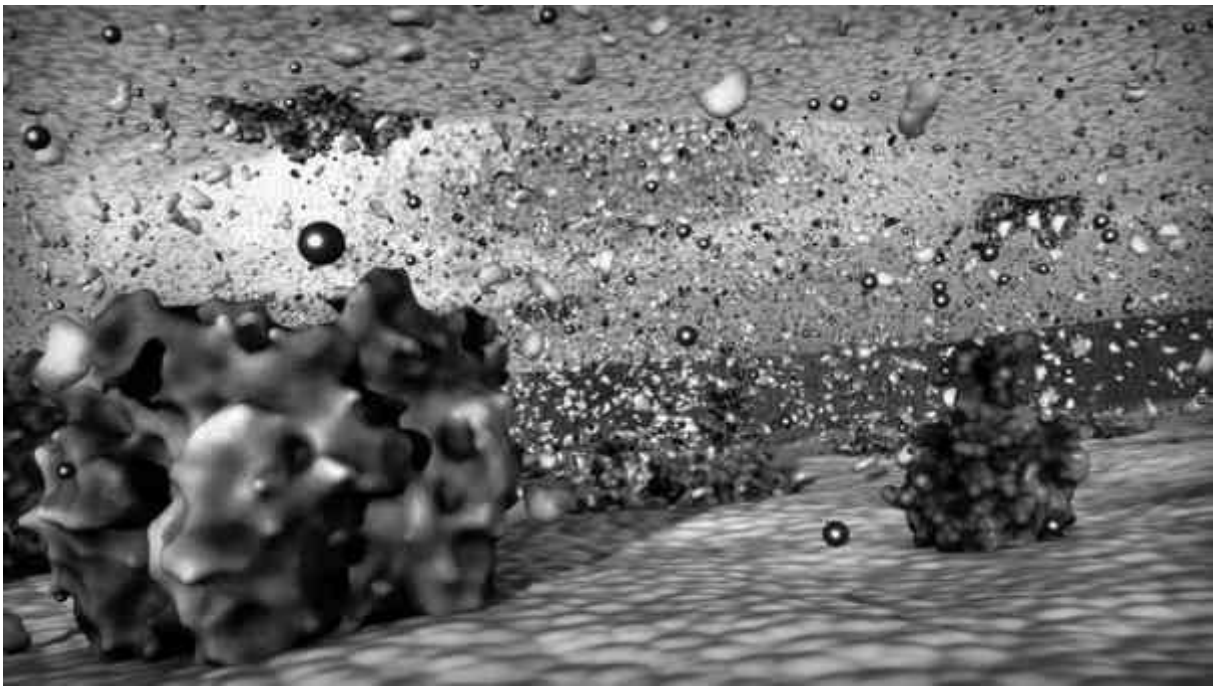
SCIENTIFIC SciVis VISUALIZATION

THE DARK ANIM
Technical Notes



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FOREWORD

The animation *The Dark Anim* was produced by request of Directors Miriam Jakobs and Gerhard Schick for their documentary film The Dark Gene, produced by Filmtank, in 2013-14.

The film follows Frank Schauder, who is both a doctor and a patient suffering from depression, in his quest for understanding, and in his struggle to fight the symptoms of his disease. The film opens a number of questions, including the possibility of a genetic cause, the likelihood that the 'responsible genes' might have passed to his son, the social determinants, the pursue of artistic expression as a way to alleviate the pain, and more.

During his search for explanation, Frank visits a neuroscientist that describes the present knowledge of how some neural features seem to be working in the state of depression. These explanations are accompanied by our animations.
The intent was to provide a view of the molecular features thought to be involved in depression, in a form that could be inserted in the movie, and that would explain to the public the current knowledge, including its treatment. Of course, the science of depression is not at all satisfactory: it is still debated the genetic *vs.* environmental origin; the effect of pharmacological interventions is only partial, with plenty of side effects, and not equally effective for all patients, and their mechanism of action is not yet completely clear.
Nevertheless, some important information is available, and some neural features specific for the disease have been investigated thoroughly. On this basis, we have built the animation that depicts the information presently available on the neural mechanism of depression.

This publication illustrates the production of the animations, starting form the scientific information, its elaboration, the technical challenges, and some other details that marked the period of work.

1. INTRODUCTION

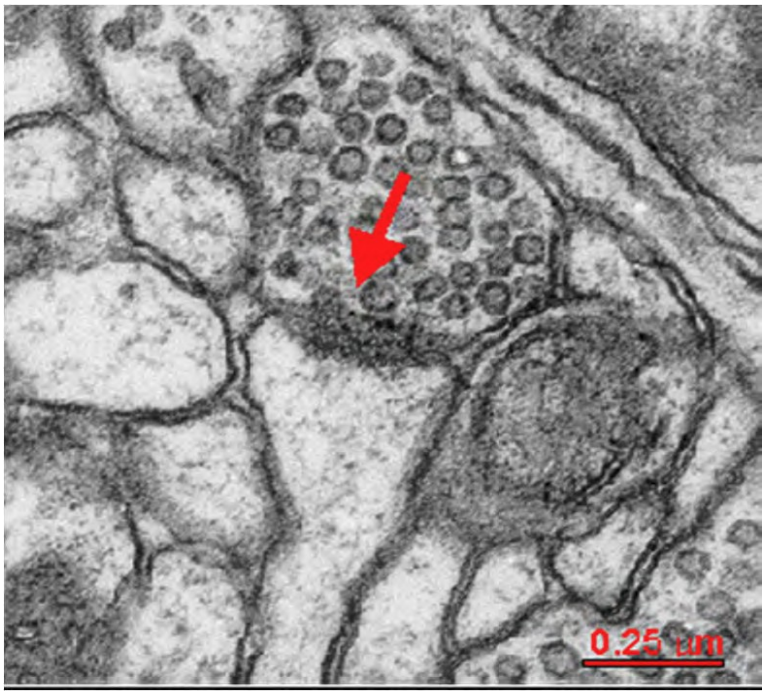
1.1 Brains, neurons, synapses and neurotransmitters

Our brains are extremely complex organs, filled with a variety of cells, including the neurons, which are the primary effectors of information elaboration. The number of neurons in a human brain is estimated to be around 100 billions, comparable to the number of stars in the entire universe, i.e. a number higher than we can imagine[1].

Each of these neurons, like a peculiar kind of tree, develops many tens to thousands of branches of varied length, which extend to connect with other neurons, in the neighbourhood or in very distant places in the body. On these branches (called neurites) there are many 'contact points', the synapses, which are the subject of intense scientific research, as well as of our movie.

At synapses, which, expanded ten million times[2], can be seen as circular 'rooms', of 3 m diameter and about only 20 cm tall, each of the two neurons brings a set of specific, dedicated proteins that contribute to build the environment in which the upstream neuron (the synapse works in mainly one direction) transmits the signal to the downstream or receiving neuron[3].

