Evolution in Simple Systems and the Emergence of Complexity

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1. Darwinian evolution in laboratory experiments
2. Modeling the evolution of molecules
3. From RNA sequences to structures and back
4. Evolution on neutral networks
5. Origins of complexity

1. **Darwinian evolution in laboratory experiments**
2. Modeling the evolution of molecules
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Three necessary conditions for Darwinian evolution are:

1. **Multiplication,**
2. **Variation**, and
3. **Selection**.

Variation through mutation and recombination operates on the **genotype** whereas the **phenotype** is the target of **selection**.

One important property of the Darwinian scenario is that variations in the form of mutations or recombination events occur uncorrelated with their effects on the selection process.

All conditions can be fulfilled not only by cellular organisms but also by nucleic acid molecules in suitable cell-free experimental assays.

	Generation time	Selection and adaptation 10 000 generations	Genetic drift in small populations 10^6 generations	Genetic drift in large populations 10^7 generations
RNA molecules	10 sec 1 min	27.8 h = 1.16 d 6.94 d	115.7 d 1.90 a	3.17 a 19.01 a
Bacteria	20 min 10 h	138.9 d 11.40 a	38.03 a 1 140 a	380 a 11 408 a
Multicelluar organisms	10 d 20 a	274 a 20 000 a	27 380 a 2×10^7 a	273 800 a 2×10^8 a

Time scales of evolutionary change

Bacterial Evolution

S. F. Elena, V. S. Cooper, R. E. Lenski. ***Punctuated evolution caused by selection of rare beneficial mutants***. Science **272** (1996), 1802-1804

D. Papadopoulos, D. Schneider, J. Meier-Eiss, W. Arber, R. E. Lenski, M. Blot. ***Genomic evolution during a 10,000-generation experiment with bacteria***. Proc.Natl.Acad.Sci.USA **96** (1999), 3807-3812

S. F. Elena, R. E. Lenski. ***Evolution experiments with microorganisms: The dynamics and genetic bases of adaptation***. Nature Review Genetics **4** (2003), 457-469

C. Borland, R. E. Lenski. ***Spontaneous evolution of citrate utilization in* Escherichia coli *after 30000 generations***. Evolution Conference 2004, Fort Collins, Colorado

Genotype = Genome

Mutation → GGCTATCGTACGTTTACCCAAAAAGTCTACGTTGGACCCAGGCATTGGAC.....G

Unfolding of the genotype:

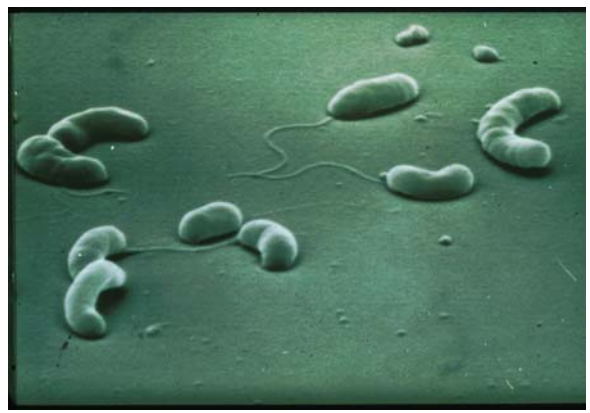
Production and assembly of all parts of a bacterial cell, and cell division

Fitness in reproduction:

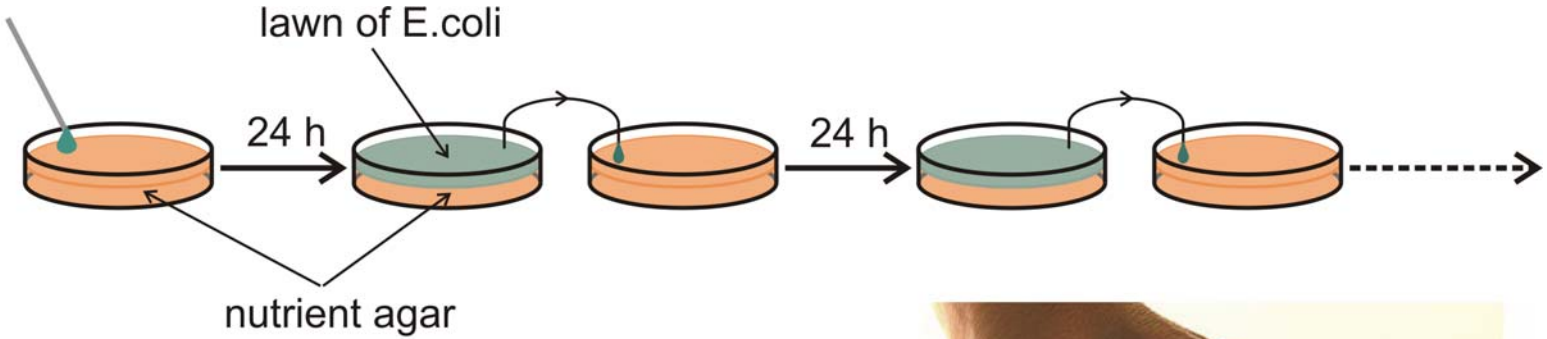
Number of bacterial cells in the next generation

Phenotype

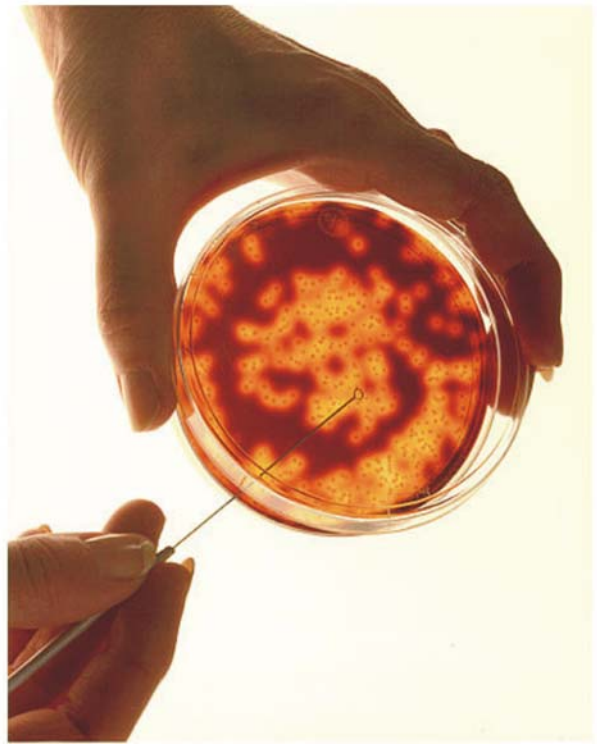
Selection →



Evolution of phenotypes: Bacterial cells



1 day » 6.67 generations
1 month » 200 generations
1 year » 2400 generations



Serial transfer of Escherichia coli cultures in Petri dishes

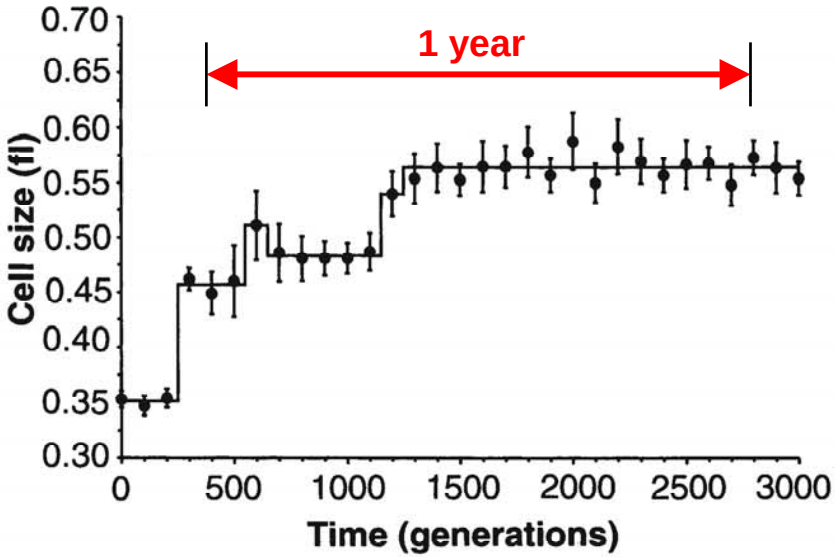


Fig. 1. Change in average cell size (1 fl = 10^{-15} L) in a population of *E. coli* during 3000 generations of experimental evolution. Each point is the mean of 10 replicate assays (*22*). Error bars indicate 95% confidence intervals. The solid line shows the best fit of a step-function model to these data (Table 1).

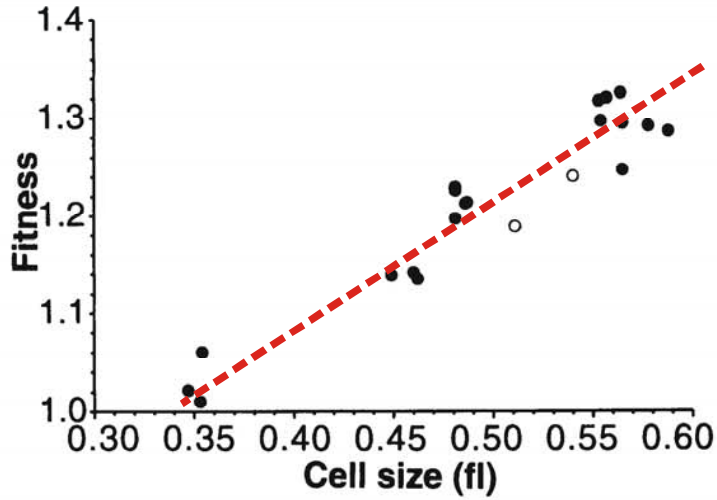
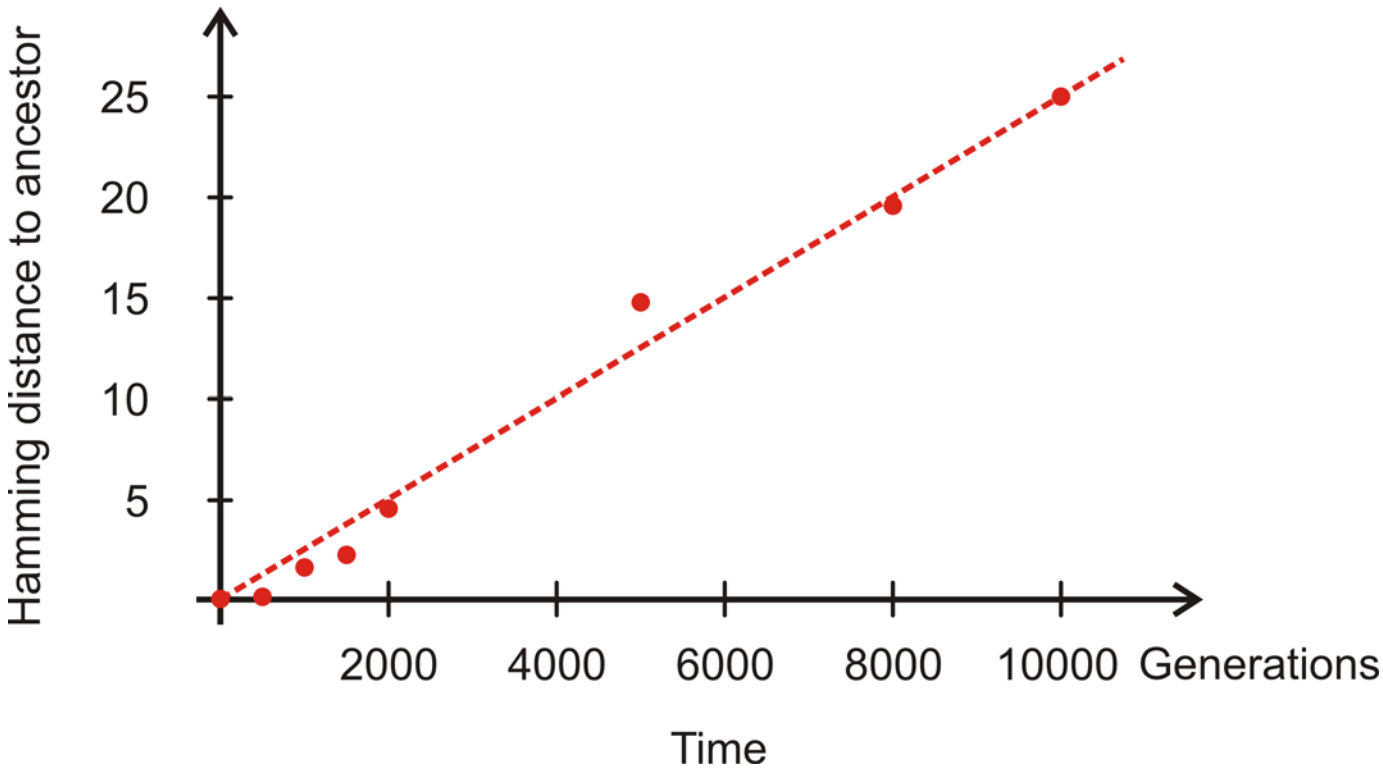


Fig. 2. Correlation between average cell size and mean fitness, each measured at 100-generation intervals for 2000 generations. Fitness is expressed relative to the ancestral genotype and was obtained from competition experiments between derived and ancestral cells (*6, 7*). The open symbols indicate the only two samples assigned to different steps by the cell size and fitness data.

Epochal evolution of bacteria in serial transfer experiments under constant conditions

S. F. Elena, V. S. Cooper, R. E. Lenski. *Punctuated evolution caused by selection of rare beneficial mutants*. Science **272** (1996), 1802-1804



Variation of genotypes in a bacterial serial transfer experiment

D. Papadopoulos, D. Schneider, J. Meier-Eiss, W. Arber, R. E. Lenski, M. Blot. *Genomic evolution during a 10,000-generation experiment with bacteria*. Proc.Natl.Acad.Sci.USA **96** (1999), 3807-3812

Innovation after 33 000 generations:

One out of 12 *Escherichia coli* colonies adapts to the environment and starts spontaneously to utilize citrate in the medium.

