. Green (EGFP ACF), red (mRFP1 ACF) and blue (CCF) solid lines indicate the

857 experimental data, whereas dashed lines are fits assuming two diffusing components (ACFs) or

858 one component (CCFs). The red horizontal line is the cross-talk-corrected red ACF amplitude and

859 the blue line is the level of cross-correlation due to cross-talk. Cross-correlation above this value

860 is due to co-diffusion of green and red molecules. B) The first two columns are fluorescence

861 intensity maps of EGFP (green) or mRFP1 (red) from a selected cell. The third column is a map

862 of the fraction of green-red dimers among all detected molecules, $c_{GR}\big/\left(c_{G-only} + c_{R-only} + c_{GR}\right)$

863 determined from the fits, and the histograms show their distributions. C) Average fraction of

864 green-red dimers and D) diffusion coefficients D_{cross} from the cross-correlation fits (mean±s.d.,

865 n>20 for each sample). Fits were carried out on a pixel-by-pixel basis, and the median of the

866 respective parameter from each cell was then averaged. ***p<0.0001 for the t-test.

867

868 **Figure 8**

869 **Both Fos-Jun and Fos-Fos complexes form stable dimers and bind to DNA**

870 Molecular dynamics simulations were carried out on small Fos-Jun (**A**) and Fos-Fos (**B**)

871 fragments bound to the DNA fragment. Ribbon representation (colored cyan) was applied for the

872 helical secondary structure of the Fos protein fragment (**A,B**). The atomic details of constituent

873 residues are shown by stick representation coloring the C, H, N, O and S atoms gray, white, blue,

874 red and yellow. For the Jun fragment (**A**) or the second Fos fragment (**B**) solvent excluded

875 surface representation was applied using the above-mentioned color codes. (**C-F**): Visual

876   representation of trajectories from MD simulations of the Leu zipper region of the Jun-Fos (**C,E**)

877   and Fos-Fos (**D,F**) dimeric structures. Wild type protein fragments (**C,D**) and virtually mutated

878   (Leu280Asp, Leu294Asp in c-Jun- and Leu165Asp, Leu179Asp in c-Fos) fragments (**E,F**) were

879   considered. Mutant residues are shown by stick representation using the same color scheme for

880   the atoms as above. Jun is represented by the orange helix, whereas Fos by green and yellow

881   ones. From each 500 ns dynamics trajectory 100 frames were saved equidistantly and

882   superimposed (after removing rotation and translation). Wild type protein fragments (**C,D**)

883   demonstrate stable coiled coil motifs with relatively low fluctuations. The mutations in the Fos-

884   Jun fragment (**E**) resulted in a somewhat distorted structure and larger fluctuations indicating

885   weakening of the interaction between the monomers. This is even more expressed for the mutant

886   dimeric Fos-Fos fragment (**F**) where hydrophobic interaction between regions affected by the

887   mutations is completely destroyed.

888