The signal coming from the upstream neuron reaches the presynapse in electric form, and is transformed into a chemical form by the activation of a vescicle release machinery: the vescicle, which is filled with neurotransmitter, opens into the synaptic cleft and pours its content into it. On the surface of the receiving neuron (the postsynaptic membrane) there are a set of proteins capable if 'reading' the signal: they can sense the presence of the neurotransmitter, and relay the information to the rest of the neuron, both by activating a new wave of electric signal that will travel along the neural membrane, and by delivering the information to the cell interior, with more long term effects.



The synapse (pictured here, from ref. 4), besides all proteins directly involved in neural signal propagation, also contains structural proteins which help in the maintenance of the structure of the cleft, keeping it stable and active. Like any other structure in living matter, the synapses are in constant dynamic equilibrium, and can form and disassemble, or become more or less tight during their life.

As it invariably happens, the more we study the details of a topic, the more complex the picture becomes.

Each synapse is in general specialized to transmit a particular signal, which is encoded in the identity of the neurotransmitter. We can think of different signal as of different words, or sounds, that, when integrated in meaningful way, can compose many different phrases, modulating the activity in very subtle ways. Furthermore, also the receptors can be modulated by several means, of genetic, physiological and artificial origin[5], or by random chance[6]. For this reason, it should be kept in mind that talking about synapses is like talking about faces: we all have two eyes, a nose, a mouth and so on, yet we are all different form one another, with shared similarities in families and groups.
If the general working of synapses in the brain is thus sketched out in some depth, the overall activity and complexity of the system is quite beyond our present state of understanding. The complete set of activities performed by the neurons, with their synapses, their electric transfer, their metabolic modulations, are the basis of our thoughts and mind, a fascinating field of research, subject of intense investigation.
Seen from a mechanistic perspective, most manifestations of ourselves are the result of brain activity, including our sentiments and moods. These latter are modulated by elaborating inputs from the external environment, which are all integrated in the complex network.
Different moods, or states of mind, have been generally associated with modulation of one or more of the neurotransmitters, and, in particular, the small molecule serotonin seems to be important in the establishment of a state of well being. Accordingly, when serotonin activity or metabolic processing is insufficient, the person experiences a situation of suffering. This can be 'physiological', if it is determined by facts that make us sad or sorrow, and usually lasts for a limited period of time. Sometimes, however, the sadness can be the result of a true disease, or unbalanced functioning of the serotoninergic system, and people become trapped in a depressed state, unable to enjoy even the smallest joy of life[7,8].
In theses cases, when the cause of depression is clearly due to serotonin dysfunction, pharmacological help can be found with the use of drugs, as described in the movie.

1.2 Plot

We have created the animation of the serotoninergic synapse, trying to interpret in visual terms the knowledge available about this very specific neural site.
The scene set (the synapse) is an environment delimited by the two membranes of the opposing neurons and includes the space defined by the presence of synaptic proteins anchored into the membranes. This space is filled with an aqueous solution (water not shown), in which are dissolved many small molecules: some which are ubiquitous in our body (glucose and ions) and some other which are more characteristic of the brain extracellular environment (some neurotransmitters like GABA and Glutamate).
We should notice that even if the synapse works in one direction, and even if we have placed the upstream neuron above the receiving one, at the scale of nanometer, and with the very small masses involved, the notion of up and down does not really make much sense: we have tried to encode this information in the initial minute of the animation, when the synaptic space is seen rotating.
When the signal arrives form the body of the upstream neuron (shown as a series of lightnings, accompanied by a specific sound), the presynapse get organized to release the content of one of its vescicles: many thousands of serotonin molecules are poured into the synaptic cleft, and float around like confetti in a box.
Given the small space of the synapse, quite immediately they are sensed by the receptors in

the post-synapse: one of them, the ionotropic receptor (or 5-HT_3), responds primarily by opening the channel to let ions go through (thus depolarizing the membrane and so propagating the electric signal), while another, the metabotropic receptor, has the primary function of transmitting the signal to the interior of the cell.

A mechanism of transmitter reuptake is also immediately activated in the presynaptic neuron: the aptly called reuptake receptors, located at the periphery of the synapse, avidly 'eat' as many serotonin molecules as they can, bringing them back into the upstream neuron, and making them available for recycling.

In the cases of depression due to low serotonin, the pharmacological inhibition of these reuptake receptors can help by letting more of the neurotransmitter lingering in the synaptic space (and probably also beyond). Among the most effective of the inhibitors is a drug known as Prozac®, which shares some chemical features with serotonin itself. When the reuptake receptor 'eats' a fluoxetine molecule, the drug sticks into the entrance and impedes further reuptake.
With higher level of serotonin in the brain, several depressed patients are able to restore the 'normal' mood, and are relieved from the condition. We have represented this 'return to normality' as a brighter light in the synapse of the treated brain.

1.3 Neurobiology and protein structure: research and tools

The representation of a complex subcellular environment in a virtual 3D scene implies the incorporation of information derived from a wide variety of sources, which has to be integrated across several orders of size and temporal frames.
Our study for The Dark Anim include:

- the collection of microscopy images, in particular EM of thin sections, from public repositories, such as the SynapseWeb[4] and the Cell Library[9];
- the consultation of textbooks of general biology and of neurobiology;
- a thorough bibliographic research on the general synaptic organization, on specific components and complexes, and on the dynamics of synaptic transmission (details in Chapter 6 BIBLIOGRAPHY);
- a detailed study of each of the proteins which are presented. These may be shown still (although environmental interactions and thermal vibrations are always present), or engaged in some activity that involves a change of conformation.

Regarding the last point, it should be noted that for each protein and for their movements we rely on the structural information deposited in the Protein Data Bank[10,11]. Structures found in the repository often belong to homologous proteins in different species (while our set is a human synapse), are sometimes incomplete (due to technical reasons), and may be of different quality. We gather the information, adapt it to the human sequence, integrate missing part (on the basis of homology modelling), add sugars and include other (known) post-translational modifications.

The elaboration of protein motion further complicates the procedure: the starting material is always data from PDB, which is adjusted in order to let BioBlender calculate the transition between the various conformations[12]. Specific elaborations are described for each protein.

1.4 Computer graphics: research and tools

Producing a scientific animation can be compared to making a documentary film, except that the 'environment' to be filmed is produced as a 3D scene in a Computer Graphics (GC) program. Once the scene is made, then we have to place lights and cameras and decide the framing of each shot.

These aspects are somehow arbitrary, determined by the artistic choices of the director; however they are also influenced by the available technological tools and available information.

We have exploited heavily many features of the CG toolset of Blender[13]: Particles, Physics simulation, Depth of field, Game Engine, Materials and Textures, and many Compositing techniques. Some of the tasks were obtained by scripting procedures that allowed, for example, the fine control of motion for the Reuptake receptor (see scene 3.6), or the composition of many serotonin molecules in the first scene, with consistent light, even if each one was filmed independently (see scene 3.1 and 3.7).

1.5 Time considerations

One very remarkable property of synaptic transmission is its speed: between the electric incoming signal and the next electric wave, the time lapse is usually less than a millisecond. The total length of The Dark Anim is about 5 minutes. However, we have not simply 'extended' the synaptic transmission time. Instead, we have used cinematographic technique to show at different times (in the film) things that happen at the same time in reality.