Evolution of RNA molecules based on Qβ phage

D.R.Mills, R.L.Peterson, S.Spiegelman, ***An extracellular Darwinian experiment with a self-duplicating nucleic acid molecule***. Proc.Natl.Acad.Sci.USA **58** (1967), 217-224

S.Spiegelman, ***An approach to the experimental analysis of precellular evolution***. Quart.Rev.Biophys. **4** (1971), 213-253

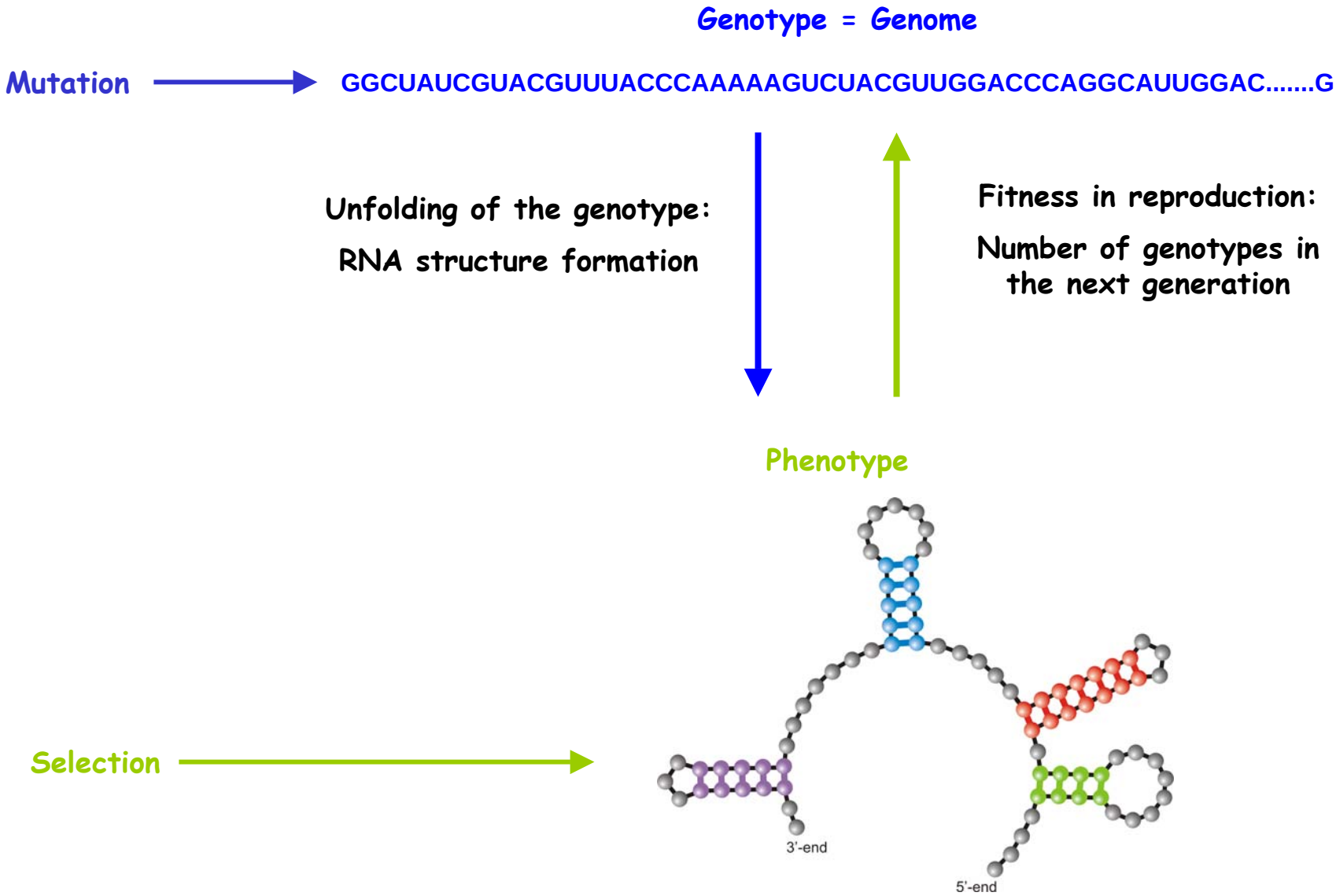
C.K.Biebricher, ***Darwinian selection of self-replicating RNA molecules***. Evolutionary Biology **16** (1983), 1-52

G.Bauer, H.Otten, J.S.McCaskill, ***Travelling waves of* in vitro *evolving RNA.*** *Proc.Natl.Acad.Sci.USA* **86** (1989), 7937-7941

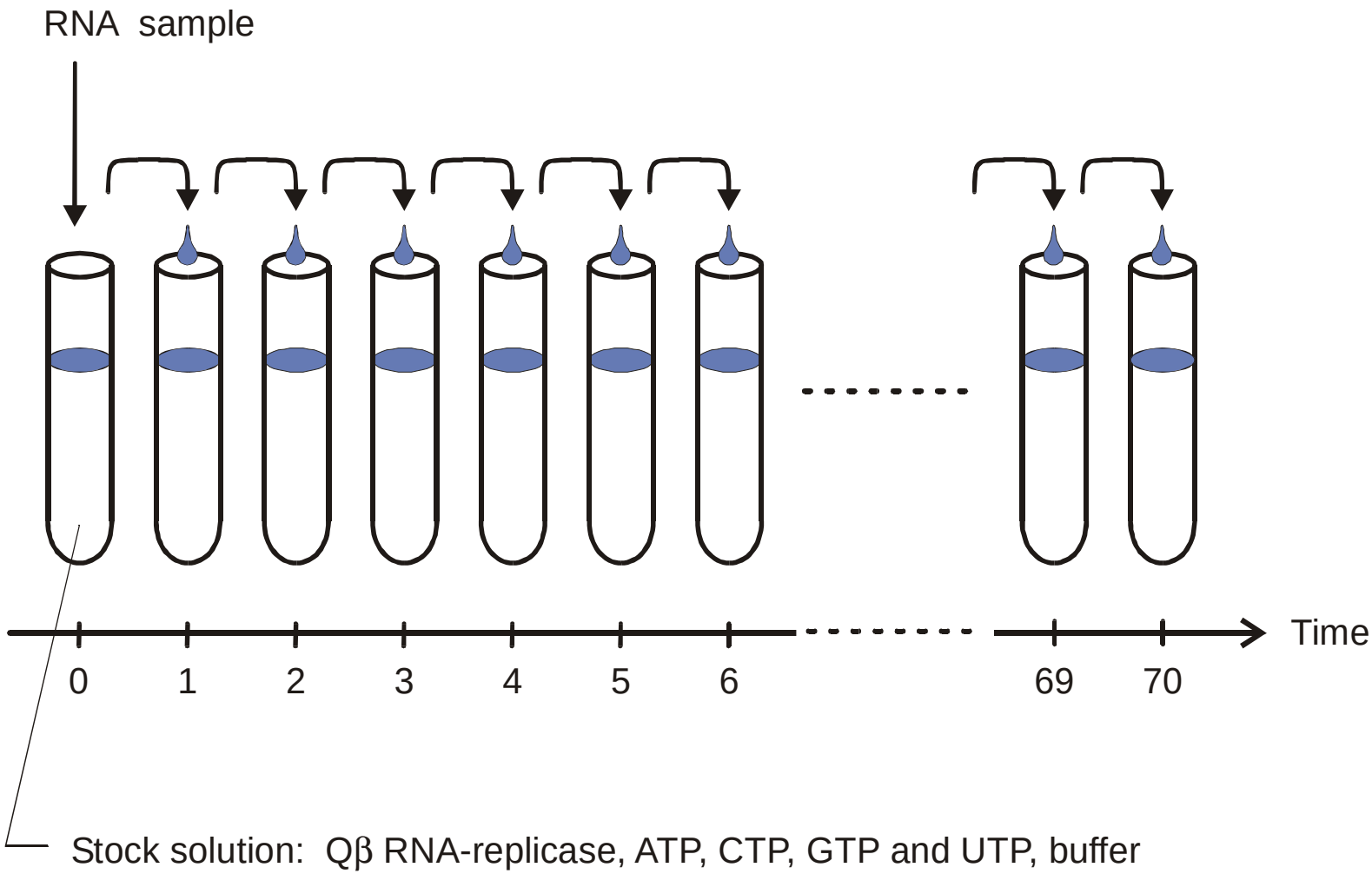
C.K.Biebricher, W.C.Gardiner, ***Molecular evolution of RNA* in vitro**. Biophysical Chemistry **66** (1997), 179-192

G.Strunk, T.Ederhof, ***Machines for automated evolution experiments* in vitro *based on the serial transfer concept***. Biophysical Chemistry **66** (1997), 193-202

F.Öhlenschlager, M.Eigen, ***30 years later – A new approach to Sol Spiegelman‘s and Leslie Orgel‘s* in vitro *evolutionary studies***. Orig.Life Evol.Biosph. **27** (1997), 437-457



Evolution of phenotypes: RNA structures and replication rate constants



The serial transfer technique applied to RNA evolution ***in vitro***

Reproduction of the original figure of the serial transfer experiment with Qβ RNA

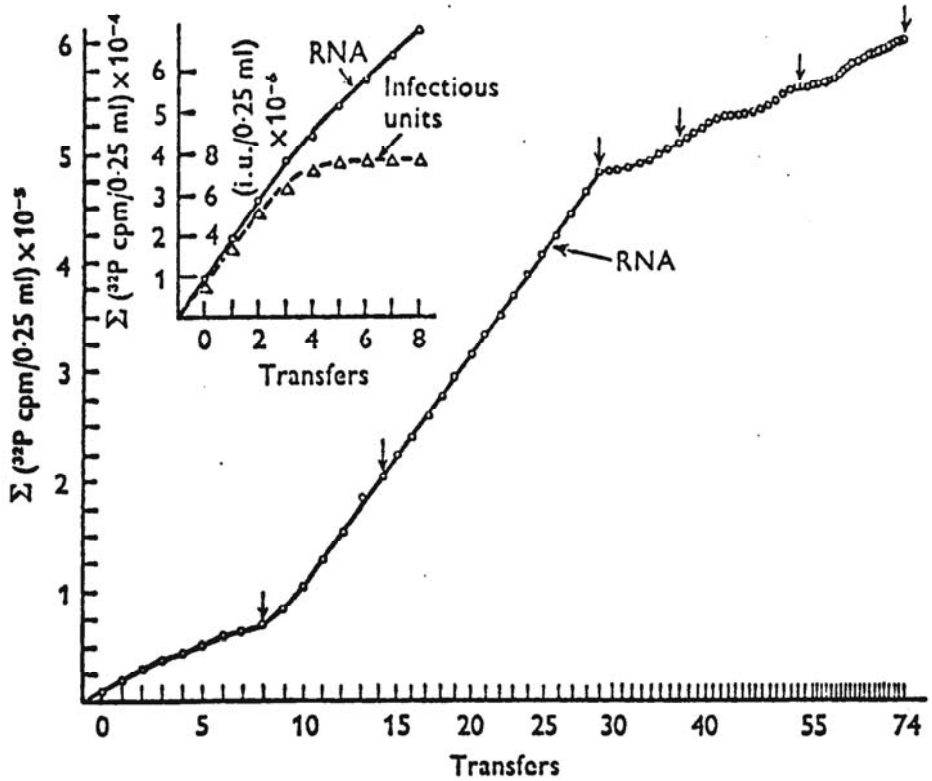
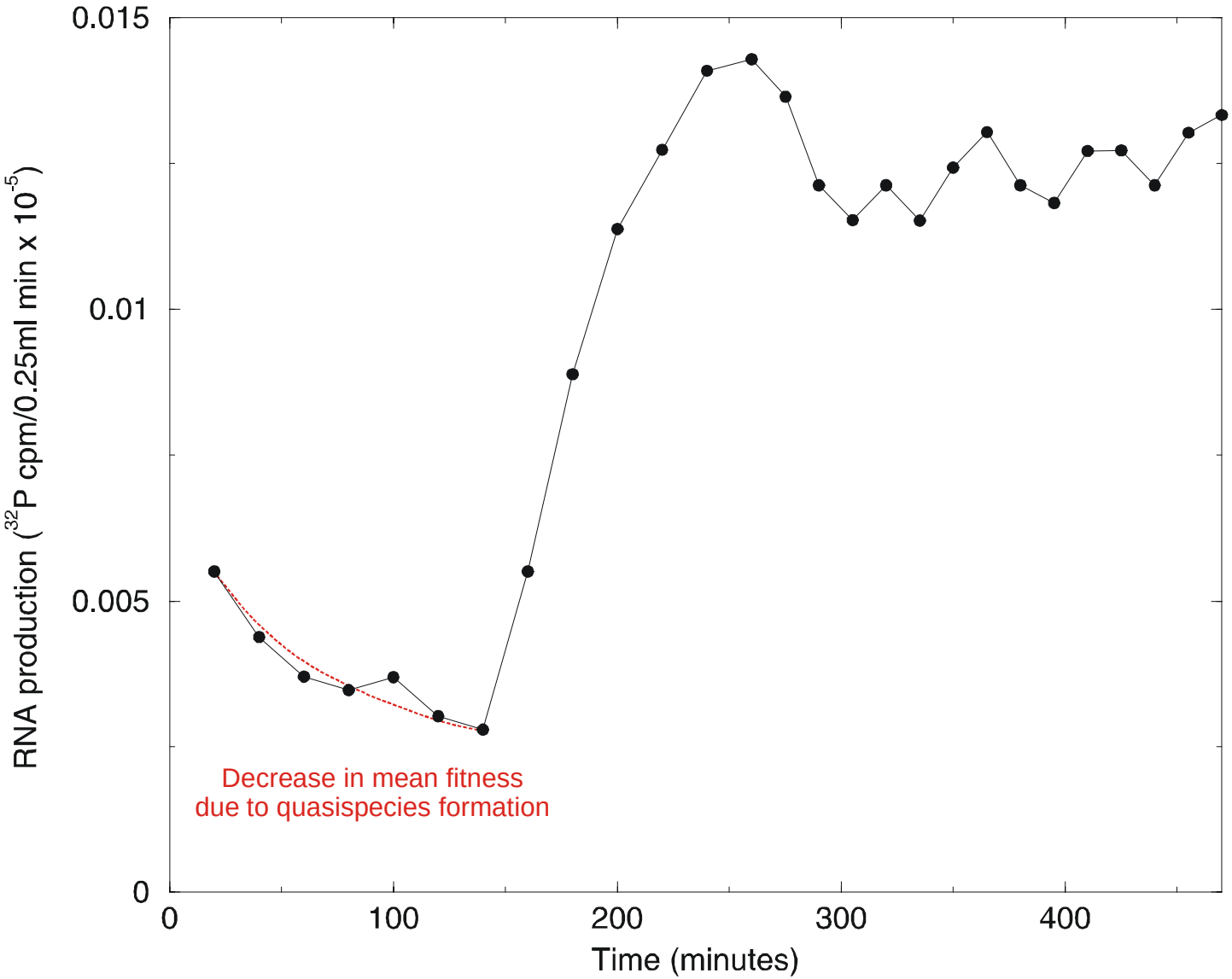


Fig. 9. Serial transfer experiment. Each 0·25 ml standard reaction mixture contained 40 μg of Qβ replicase and ³²P-UTP. The first reaction (0 transfer) was initiated by the addition of 0·2 μg ts-1 (temperature-sensitive RNA) and incubated at 35 °C for 20 min, whereupon 0·02 ml was drawn for counting and 0·02 ml was used to prime the second reaction (first transfer), and so on. After the first 13 reactions, the incubation periods were reduced to 15 min (transfers 14–29). Transfers 30–38 were incubated for 10 min. Transfers 39–52 were incubated for 7 min, and transfers 53–74 were incubated for 5 min. The arrows above certain transfers (0, 8, 14, 29, 37, 53, and 73) indicate where 0·001–0·1 ml of product was removed and used to prime reactions for sedimentation analysis on sucrose. The inset examines both infectious and total RNA. The results show that biologically competent RNA ceases to appear after the 4th transfer (Mills *et al.* 1967).