To give an idea of the speeds involved, note that protein motion (vibration) is usually measured in terms of nanoseconds (sec x 10^{-9}), and conformational changes are typically performed in the microsecond timescale, although there is no clear distinction between the two types of motion. Calcium entry through the Ionotropic receptor and the VGCC is estimated around 10 and 100 million ions per second, respectively, for each open channel. Considering that a single 5-HT_3 receptor may stay open less than half millisecond, in this time it should let through up to 5 thousands single ions. In the scene, we show several hundreds ions passing through, in about 1 minute (see section 3.3).

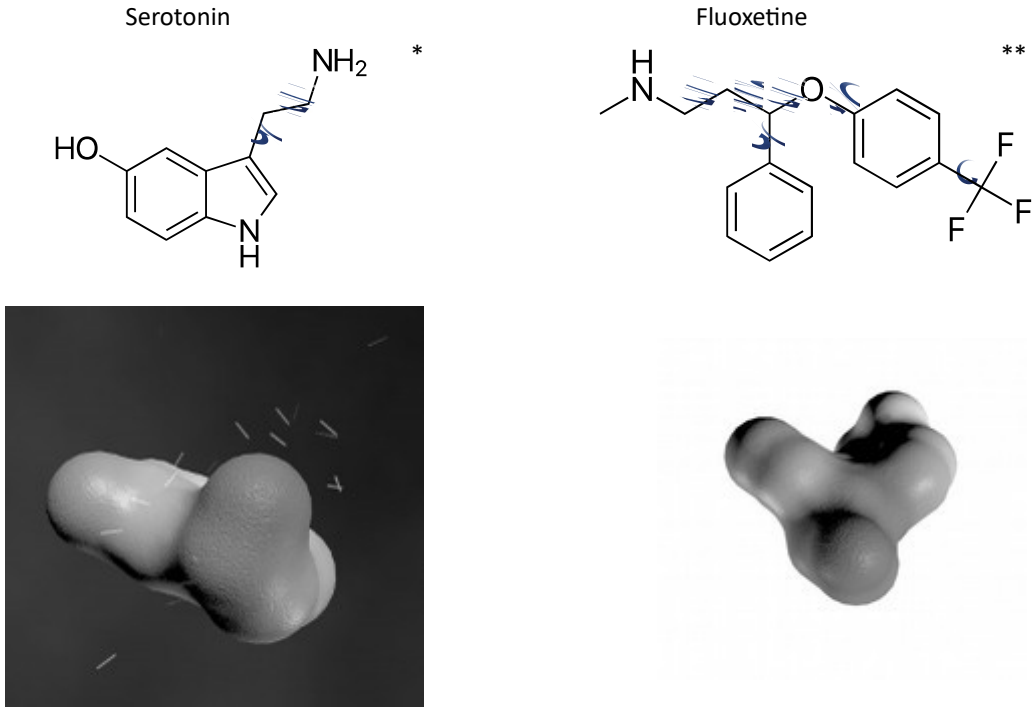
1.6 Music

The entire film The Dark Gene is graced by a sound track created by musician Daniel Almada. For the part of our animation, he used a non-diegetic sound that accompanies the viewer in the various steps of neural signal transmission.

2. MOLECULES

2.1 Serotonin and Norfluoxetin

The two small molecules, illustrated here, are the main characters of the animation:



Top: *chemical structures (Modified from *https://commons.wikimedia.org/w/index.php?curid=18074053 and ** https://commons.wikimedia.org/w/index.php?curid=50604522);* ***Bottom:*** *view of the molecules from the video.*

Serotonin (whose chemical name is 5 Hydroxy-triptamine, or 5-HT) is the main character of the animation. It is a neurotransmitter involved in several functions, and in particular in mood determination in the human brain. It is composed of 13 heavy atoms (C_{10}, N_2,O), plus 12 H, and we used PDB file 2YMD[14] as basis for the model imported in BioBlender (BB).

Animation: The only chemical bonds that rotate are those indicated in the figure. Rotation was set in BioBlender by using the Game Engine: a spin was impressed independently for each of the two bonds.

Render: Meshes (calculated with pyMOL, through BioBlender), Textures[15] (calculated by pyMLP, modified in BioBlenderSerot, described in Chapter 4, SOFTWARE) and EP[16] for each frame were calculated and saved. Textured meshes were rendered as sequence of PNG (RGBA), and EP was rendered as sequence of PNG (RGB), and then mounted with EP included as Add value. A single serotonin molecule per scene was rendered, with alpha background, and they were finally composited with a background of procedural Cloud.

Fluoxetine, also known by its commercial name Prozac®, is a drug that interferes with

serotonin metabolism, and is effective in many patients to reduce symptoms of depression. Its principal target is SERT, the re-uptake transporter that clears the synaptic space of the neurotransmitter. Fluoxetine binds to the receptor and stops its activity (scene 3.8).

The molecule is composed of 22 heavy atoms (C_{17}, F_3, N, O) plus 18 H, including 3 Fluorine atoms; it is a mixture of R and S forms (enantiomers), whose coordinates were derived from PDB files 3GWV and 3GWW respectively[17]. Data on the partial charges were kindly calculated by Fabrizio Santoro, of IPCF-CNR; values for radius of Fluorine from other PQR files, and values for fi (MLP) were adjusted from other files and Wikipedia. Fluoxetine is de-methylated *in vivo*, and is converted into Norfluoxetine .

Animation was made using BioBlender and its Game Engine, by imposing random rotations along the 6 bonds indicated in the figure.

Render: Meshes, Textures and EP for each frame were calculated (pyMOL, pyMLP and BioBlenderFluoxetine, modified with the data kindly provided by Susanna Monti of IPCF - CNR see p. 18), saved and used in compositing similarly to Serotonin.

2.2 5-HT_3, ionotropic receptor / Channel Receptor

5-HT_3, or serotonin receptor channel, belongs to the family of Pentameric Ligand Gated Ion Channel (PLGIC, of the Cys-loop transmitter-gated superfamily, such as nicotinic acetylcholine-, GABA-, Gly-receptors)[18,19].

It is composed of 5 subunits, with the serotonin binding sites at the interfaces of any two adjacent subunits and the cation (Sodium) channel opening through the center of the pentamer.

The model is based on PDB file 2YMD (pentameric crystal structure of mutated Ac-choline receptor from Aplysia, an experimental animal, in complex with serotonin[14]) for the open structure, and 4AQ5 (pentameric Ac-choline receptor from Torpedo[20]) for the closed structure. The human sequence of the 5-HT_{3beta} (UniProt accession n. O95264) was used to derive the monomer by homology modelling of the two structures**.**

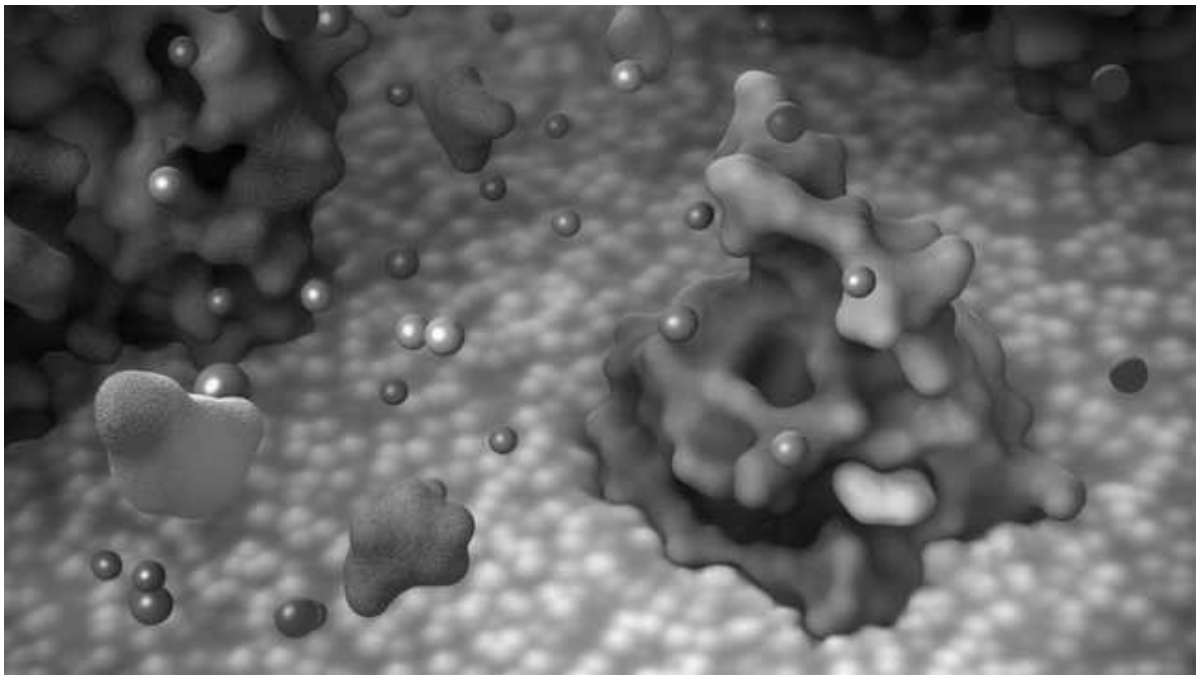
The functional receptor in humans is a hetero-pentamer of 5-HT_{3alpha} and 5-HT_{3beta} subunits of unknown stoichiometry[21,22]. Unfortunately the homology modelling of the 5-HT_{3alpha} on the 4AQ5a didn't work. The automatic threading of SPDBV[23] removes 80 aa C-ter (intracellular) and aligns the large cytoplasmic loop, (aa 322-455, UniProt n. P46098) on the transmembrane portion of the target model. The loop contains the 'HA-stretch'[24], a putative amphipathic helix important for the channel conductance. Even after BLAST alignment and manual threading of the raw sequence on the target structure, the homology modelling didn't return any result. For these reasons we decided to build a homo-pentamer composed only of beta chains. (This type of receptor showed low conductance in *in vitro*

experiments[22]).
In order to have the same aa in the closed and open structures, we built the models using aa Leu35 - Leu247, comprising almost the entire extracellular portion of the protein.
The torsion angles of the side chains of Tyr 146 and 148 of the homology model based upon 2YMDa, were modified because of the steric hindrance with serotonin.

Animation: the two models were imported in BB_0.6 at 200 frames interval between the two. The transition was calculated with the Game Engine[12] and all models (i.e. all the interpolated frames) were exported, resulting in 201 models in the final pdb file.
The 201 meshes were calculated with pyMOL, and handled through BB_meshes (see p. 19), which collects the meshes in a data base, and outputs a .blend file that assign every mesh to the same object. We calculated the meshes with a radius of 2.0 Å for the rolling probe, because, due to the complexity of the mesh, pyMOL returned bad meshes. Nevertheless, meshes had to be smoothed using the Object Tool (interpolating Vertex Normals), by means of the script Smooth.py, and then adjusted manually in the Blender space (inverted normals, or crossing faces).

Render: The mesh data-base included also values for lipophilicity, according to BioBlender code, in the scale from light/smooth/reflective to dark/rough/dull, from lipophilic to hydrophilic[15].

2.3 5-HT$_{1B}$, metabotropic receptor



Also called metabotropic receptor and member of the G-protein coupled receptor 1-family, 5-HT$_{1B}$ has only a small portion on the ectracellular surface in the synaptic cleft. This is a monomeric receptor of serotonin, whose signalling inhibits adenylate cyclase activity. (UniProt n. P28222)
For the human 3D structure we used the theoretical model 2G1X, because the 4IAR

experimental model lacks most of the extracellular portion. The serotonin binding site was elucidated from the 4IAR file and the relative article[25]. Serotonin binds into a deep cleft in the transmembrane portion of the protein.

Animation. 5-HT_{1B} movement is not elucidated (yet), and we have been able only to calculate a little motion using NMA in BioBlender. After the NMA analysis, 66 models were generated, and all meshes were calculated. These were then smoothed using the Object Tool in the same way as we did for the 5-HT_3 receptor.
Serotonin binding was animated manually in order to reach the binding pocket. The binding triggers a metabolic cascade inside the cell; this is marked by sound, and is described using a scene from one of our previous productions, Protein Expressions – Study N.3.

Render: All scene was rendered as TGAs. Then modified in post, using filters, rendered again only Z values for depth blur, then again only serotonin for DOF mask.