D.R.Mills, R,L,Peterson, S.Spiegelman, *An extracellular Darwinian experiment with a self-duplicating nucleic acid molecule*. Proc.Natl.Acad.Sci.USA **58** (1967), 217-224



The increase in RNA production rate during a serial transfer experiment

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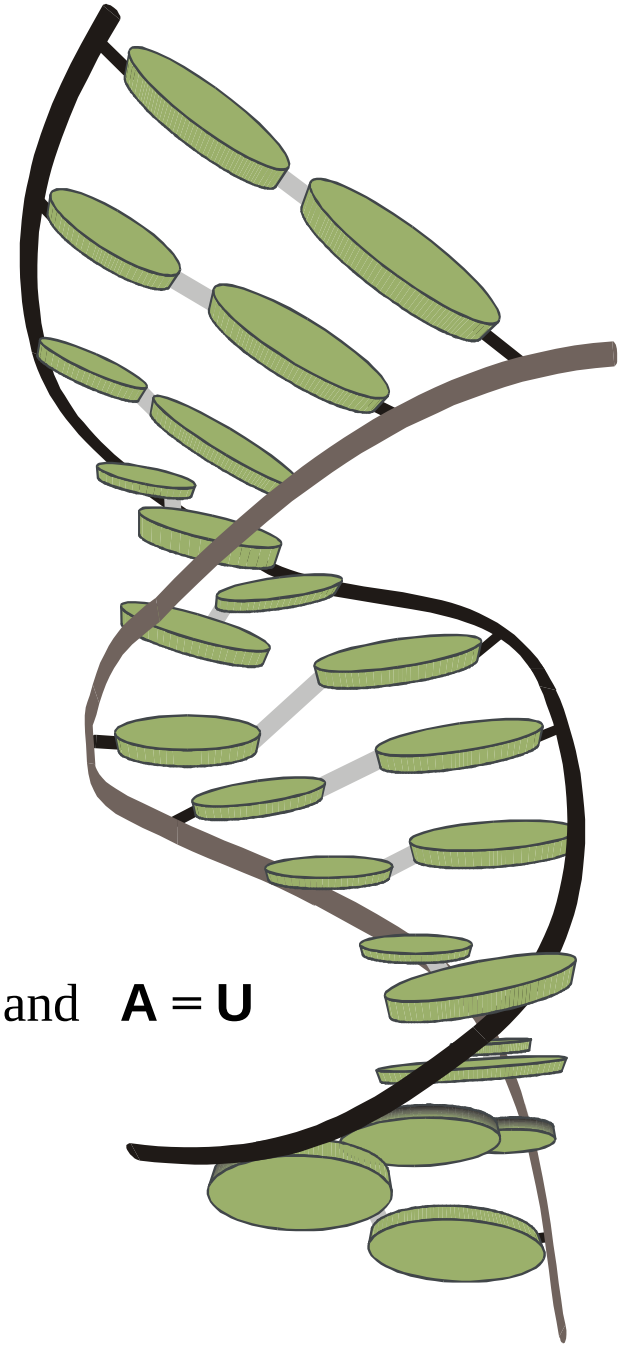
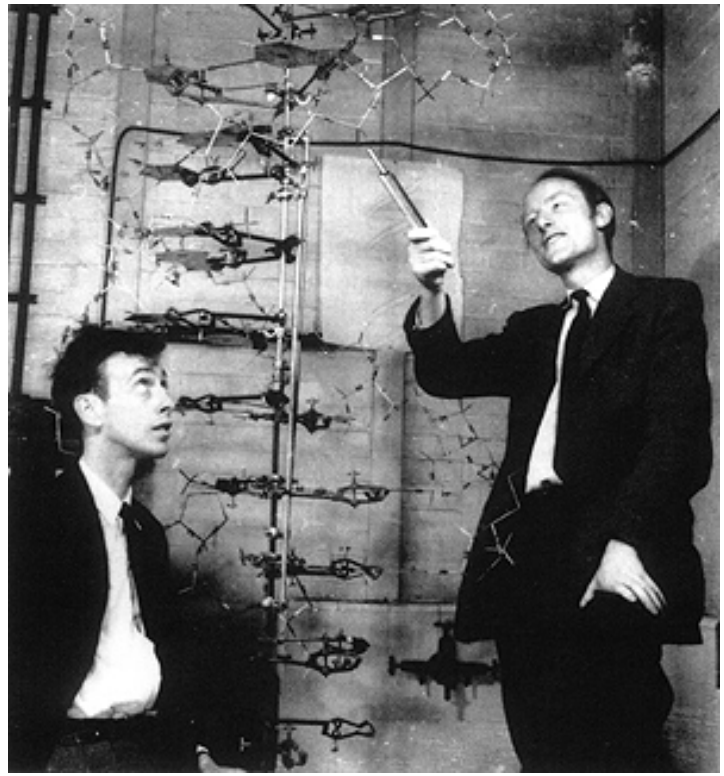
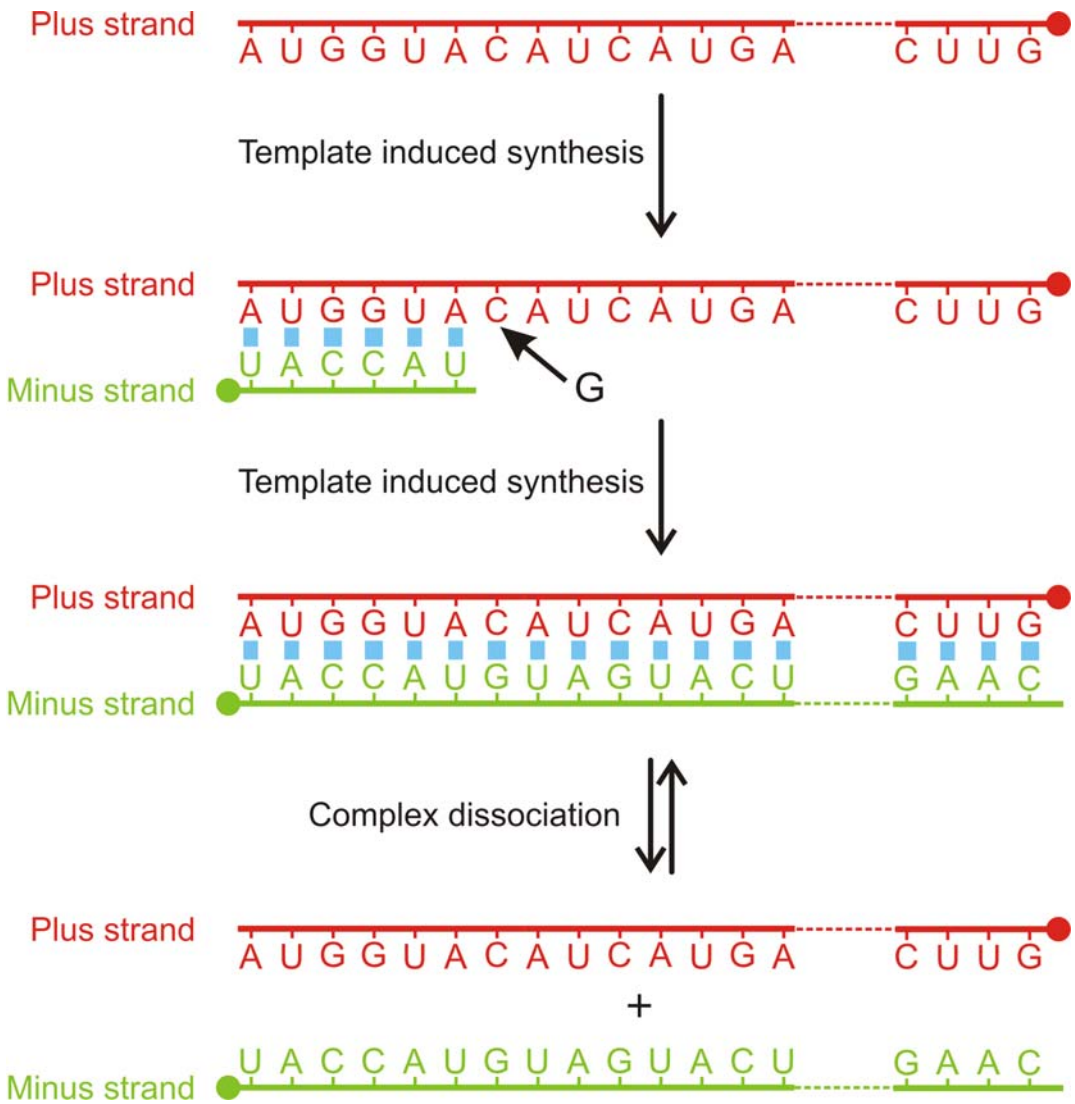


G ≡ C and **A = U**



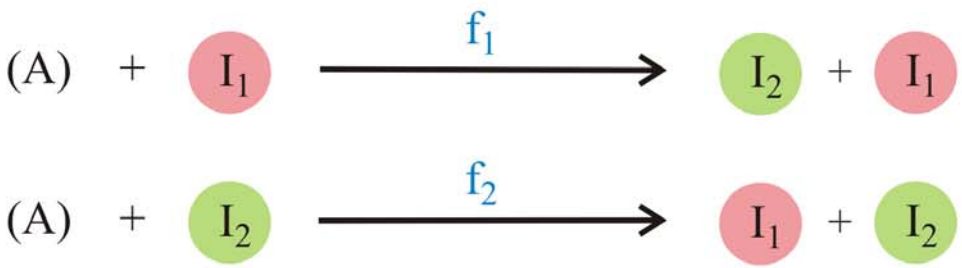
James D. Watson, 1928- , and Francis Crick, 1916-2004,
Nobel Prize 1962

The three-dimensional structure of a short double helical stack of B-DNA



Complementary replication is the simplest copying mechanism of RNA. Complementarity is determined by Watson-Crick base pairs:

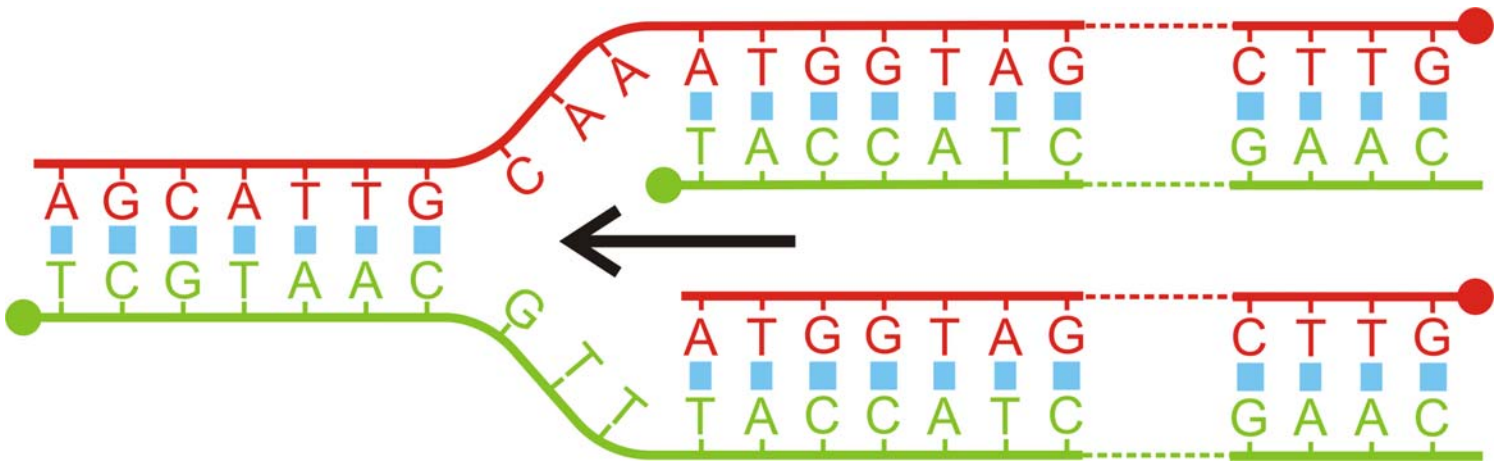
G≡C and **A=U**



$$dx_1 / dt = f_2 x_2 - x_1 \Phi$$
$$dx_2 / dt = f_1 x_1 - x_2 \Phi$$

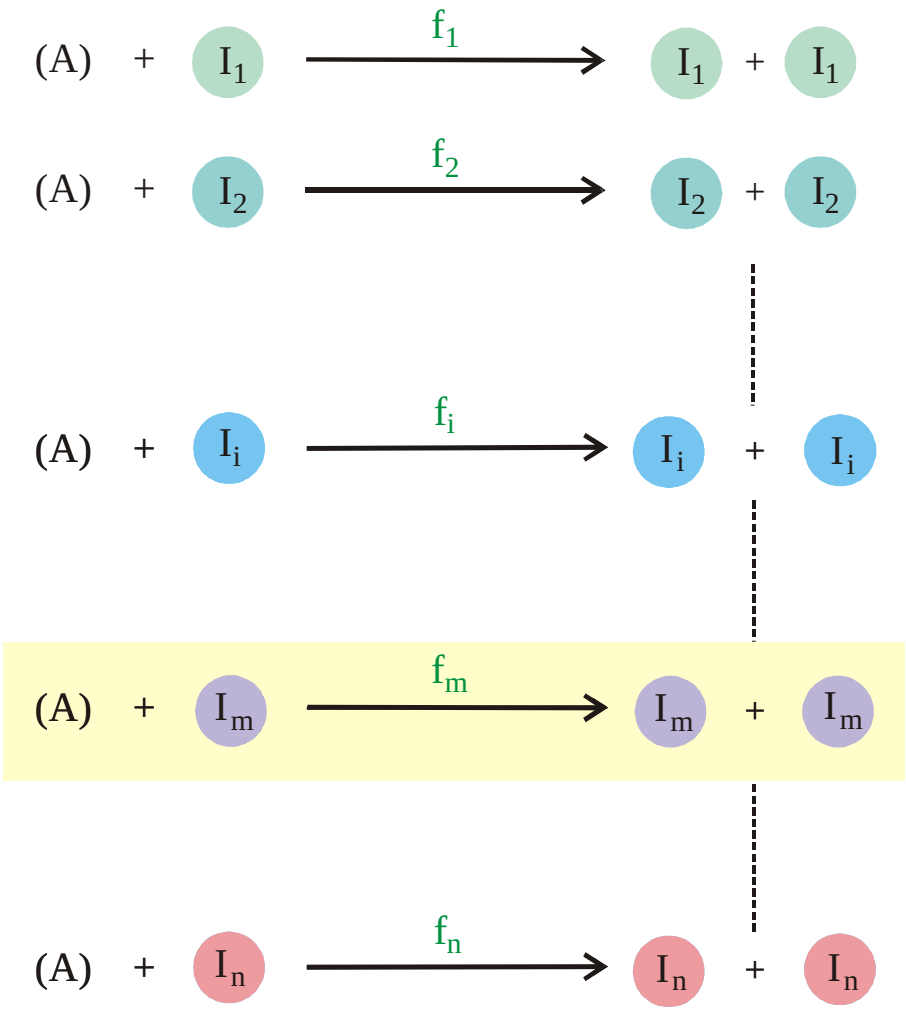
$$\Phi = \Sigma_i f_i x_i ; \quad \Sigma_i x_i = 1 ; \quad i = 1,2$$