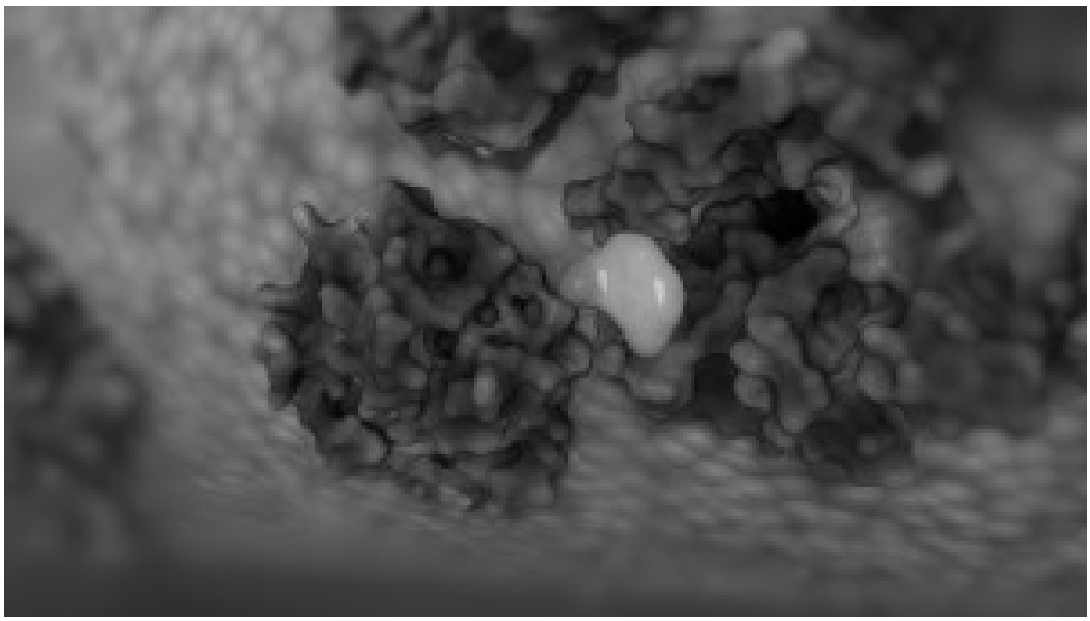
2.4 SERT, or re-uptake receptor

The **Sodium-dependent serotonin transporter**, the fourth member of the Solute Carrier Family 6, is also called *reuptake receptor*, or 5-HTT or SERT. This type of monoamine transporter mediates the reuptake of serotonin into the presynaptic neuron from the synaptic cleft, thus halting the action of serotonin and recycling the molecule, in a sodium dependent manner (co-transport of Sodium ions and serotonin, in a stoichiometry of 2:1).
The human protein (UniProt P31645) is homologous to the bacterial LeuT transporter and to other monoamine transporters, like the Dopamine transporter 4M48; on the basis of this homology we elaborated the protein movement. Among of the very many deposited structures, we chose three structures representative of the three states of the transporter:

substrate free, outward open	3TT1a
substrate bound, occluded state	2A65
inward open	3TT3a

We considered the sequence comprised between Arg11 and Leu495, in order to have, for every structure, the same number of aminoacids. All the structures were fitted in the space on the 2A65 model and some aa were mutated in order to have the same sequence among the three.

3TT1a	residue 268, Ala to Tyr residue 355, Ala to Ser
2A65	residue 288, Lys to Ala
3TT3a	residue 108, Phe to Tyr



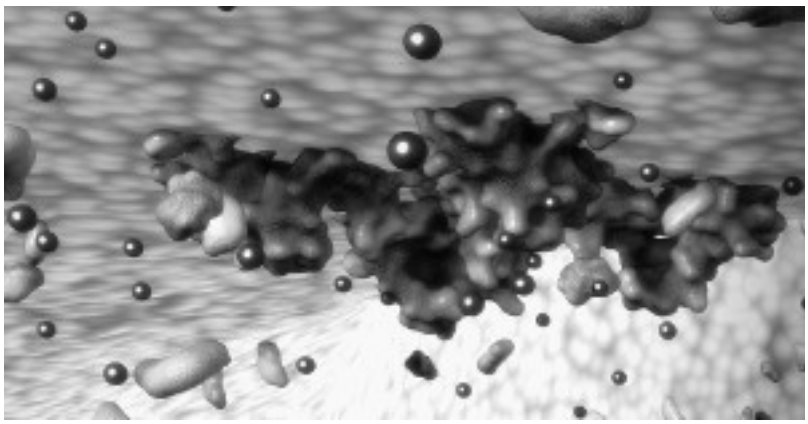
3TT1a and 2A65 were also partially reconstructed because they miss aa $_{130}$PPPNA$_{134}$ and $_{133}$NA$_{134}$, respectively, and the missing parts were modelled using the structure of the loop of 3TT3a.
After the mutations and the structure reconstruction, they were all minimized using Swiss PDB Viewer[23].
For the inhibited (fluoxetine bound) state we did consider the 3GWV pdb file, crystallized with R-fluoxetine, but we observed that the structure was most superimposing on the substrate bound, occluded state.

Animation. We created a pdb file with all the three sequential states (from substrate free outward open, to substrate bound occluded state, to inward open) plus the substrate free outward open again, in order to have the complete cycle.
We imported the pdb file in BioBlender and elaborated the movement (1 key-frame every 20 frames), then exported all frames.

2.5 VGCC, Voltage Gated Calcium Channel

The Voltage Gated Calcium Channel is ubiquitous in the membrane of excitable cells (N-type High-voltage-activated, $Ca_V2.2$). In the post-synapse the channel is opened as a reaction to the depolarization due to the arrival of the chemical signal and allows a rapid flux of Ca^{++} ions (~10^6 ions per second) from outside to inside the cell (passive diffusion): in this way the membrane is strongly depolarized, and the signal is propagated. In the pre-synapse the channel is opened as a reaction to the arrival of an action potential, where it contributes to the cascade leading to the fusion of the vesicle with the cell membrane and the release of the neurotransmitter in the synaptic cleft.
The channel is a tetramer of 4 similar polypeptides, each one slightly longer than 1000 aa, and is related to the Na^+ and the K^+ gated ion channels. Most of the protein is located either in the cytoplasm, or in the membrane thickness, and only some loops extend to the external

surface of the cell. The VGCC depicted was modelled on the basis of the tetrameric K^+ channel, pdb 2R9R (not animated)[26].



2.6 Neurexin/Neuroligin

Neurexin, NRXN (UniProt Q9ULB1) is a presynaptic cell-surface protein whose presence/activity affects cell-cell interactions, exocytosis of secretory granules and regulation of signal transmission[27,28]. It is involved in the stabilization of neural networks, through its role in enforcing synapse specificity, by clustering with postsynaptic scaffolding protein[29]. The C-terminal of the short intracellular section binds to synaptotagmin and PDZ thus connecting with the fusion proteins of intracellular synaptic vesicles.
NRXNs are a group of trans-synaptic cell-adhesion molecules that participate in a modular organization of presynaptic terminals by mediating interaction with Neuroligins and with other synaptic proteins[30,31]. NRXN, which contain multiple domains, also associate with other partners in the intracellular space.
The α-neurexin extracellular portion is composed by three globular repeats, each containing LNS-EGF-LNS domains, where LNS stands for Laminin, Neurexin, Sex-hormone-binding globulin and EGF for Epidermal Growth Factor. The LNS has a lectin, jelly roll fold comprising a Ca^{++} binding site. These three repeats are followed by a single stalk domain, connecting to a single transmembrane domain and a short cytoplasmic tail.
Two monomers of Neurexin interact with its main partner, the Neuroligin homodimer, anchored to the post-synaptic membrane.

Neuroligin, NLGN (UniProt Q8N0W4) is a postsynaptic cell-adhesion protein.
The extracellular, N-terminal domain is homologous to AchE (α/β-hydrolase fold superfamily), without esterase activity. It forms a dimer that binds two NRXN1 β monomers, forming a heterotetramer with the mediation of Ca^{++} [32].
NRXNs and NLGNs are thought to form a trans-synaptic complex, meeting near the center of the synaptic cleft, with the C-terminal sequences of either protein extending in opposite directions, tethering them to the pre- and postsynaptic membranes, respectively[30,33].
For the 3D model we started from the 2XB6 file[34], in which the complex between human NLGN4 and rat NRXN1 β were crystallized. After the homology modelling of the NRXN1β on the human sequence, we added the stalk domain of the two proteins on the basis of a file kindly provided by D. Comoletti.

The variety of forms and interactions between Neurexins and Neuroligins, and of their association with pther synaptic proteins, are still to be completely elucidated, and are likely to play an important role in the modulation of synaptic activity[35].

2.7 CaATPase / Plasma Membrane Calcium Pump (ATPase)

Human sequence modelled on the structure of bovine SERCA 3TLM. The protein is present on the post-synaptic membranes, and is a transporter devoted to expel Calcium from the cytoplasm, to restore the resting potential after the signal has been transmitted. The protein, whose ATPase activity is deployed in the inner side of the cell membrane, is seen as a still object.

2.8 NaK-ATPase/ Sodium-potassium pump

The Sodium-potassium pump is an abundant and ubiquitous protein present on all cellular membranes. The Blender file used derives from previous work, Protein Expressions – Study N.3, based on pdb file 3KDP. It has OS chains moving through a skeleton, but no texture (we used a Cloud texture for both protein and the sugars).

2.9 Other small molecules (ions, glucose, transmitter mix)

Glucose: mesh textured with highly hydrophilic texture (dark, rough and opaque). The molecule is rigid.
GABA: Gamma Amino Butyric Acid, is a neurotransmitter, included in the synaptic vescicle, since vescicles are not pure. Mesh obtained using file pdb 3IP9.
Glutamate, besides being an aminoacid present in proteins, it also has a role as an isolated compound in the transmission of signals in the brain. Its structure is quite simple, with 9 heavy atoms, and 8 H.
Ions: all ions modelled as spheres with VdW radius, in Å:

Ca^{++}	114
K^{+}	152
Na^{+}	116
Mg^{++}	86

3. SCENES

3.1 Serotonin **20 sec**

Serotonin is seen floating in space. Focus is on a single serotonin molecule, while others fly about in the background. The molecule is seen in full BioBlender mode (mesh with MLP and EP[15,16]).
The background is made with procedural clouds.

3.2 Synaptic space **1 minute**

The **serotoninergic synapse** is modelled on the basis of the typical Glutamate- or GABA-ergic synapses: a presynaptic spine is juxtaposed to the postsynaptic membrane, forming a circular button of about 300 nm diameter and 20 nm high. The scene was made in Blender 2.68.
The only proteins in the **presynaptic membrane** are the Voltage Gated Calcium Channels (VGCC) and the stems of Neurexin, seen in the distance. Several VGCC tetramers move around randomly before Action Potential (AP), and after AP some of them move to get close to the place where the vescicle will open.

Action Potential, rendered as flashes of light travelling fast along the membrane (made using point lights travelling on a Nurbs Path curve), lasts about 2 seconds, and the vescicle opens about 2 sec after the end of AP, releasing 6 thousands molecules (almost all serotonin, with some Glutamate and GABA). The accompanying sound helps in decoding the vision.

Membranes were built similarly to other rendering of membrane in previous movies: each membrane is generated as a simple plane, to which a Voronoi texture is applied both as bump map and as texture. This provides viewers with the impression of stacked lipids, which is the composition of biological membranes.

The **post-synaptic membrane** hosts numerous proteins:
The two receptors (5-HT_3, and 5-HT_1, see above), VGCC tetramers, Na/K Exchanger, Plasma membrane Calcium ATPase and Cadherin. Some of these proteins are grouped by type. For example, 5 molecules of 5-HT_3 are placed in strict vicinity, with few VGCC surrounding them, in correspondence with the site of vescicle opening, a position confirmed by results published after we made the animation[36].
Connecting the two membranes are the Neurexin/Neuroligin complexes. We have placed 3 clusters in the space of the synapse, in groups of 4 (2 Neurexin and 2 Neuroligins), although they are barely visible.

For all proteins, the surface mesh, the UV map and the MLP textures (1 bump map, 1 specular map used also for color) were created using BioBlender 0.6. Several levels of details (LOD) have been used for each protein (low and high resolution) depending on the distance from the camera. Only Hi Res meshes are equipped with textures and some are animated (see chapter 1. Molecules).

Bounding boxes were also built for each protein, to collide with the floating ion particles and avoid nonphysical closeness and/or interpenetration.
The b-boxes were created with the Convex Hull operator to get a clean and simple mesh for collisions, in order to reduce the calculation time during particle simulations (see below). All proteins, thus, are present as objects with 3 meshes: high resolution (HR, with complete MLP texture), low resolution (LR, reduced using the Modifier), and the bounding box (convex hull).

Particles: Ions (Na^+, Ca^{++}, K^+, Mg^{++}) and Glucose are in the scene from the start.
The volume of synapse is calculated to be 1,413 x 10^{-18} l, and the particles were included in the scene as shown in the following table.

particle	**Cell conc. (mM)**	**Amount in synapse volume**	**Amount in scene 2 (synaptic space)**	**Amount in scene 3 (closeUp 5-HT_3)**
Na^+	145	123500	35100	6500
K^+	5	4255	6500	1200
Ca^{++}	5	4255	6500	1200
Mg^{++}	1	850	2900	55
Glucose	5	4255	6500	1200

The relative amount of sodium and the other small molecules is such that we could not appreciate by eye the presence of anything but Na^+ ions. Therefore we tried to find a formula capable of providing information about the fact that, although the vast majority of what fills the space is sodium, also other molecules and ions are present. After several trials, we finally opted for: Final N. of particle in scene = (Amount in synapse volume)$^{-2}$ x 100

Floating particles are generated by several **emitters** during the initial 3 frames: Ca emitter, Mg_K_gluc emitter, Na emitter, and the vescicle emitter (this last activated later); they are equilibrated for 200 frames with Brownian motion, with colliders at membranes, around proteins and in front of camera before scene render. An invisible container roughly of the size of the synapse is used for keeping particles within the synaptic space. The ions are continuously scattered around with Brownian motion.

The **lighting** setup is composed of several spot lights placed outside the presynaptic space, just above the horizon, aiming towards the synaptic space so that they cast long and soft shadows and instill a dark and cloudy mood to the ambient.
The camera movement rotating at first, has been set to convey the impression of the non existence of the upward and downward concepts when we are at this scale.
Rendering was done in batches, and in layers: particles and scene were rendered as .exr and composed. For the 5-HT_3 close-up, particles were cached and rendered in two parts: front (foreground) and back.

Serotonin release. The action potential triggers the VGCCs on presynapse to get close to

vescicle borders (3 seconds) leading to vescicle opening (12 seconds) and serotonin (6000 particles, including 10% GABA and 10% Glutamate) release. The average content of serotonin in a synaptic vescicle is estimated to be 12000 molecules. We use 4800 serotonin particles (+600 GABA, and 600 Glutamate) to show that the entire space is flooded with the neurotransmitter. Particles receive a random rotation and translation push, and stay within the boundary of the synaptic box. They bounce against all objects (convex hulls), and modify their trajectories. The movements are recorded in a scene, and cached in order to check them before rendering, and also to allow rendering in batches on several pc.
For the vescicle opening, a set of animated shape keys on the membrane mesh has been used. The emitter is initially locked inside the vescicle, then the animation causes the membrane to open. At the same time, the particle system for the serotonin molecules starts working.
The membrane mesh has a collider applied, so that the particles are pushed outside by the walls of the membrane, giving a more realistic appearance. The three VGCC that approach the vescicle as it opens are moved manually, by key-frame animation.
After serotonin has been released, it floats in the synaptic space, and some molecules become bound to the serotonin receptors, the Ionotropic (5-HT_3) and the Metabotropic (5-HT_1).