Complementary replication as the simplest molecular mechanism of reproduction



‚Replication fork' in DNA replication

The mechanism of DNA replication is ‚semi-conservative'



$$dx_i / dt = f_i x_i - x_i \Phi = x_i (f_i - \Phi)$$

$$\Phi = \Sigma_j f_j x_j ; \quad \Sigma_j x_j = 1 ; \quad i,j = 1,2,...,n$$

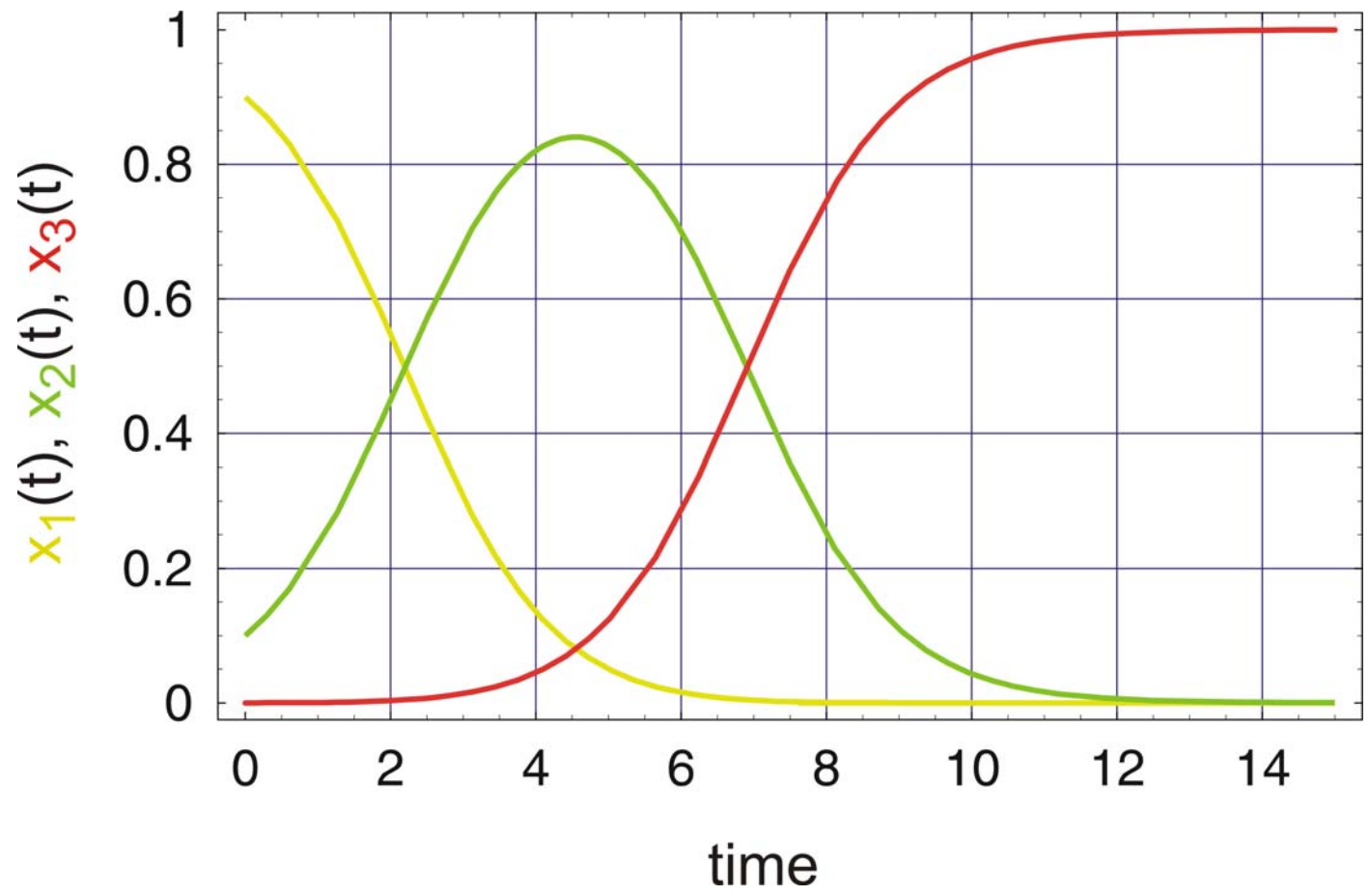
$$[I_i] = x_i \geq 0 ; \quad i = 1,2,...,n ;$$

$$[A] = a = \text{constant}$$

$$f_m = \max \{f_j; j=1,2,...,n\}$$

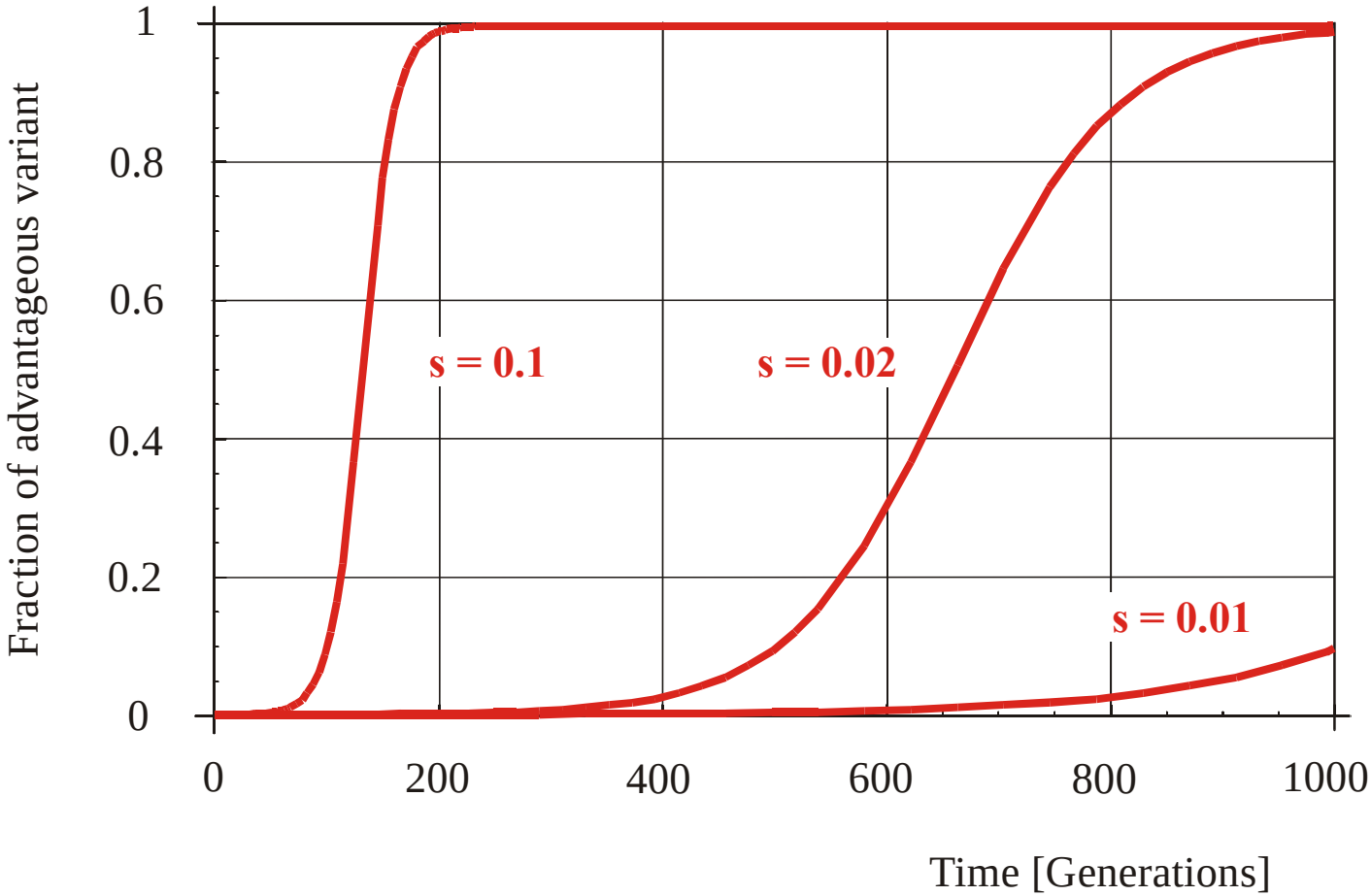
$$x_m(t) \rightarrow 1 \quad \text{for} \quad t \rightarrow \infty$$

Reproduction of organisms **or replication** of molecules as the basis of selection

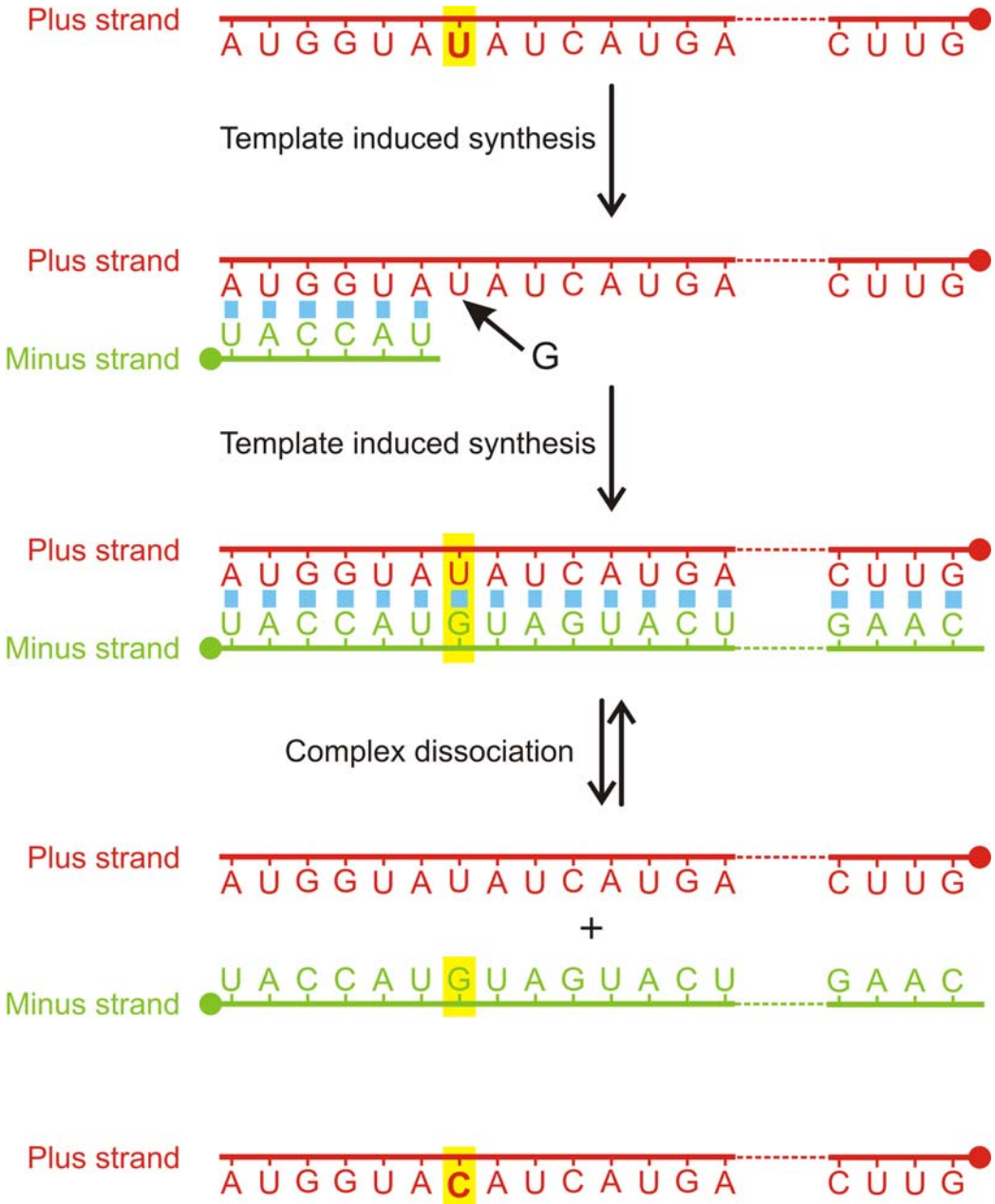


Selection between three species with $f_1 = 1, f_2 = 2$, and $f_3 = 3$

$s = (f_2 - f_1) / f_1;\ f_2 > f_1\ ;\ x_1(0) = 1 - 1/N\ ;\ x_2(0) = 1/N$



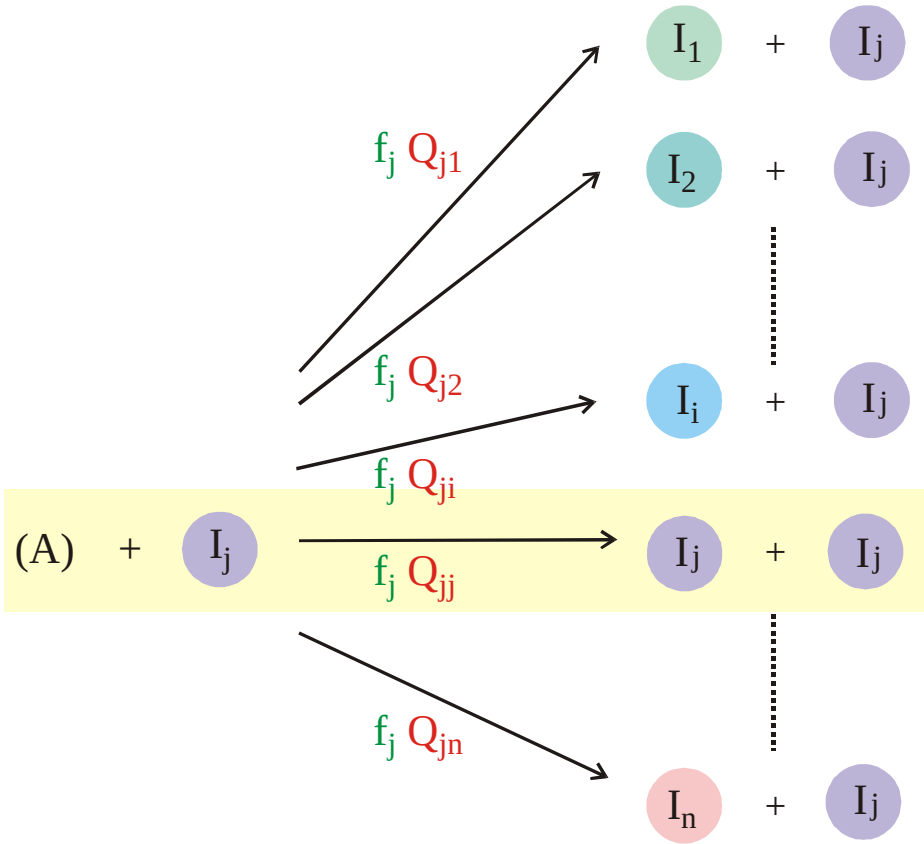
Selection of advantageous mutants in populations of N = 10 000 individuals



Point mutation is the most common error in RNA replication. Its mechanism is based on mispairing of nucleotides, here

U≡G instead of **U=A.**

The result is a replacement **A ⇒ G** in the minus strand and **U ⇒ C** in the plus strand.