3.3 Serotonin binding to 5-HT_3 **1 minute**

Serotonin binds to the 5-HT3 receptor at the interface between subunits. It is not clear how many serotonin molecules bind to each pentameric receptor, nor if there is a minimum N to induce opening of the channel. This scene is a close-up of the serotonin binding to 5-HT_3 and takes place in the synaptic cleft as the previous one. We show this binding focusing on a detail of the pentamer, and then zooming out to see Calcium flowing through many receptors in the synapse.
The 5-HT_3 receptor animation was modelled in great detail, with BioBlender using several conformations (see above 5-HT_3).
Serotonin (animated by key-frames in order to get to the right spot) reaches the binding site in the position from 2YMD.pdb. The other small molecules travelling in the synaptic space are moved by a particle system simulation and kept in place by mesh colliders.
This scene was split in foreground and background in order to get smaller scenes to work with, and then was recomposed in one blend file for render. The scene has particle emitters for Ca^{++}, Na^{+}, K^{+}, Glutamate, GABA, Serotonin, Glucose.
The closest emitter for Ca^{++} is shaped in a way that helps to give the sense of a flow of ions entering the channels, which are equipped with a magnetic force that attracts ions. This force is activated after serotonin binding. During the 30 seconds of flow, several hundred Calcium ions are seen entering the channel, a number sufficient to provide information about what's happening, but far form the real number (few thousands). Once again, we had to compromise between 'readability' of the scene, and strict attendance to the scientific evidence.

3.4 Serotonin binding to 5-HT$_1$ **12 seconds**

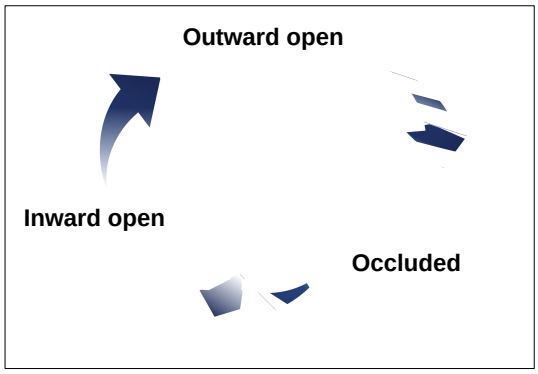
The metabotropic receptor is supposed to be a monomer whose activity, triggered by serotonin binding, is mediated by a conformational change that transmits the signal to the interior of the cell. This will activate a trimeric G protein, which, after the exchange of GTP for GDP, dissociates into the α and the $\beta\gamma$ subunits. The series of events eventually leads to a number of intracellular processes, including, in some cases, gene transcription.
The scene setup is similar to the one made for 5-HT$_3$: the structure for this protein is obtained from pdb files listed in section 1.4, and the movement of the protein itself is made from NMA calculated models, swapped in the Blender scene with a custom swap script.
Serotonin is animated by key-frames, and reaches the binding spot accordingly to PDB 4IAR.
A particle system provides the ions and proteins for the synaptic space, while a system of colliders avoids compenetrations and particle dispersion.

3.5 Cellular interior. Microtubules **20 sec**

The activity in the cytoplasm is represented by a scene derived from one of our earlier animations (Protein Expressions – Study N.3). The intent is to provide the viewer with a glimpse of how complex and apparently chaotic is the cytoplasm of an active cell.

3.6 ReUptake - SERT **25 sec**

Serotonin, after being released by the presynaptic vescicle, can diffuse, but its actual diffusion in the brain is limited. Most of the non-bound serotonin is recuperated by the presynapse, thanks to a series of ReUptake receptors placed at the boundary of the synaptic button. The transporter is the target of several important drugs, and has been studied in deep details: it transports one serotonin molecule together with two Na^{++} ions, and it has been crystallised in several conformations, as described in section 2.4.
In the scene, the only protein present is a small group of SERT units, each one animated independently, along the cycle shown in the figure.



Many serotonin molecules are floating around; the ones that actually enter the SERT and the ions of the co-transport were animated 'in reverse', i.e. the scene was set up with emitters of serotonin and ions inside the SERT, and played backwards.

Animation of the SERT was done using the mesh swap script in the Blender file, modified for this particular case: the script loads the sequential conformations frame by frame according to the times specified. In the close-up, serotonin molecules enter the channels passing trough a bound position as in pdb file 2A65, and this is achieved by key-frame animation, in sync whit the mesh swap, to get the channel in the right conformations as the small molecule goes through it. A complex particle and collision setup shows the serotonin molecules to decrease in number, while only some of them actually enter the SERT.

3.7 Fluoxetine (Prozac®) **25 sec**

Scene made in BB_0.6, similar to serotonin (2.1), with (nor)fluoxetine shown in foreground, together with other serotonin molecule, so that both the similarity and the difference between the two can be observed. The Prozac® molecule is seen in full BioBlender mode (mesh with MLP and EP), with EP rendered separately, with particles only and the molecule in alpha mask. PNGs are combined in post as well, whit a procedural cloudy background.
The background of clouds is the same as in scene 2.1, shifted to white using the color correction function of Blender; this makes the scene brighter, with soft and sparse clouds, to underline the positive change in mood that is consequent to the use of the drug.
The final scene was composited with (from the bottom layer):
- background (cloud texture, same as used for serotonin scene, skewed to white),
- several serotonins (alpha compositing)
- fluoxetine with MLP
- EP of fluoxetine as Add

3.8 Reuptake – treated **25 seconds**

The drug fluoxetine works by entering the re-uptake receptor, and locking it so that it cannot remove serotonin form the synaptic space. The scene, in which fluoxetine is placed in SERT according to file pdb 3FWV, shows some serotonin molecules and ions bouncing into the nested fluoxetine molecule, and unable to get access to the clogged transporter. The amount of serotonin in the environment stays high.

3.9 Synapse after treatment **33 seconds**

In the synaptic space, after treatment with the SERT inhibitor, some level of serotonin is constantly present. This presence results in a mild stimulation of the serotonin receptors, and to a gradual brightening of the mood of the patient. We have conveyed the amelioration of depression symptoms by using a brighter light, with sharper shadows, similar to the light of a sunny day.

4. SOFTWARE

Blender (www.blender.org)
Blender is a complete package for 3D work, from modelling to video editing, developed by the Blender Foundation, Amsterdam (NL). Blender is the only program for 3D work of high level which is completely Open Source, and free. Please refer to the website.

BioBlender 0.6 and BioBlender 1.0
BioBlender 0.6 (BB_0.6) is a version of Blender that contains features for molecular work, as described in details elsewhere. It contains a collection of scripts and other programs assembled to work with Blender and elaborate biological data. BB can fetch and read PDB files, elaborate conformational changes between different states, calculate lipophilic and electrostatic potentials, and render them according to the special visual code elaborated by us at SciVis.
BioBlender 1.0 is being developed as an AddOn for Blender, and can be downloaded from the website Bioblender.eu, where users can also find explanations on its use. *Please note: at present BB1 is not available. Work is in (slow) progress to fix it.*

For the production of The Dark Anim, we used both versions of BioBlender, and also introduced some special features to the code of BB_0.6 in order to deal with special molecules such as serotonin and fluoxetine, as follows:

BioBlenderSerot is based on BB_0.6 and modified to consider serotonin as a 'modified Tryptophan'.