$$dx_i / dt = \Sigma_j f_j Q_{ji} x_j - x_i \Phi$$

$$\Phi = \Sigma_j f_j x_i ; \quad \Sigma_j x_j = 1 ; \quad \sum_i Q_{ij} = 1$$

$$[I_i] = x_i \geq 0 ; \quad i = 1,2,...,n ;$$

$$[A] = a = \text{constant}$$

$$Q_{ij} = (1-p)^{\ell - d(i,j)} \, p^{d(i,j)}$$

p ..... Error rate per digit

ℓ ..... Chain length of the polynucleotide

d(i,j) .... Hamming distance between I_i and I_j

Chemical kinetics of replication and mutation as parallel reactions

Mutation-selection equation: $[\mathrm{I}_i] = x_i \geq 0,\ f_i > 0,\ Q_{ij} \geq 0$

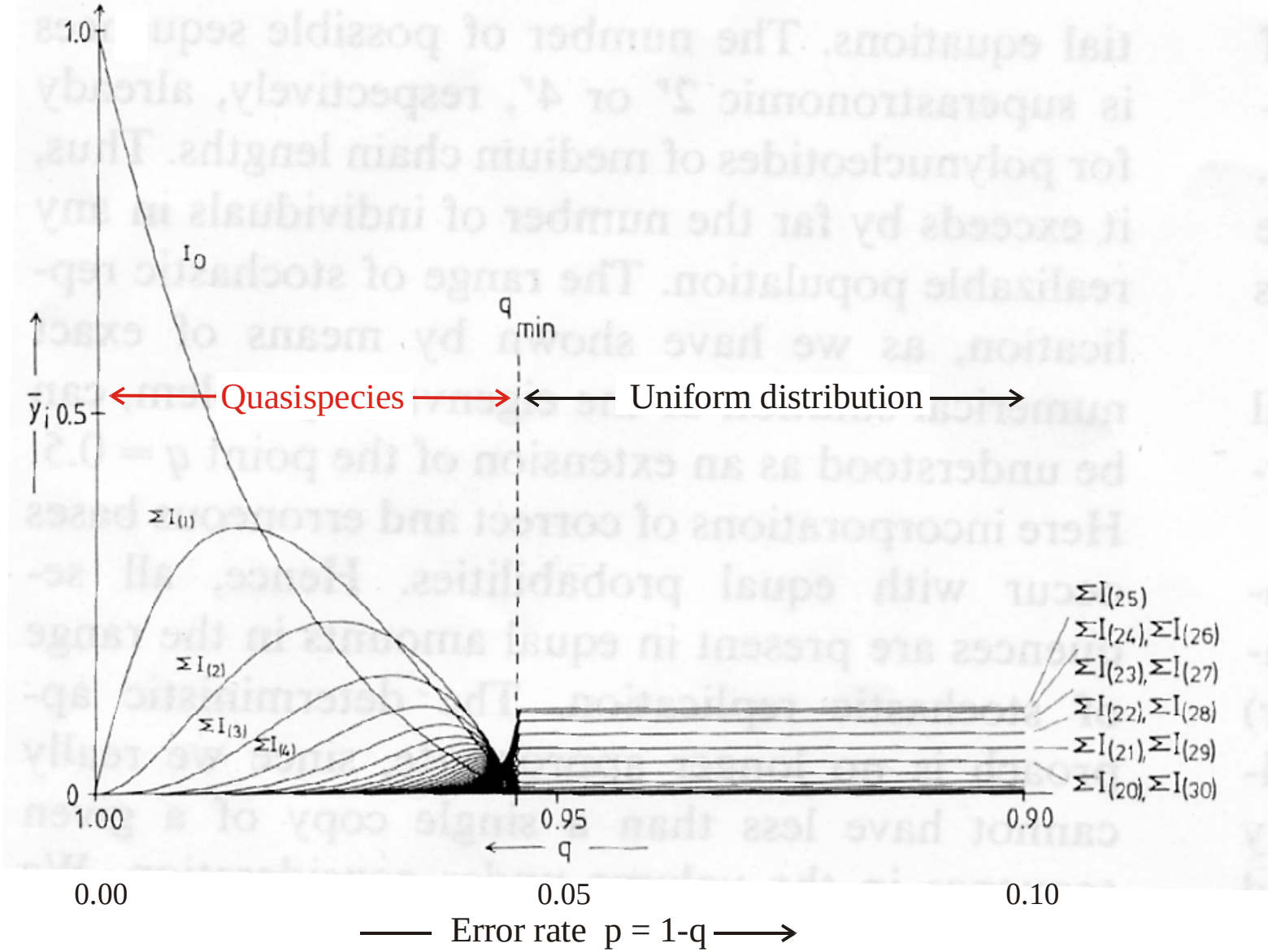
$$\frac{dx_i}{dt} = \sum_{j=1}^{n} f_j Q_{ji}\, x_j - x_i\, \phi, \quad i = 1,2,\cdots,n; \quad \sum_{i=1}^{n} x_i = 1; \quad \phi = \sum_{j=1}^{n} f_j x_j = \overline{f}$$

Solutions are obtained after integrating factor transformation by means of an eigenvalue problem

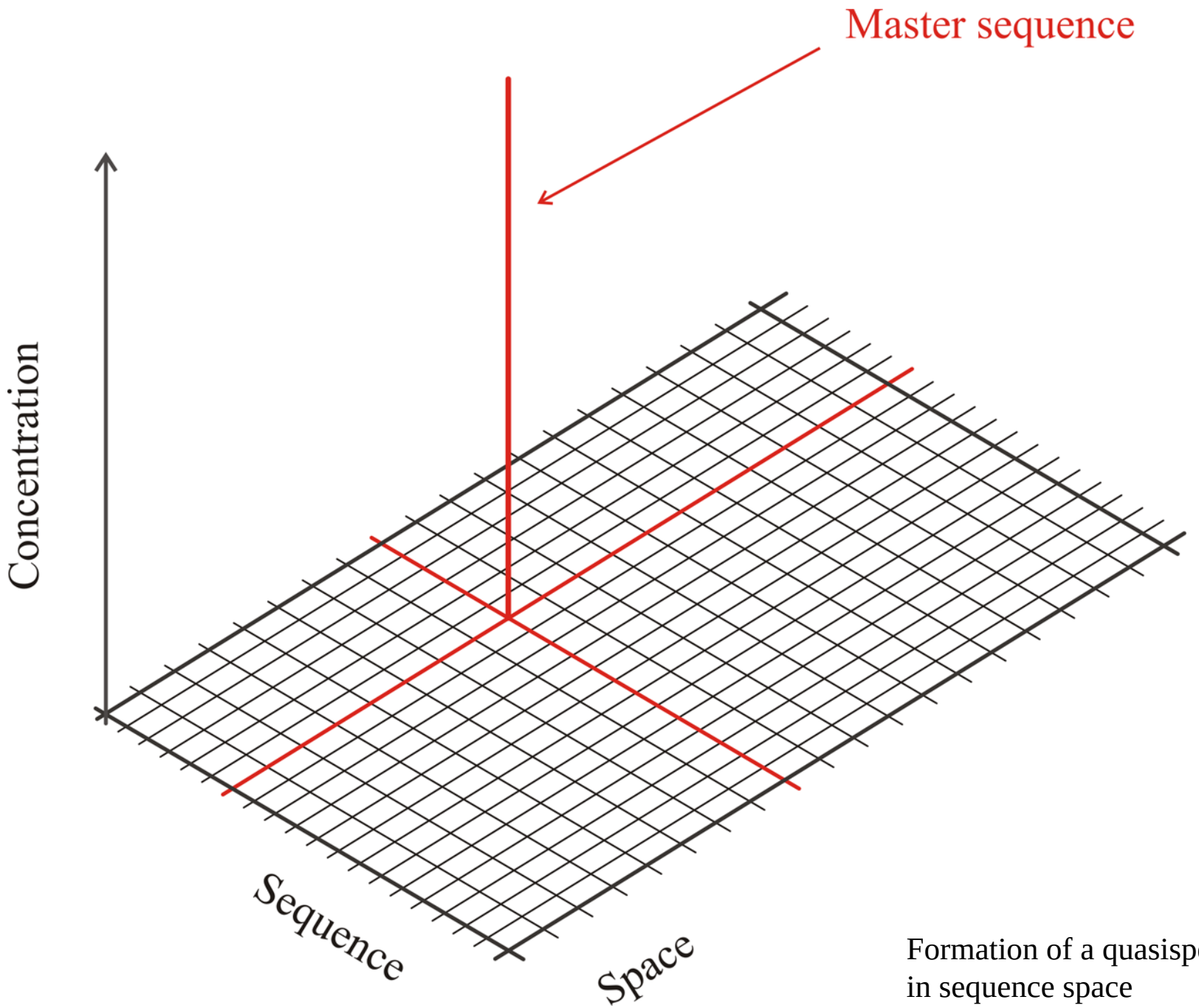
$$x_i(t) = \frac{\sum_{k=0}^{n-1} \ell_{ik} \cdot c_k(0) \cdot \exp(\lambda_k t)}{\sum_{j=1}^{n} \sum_{k=0}^{n-1} \ell_{jk} \cdot c_k(0) \cdot \exp(\lambda_k t)}; \quad i = 1,2,\cdots,n; \quad c_k(0) = \sum_{i=1}^{n} h_{ki}\, x_i(0)$$

$$W \div \left\{ f_i Q_{ij};\ i, j = 1,2,\cdots,n \right\};\ L = \left\{ \ell_{ij};\ i, j = 1,2,\cdots,n \right\};\ L^{-1} = H = \left\{ h_{ij};\ i, j = 1,2,\cdots,n \right\}$$

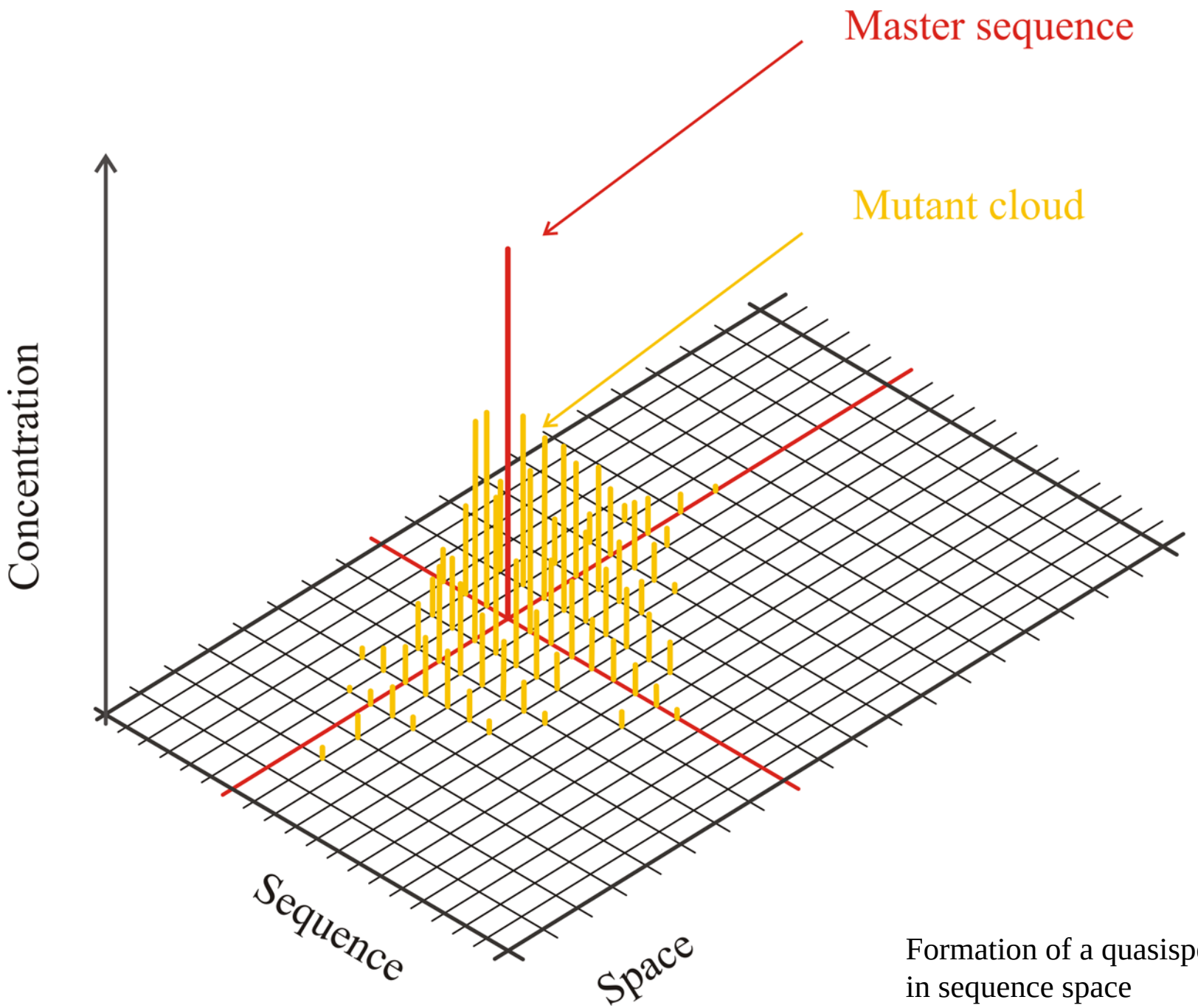
$$L^{-1} \cdot W \cdot L \quad = \quad \Lambda \quad = \quad \left\{ \lambda_k; k = 0,1,\cdots,n-1 \right\}$$



Stationary mutant distribution – called „quasispecies“ – as a function of the error rate p



Formation of a quasispecies in sequence space



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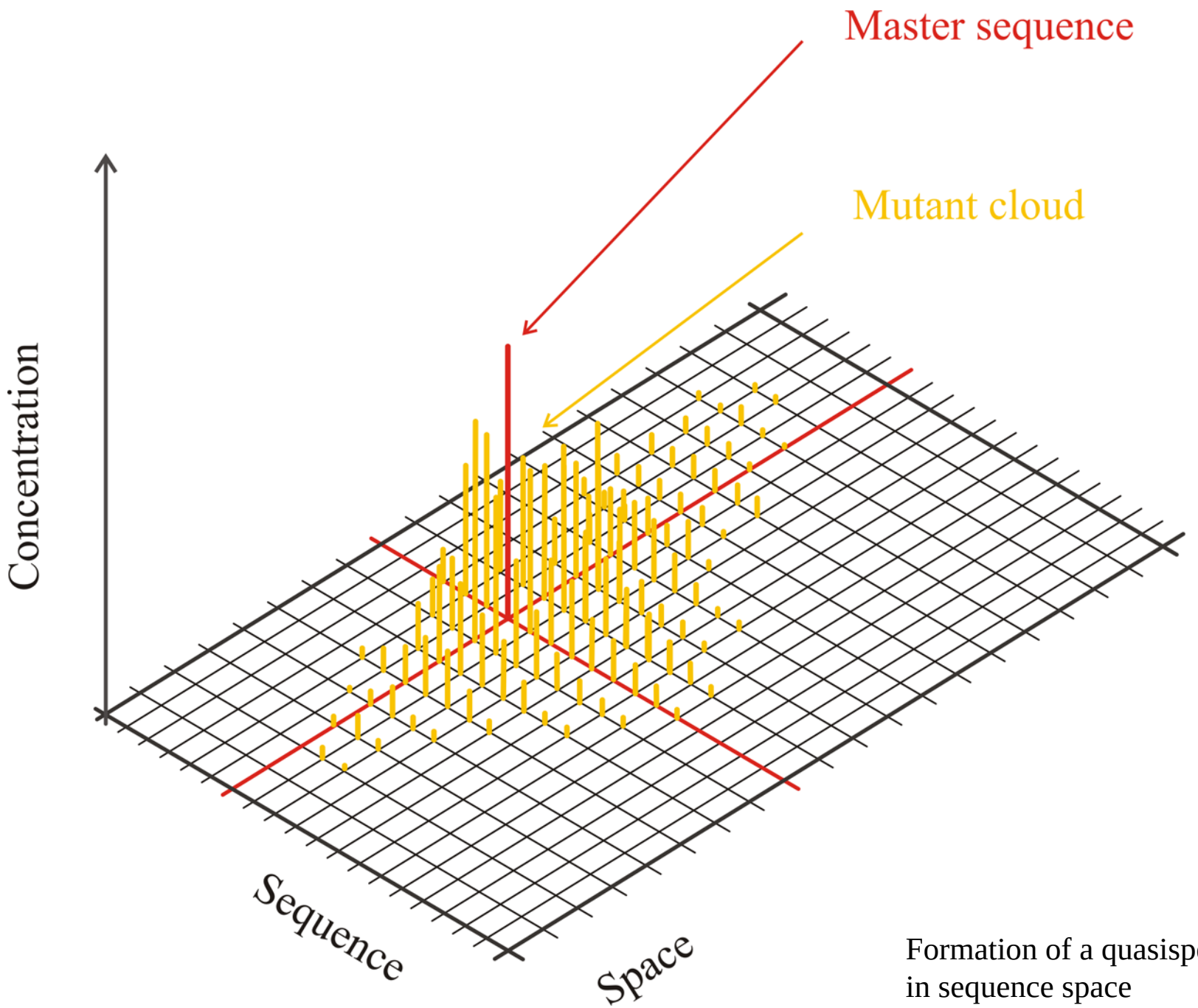

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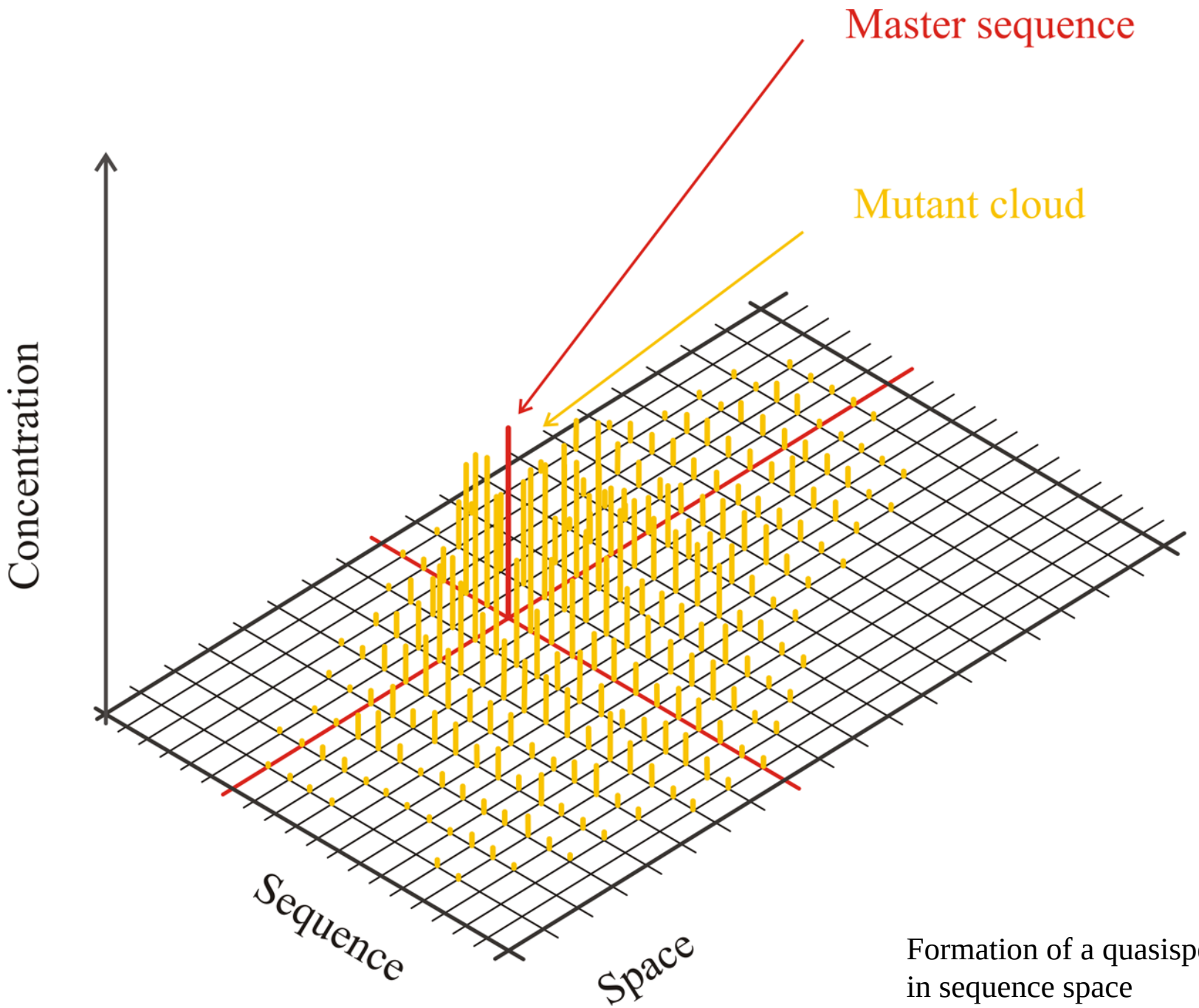

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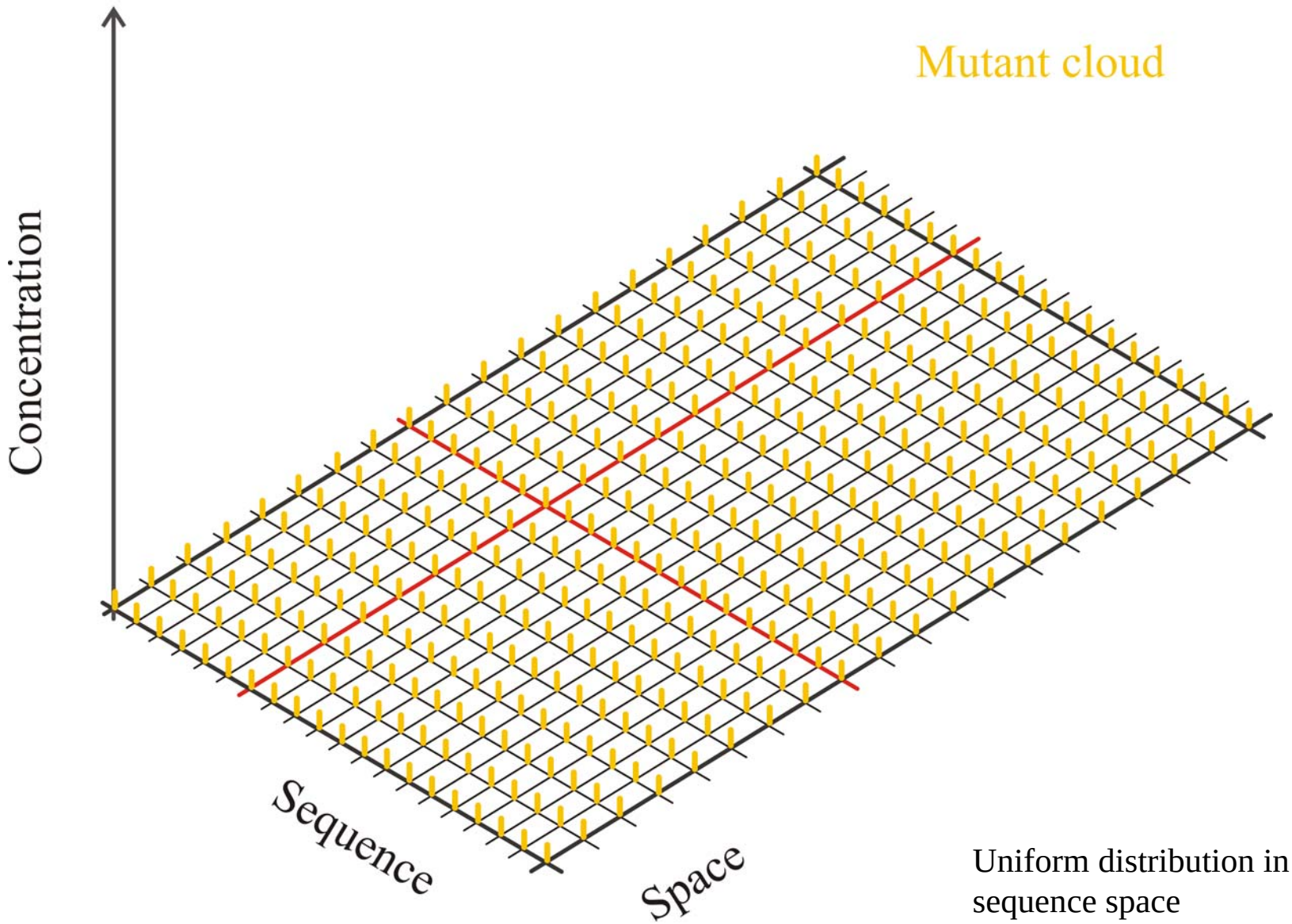

Uniform distribution in sequence space

Chain length and error threshold

$$Q \cdot \sigma \;=\; (1-p)^n \cdot \sigma \;\geq\; 1 \;\Rightarrow\; n \cdot \ln(1-p) \geq -\ln\sigma$$

p … constant : $n_{\max} \approx \dfrac{\ln\sigma}{p}$

n … constant : $p_{\max} \approx \dfrac{\ln\sigma}{n}$

$Q = (1-p)^n$ … replication accuracy

p … error rate

n … chain length

$\sigma = \dfrac{f_m}{\sum_{j \neq m} f_j}$ … superiority of master sequence

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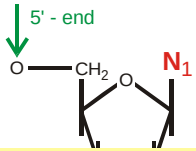
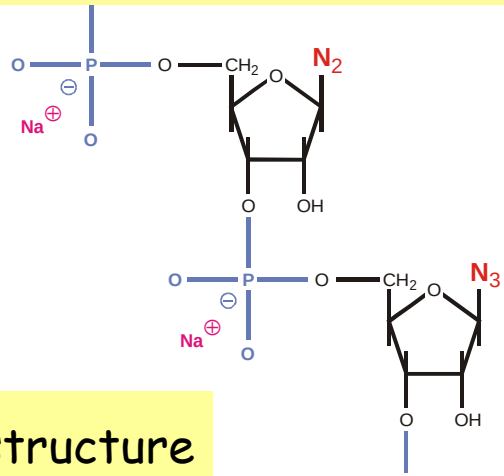
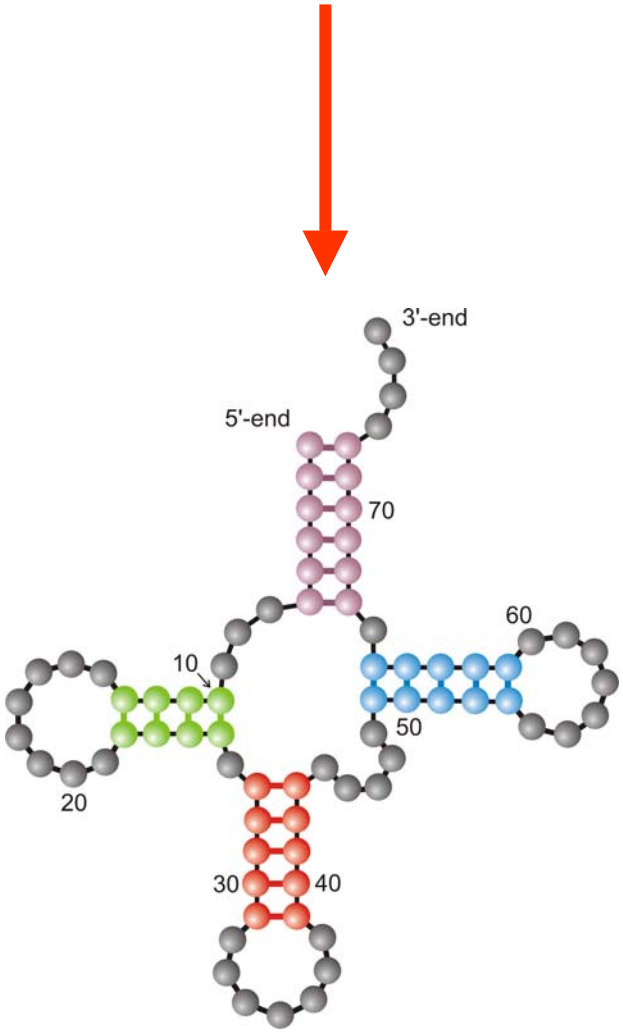
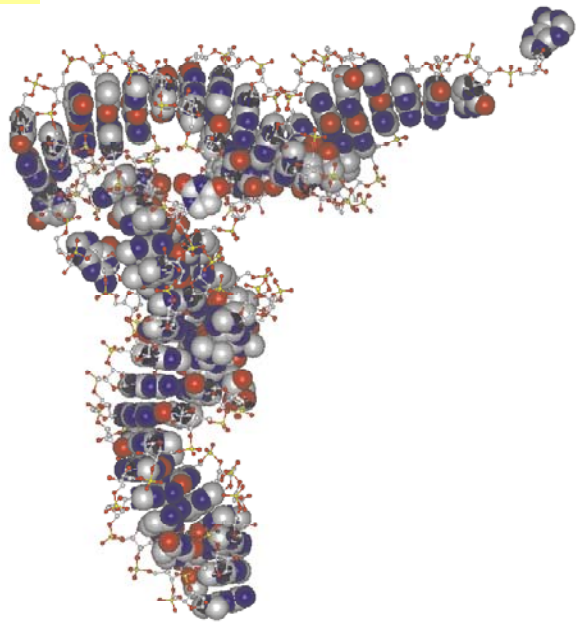