BioBlenderFluox, similar to BioBlenderSerot. Changes were made in order to include Norfluoxetine, the *in vivo* active metabolite of fluoxetine, as additional molecule. The Fluorine atom was added, with value for its radius and for lipophilicity, derived from literature and occasionally guessed with criterion.

Other scripts:
Combine.sh Command script to combine N files PDB exported by BioBlender as sequence of files into a single file containing N models.
BB_meshes.py Python script that collects meshes (protein surfaces) calculated in BioBlender, and assign them to a single object in a new .blend file, to be imported in the rendering scene.
Mesh_swap uses the meshes in the file to assign to a single object each mesh at each frame in the animation.
Smooth.py Python script that automatically smooths all meshes produced in previous steps, using the Object Tool (interpolating Vertex normals) in Blender.

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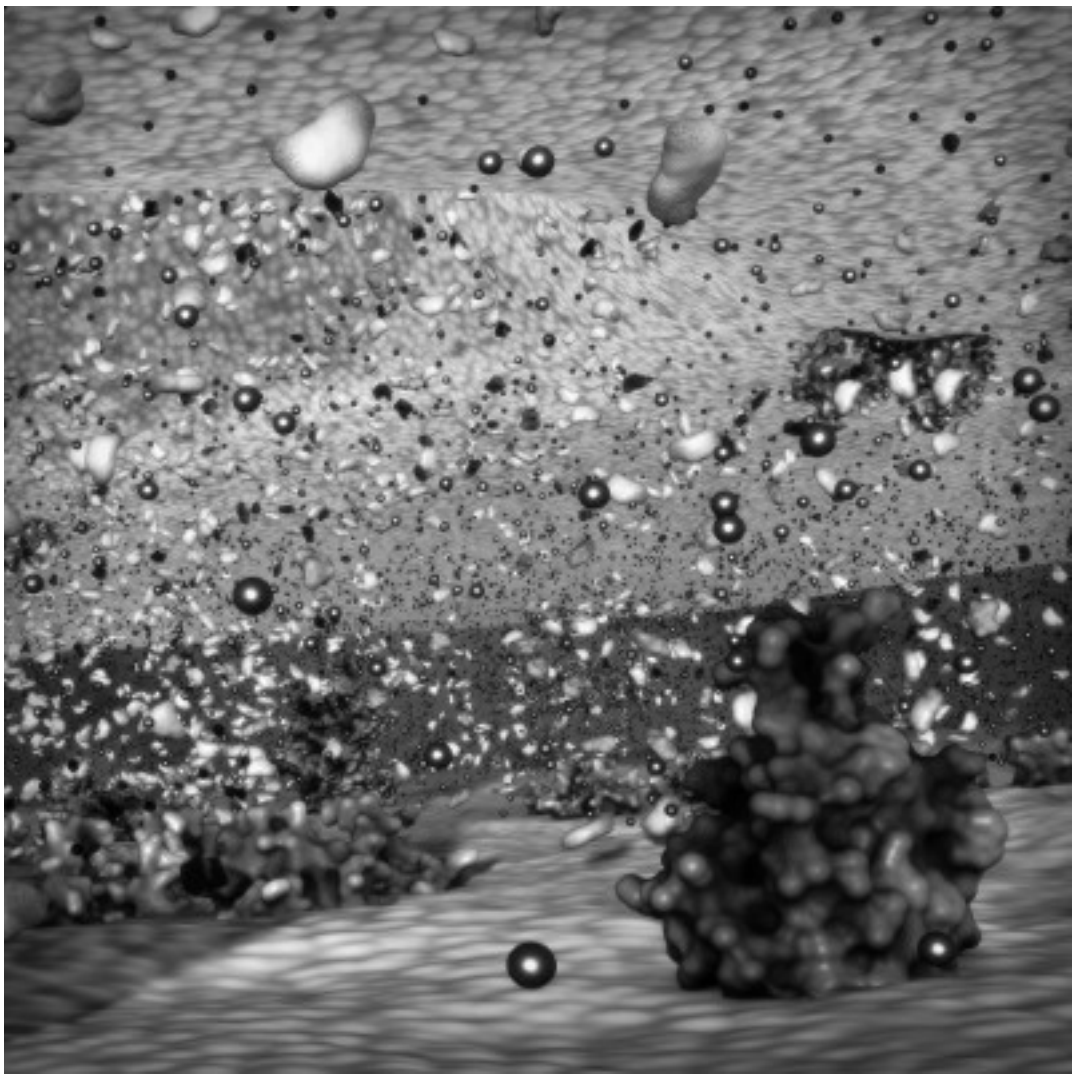
The Dark Anim is the result of work conducted as a part of the movie The Dark Gene, directed by Miriam Jakobs and Gerhard Schick, and produced by Filmtank, Hamburg, Germany.



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For all molecules and for the set, an initial acquaintance with the components was obtained by consulting textbooks, Wikipedia, Uniprot, KEGG and PDB pages.
Visual references were mostly obtained through searches with the Google Image service, and following the threads leading to the original scientific research/lab.
Many of the relevant pages are linked directly in the present document.

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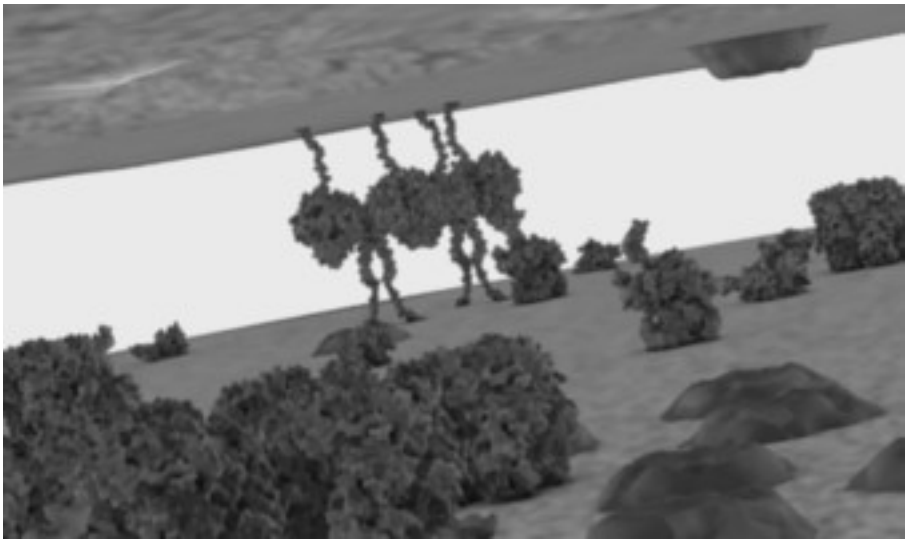

The Dark Anim was made by the SciVis group of IFC – CNR in Pisa Italy.
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