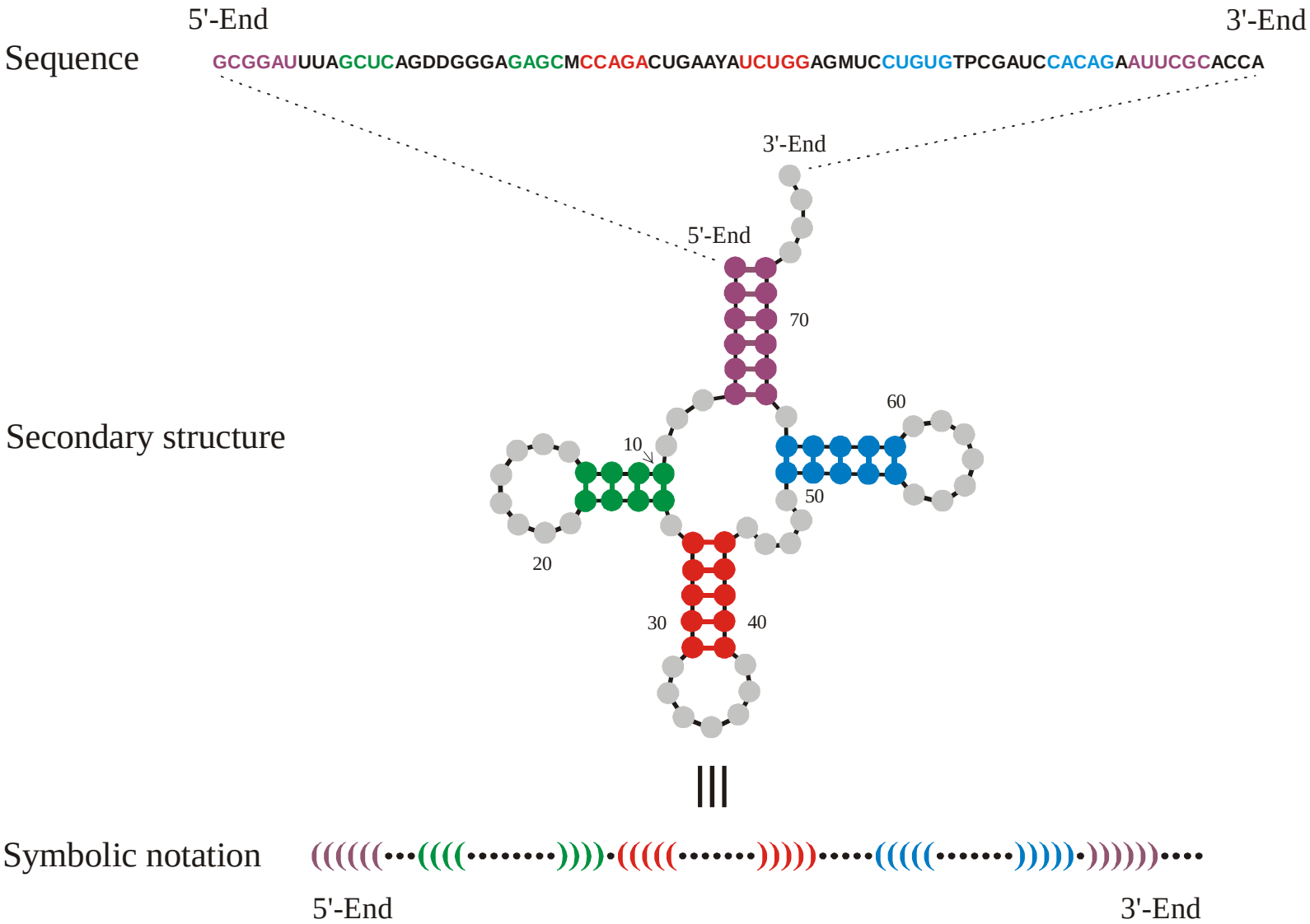
5'-end GCGGAUUUAGCUCAGUUGGGAGAGCGCCAGACUGAAGAUCUGGAGGUCCUGUGUUCGAUCCACAGAAUUCGCACCA 3'-end





Definition of RNA structure





A symbolic notation of RNA secondary structure that is equivalent to the conventional graphs

RNA sequence GUAUCGAAAUACGUAGCGUAUGGGGAUGCUGGACGGUCCCAUCGGUACUCCA

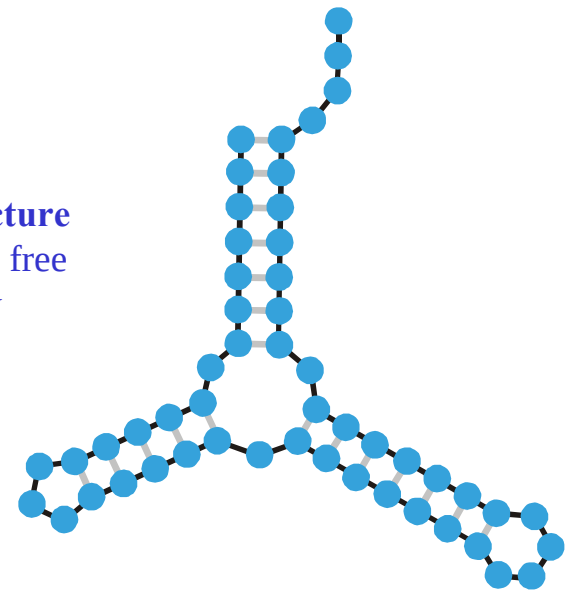
RNA folding:

Structural biology, spectroscopy of biomolecules, understanding **molecular function**

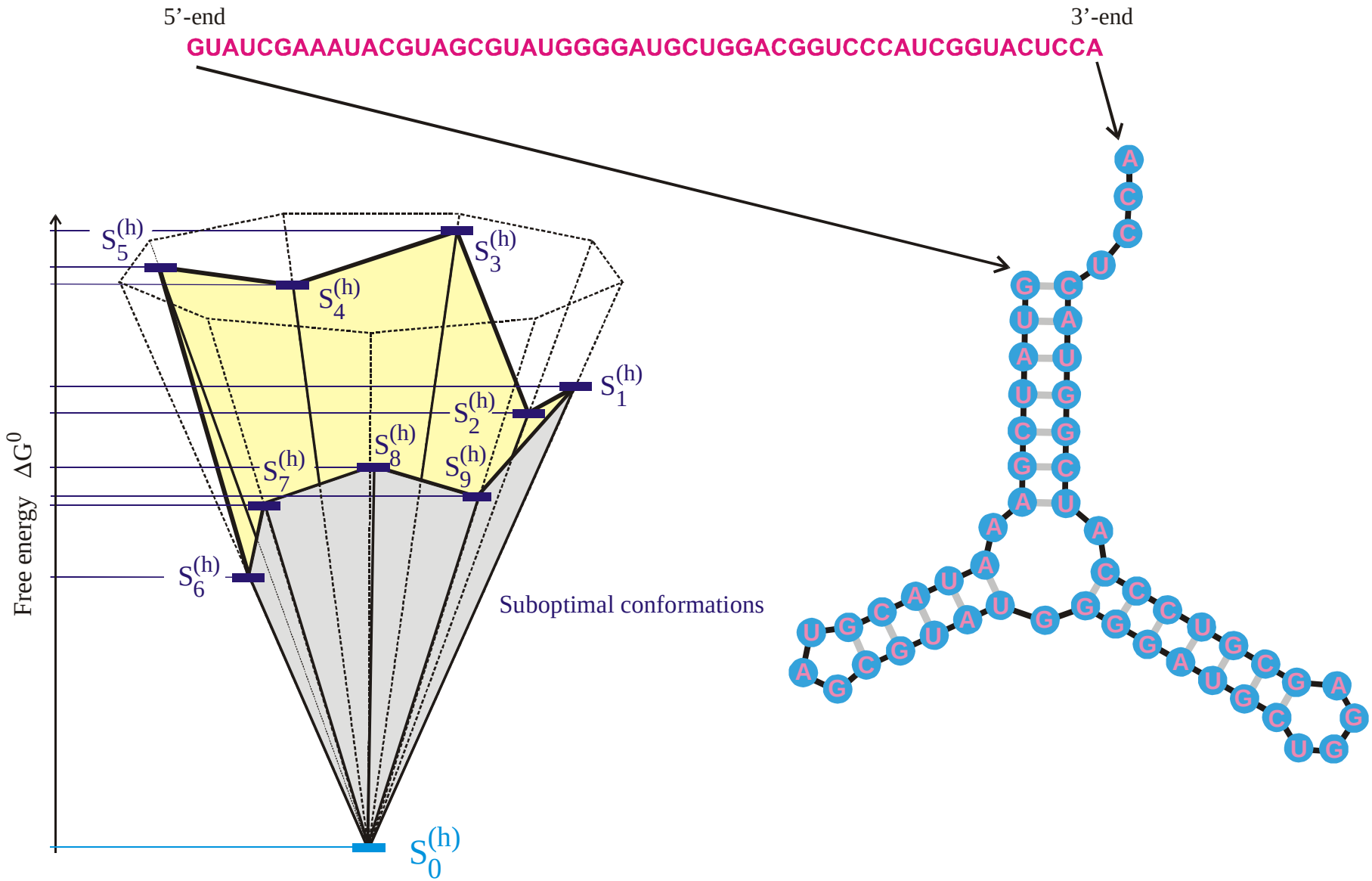
Biophysical chemistry: thermodynamics and kinetics

Empirical parameters

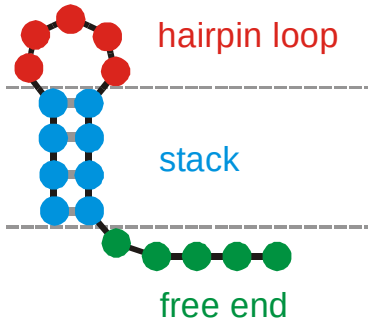
RNA structure of minimal free energy

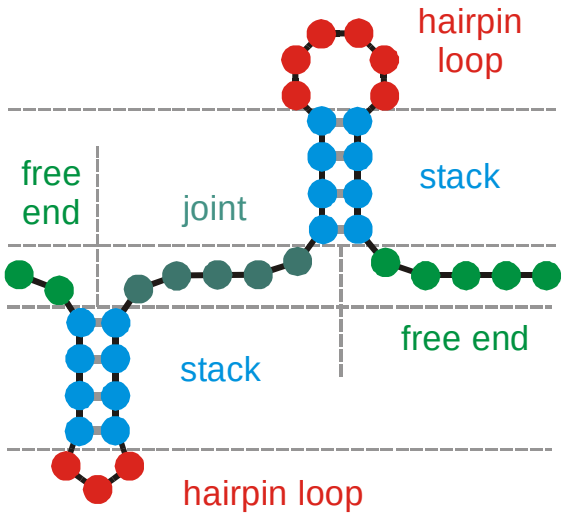


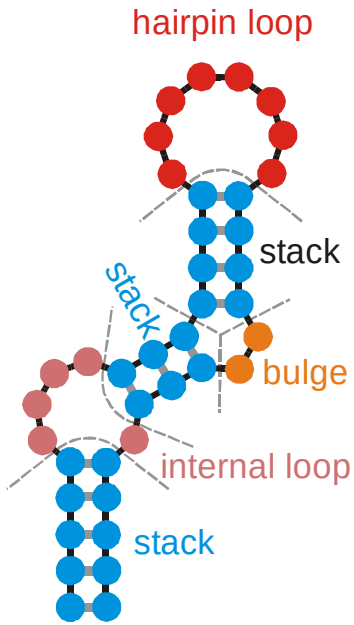
Sequence, structure, and design



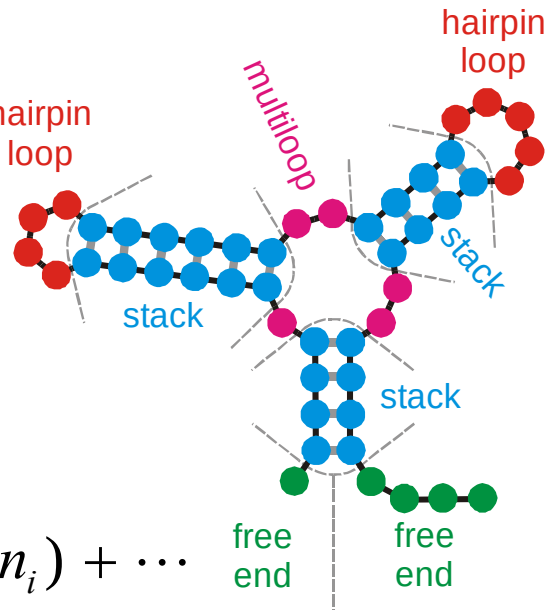
The minimum free energy structures on a discrete space of conformations







Elements of RNA secondary structures as used in free energy calculations



$$\Delta G_0^{300} = \underbrace{\sum g_{ij,kl}}_{\substack{\text{stacks of} \\ \text{base pairs}}} + \underbrace{\sum h(n_l)}_{\substack{\text{hairpin} \\ \text{loops}}} + \underbrace{\sum b(n_b)}_{\text{bulges}} + \underbrace{\sum i(n_i)}_{\substack{\text{internal} \\ \text{loops}}} + \cdots$$

RNA sequence GUAUCGAAAUACGUAGCGUAUGGGGAUGCUGGACGGUCCCAUCGGUACUCCA

RNA folding:

Structural biology, spectroscopy of biomolecules, understanding **molecular function**

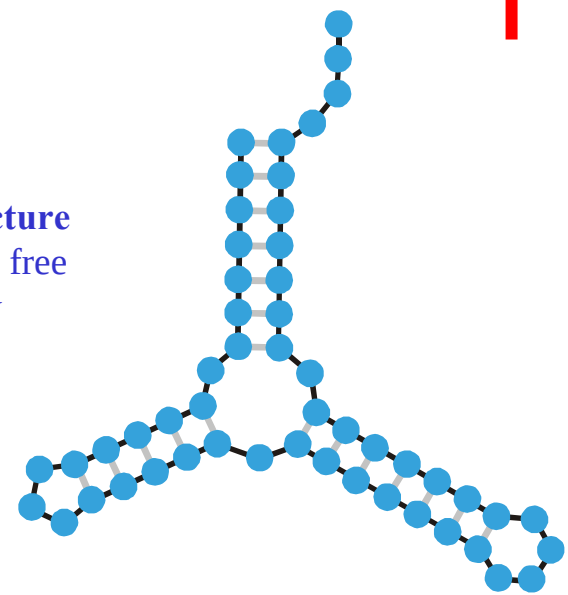
Iterative determination of a sequence for the given secondary structure

Inverse Folding Algorithm

Inverse folding of RNA:

Biotechnology, **design of biomolecules** with predefined structures and functions

RNA structure of minimal free energy



Sequence, structure, and design

Inverse folding algorithm

$\mathbf{I_0} \rightarrow I_1 \rightarrow I_2 \rightarrow I_3 \rightarrow I_4 \rightarrow \ldots \rightarrow I_k \rightarrow I_{k+1} \rightarrow \ldots \rightarrow \mathbf{I_t}$

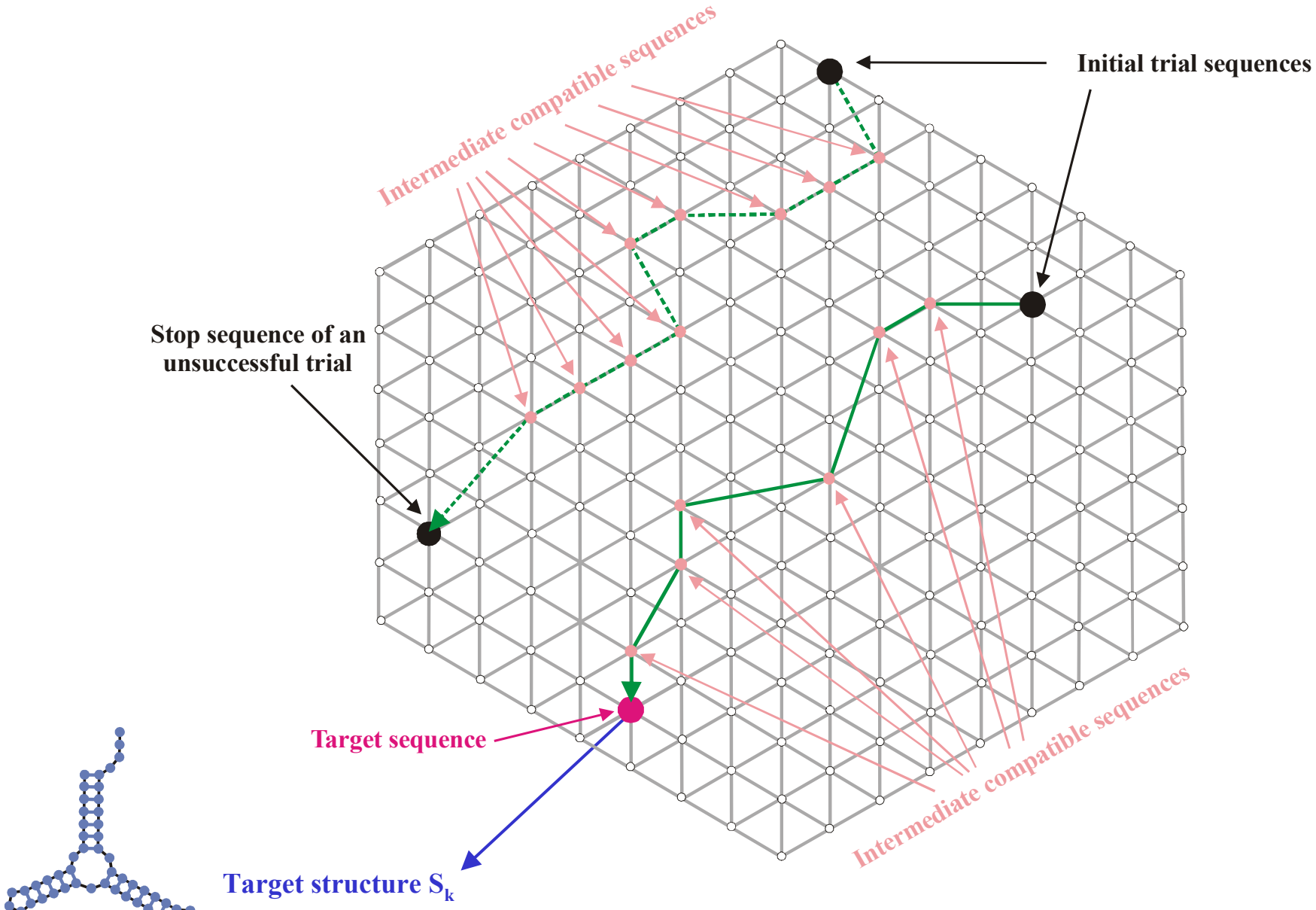
$\mathbf{S_0} \rightarrow S_1 \rightarrow S_2 \rightarrow S_3 \rightarrow S_4 \rightarrow \ldots \rightarrow S_k \rightarrow S_{k+1} \rightarrow \ldots \rightarrow \mathbf{S_t}$

$I_{k+1} = \mathfrak{M}_k(I_k)$ and $\Delta d_S(S_k,S_{k+1}) = d_S(S_{k+1},S_t) - d_S(S_k,S_t) < 0$

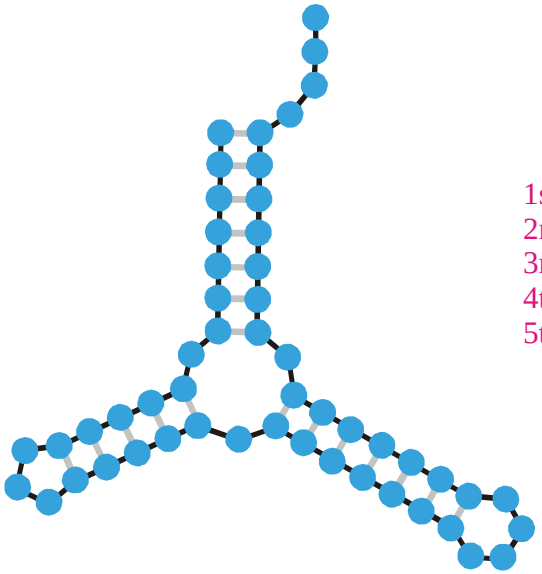
$\mathfrak{M}$ ... base or base pair mutation operator

$d_S\,(S_i,S_j)$ ... distance between the two structures S_i and S_j

‚Unsuccessful trial' ... termination after n steps



Approach to the **target structure S_k** in the inverse folding algorithm



Minimum free energy criterion

1st
2nd
3rd trial
4th
5th

GUAUCGAAAUACGUAGCGUAUGGGGAUGCUGGACGGUCCCAUCGGUACUCCA

UGGUUACGCGUUGGGGUAACGAAGAUUCCGAGAGGAGUUUAGUGACUAGAGG

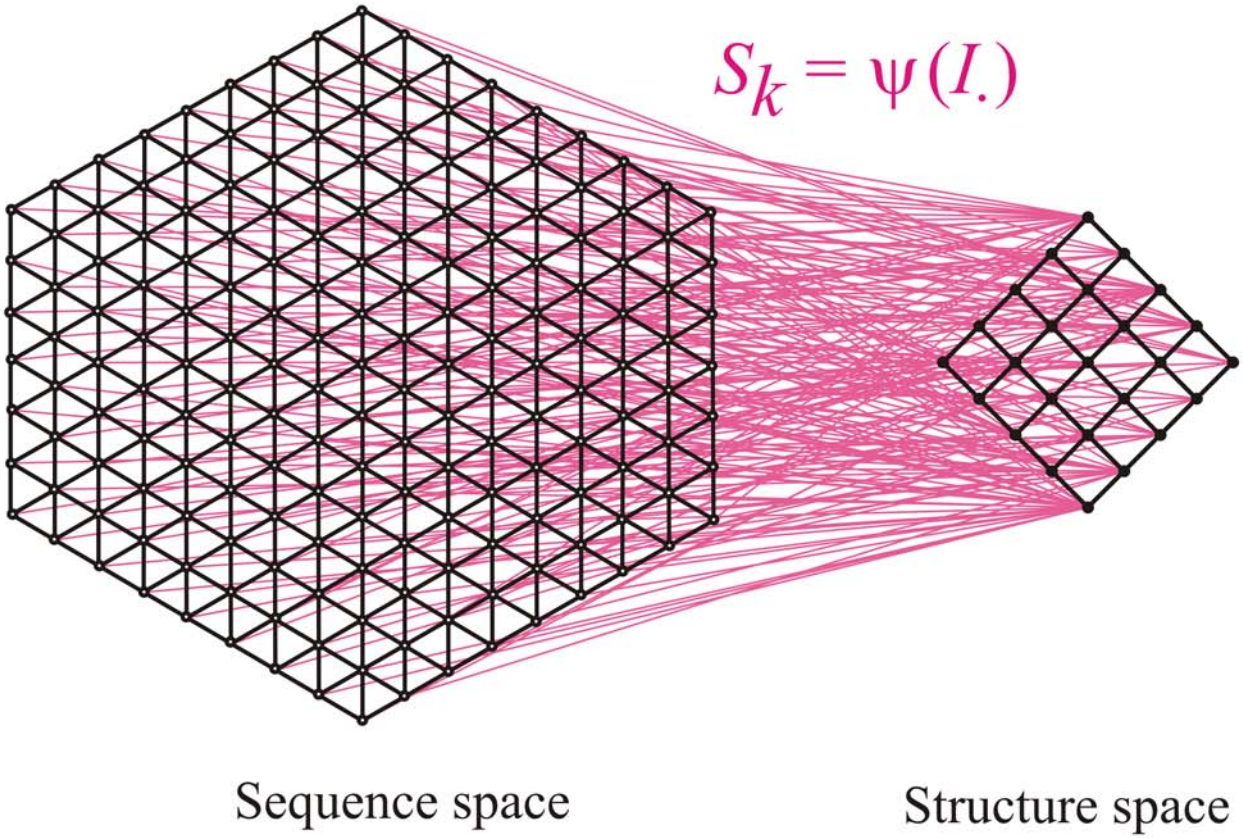
CUUCUUGAGCUAGUACCUAGUCGGAUAGGAUUUCCUAUCUCCAGGGAGGAUG

CUUUUCUUCACGUUAGAUGUGUAAUGGACAUGUGUUUAUUUAGGAAAGGCGC

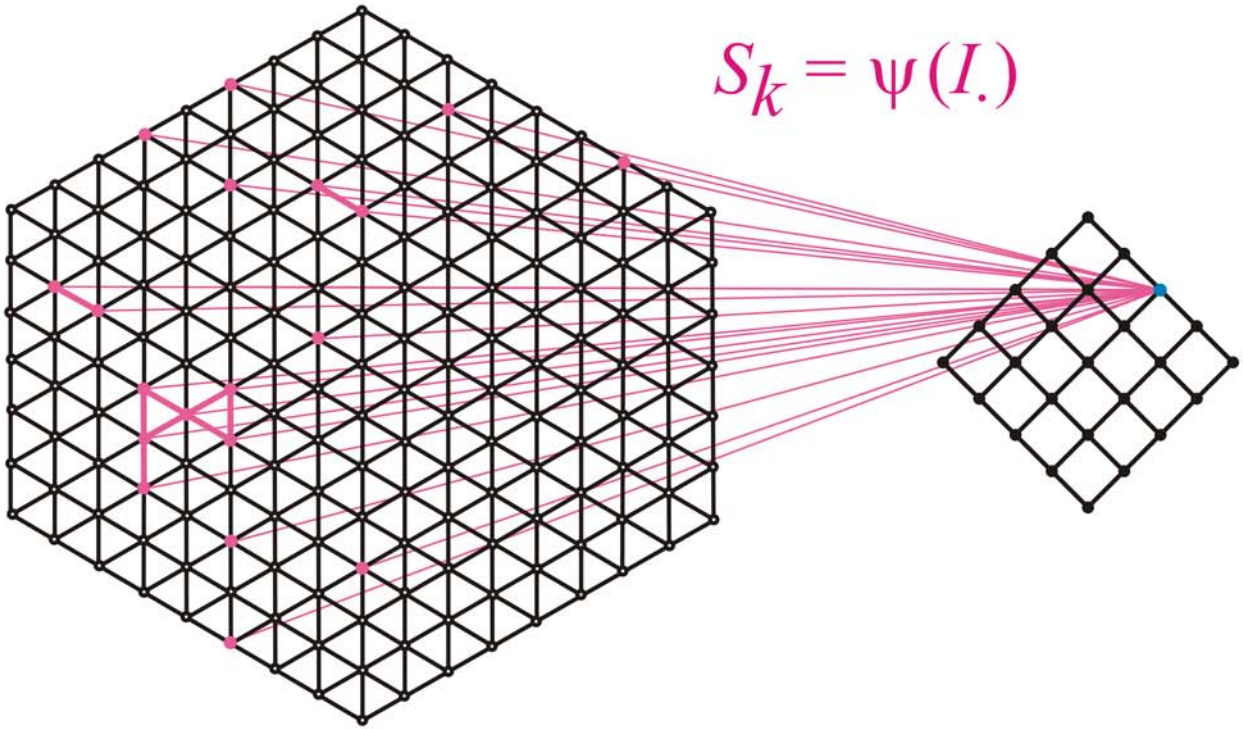
AUAACGUGAGUGUCUAAUACUGAUCGCUCCGGAGGGUGGUGGCGUUGUUAAU

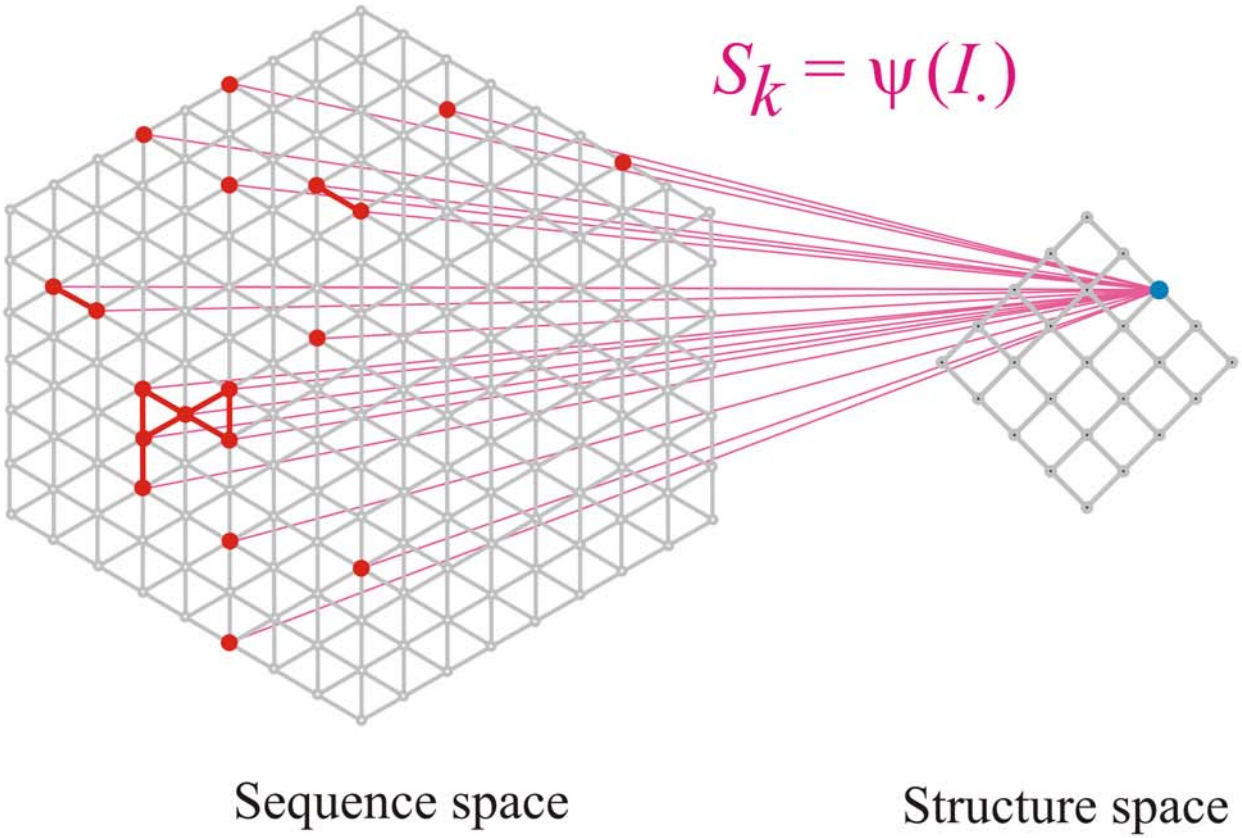
Inverse folding of RNA secondary structures

The inverse folding algorithm searches for sequences that form a given RNA secondary structure under the minimum free energy criterion.



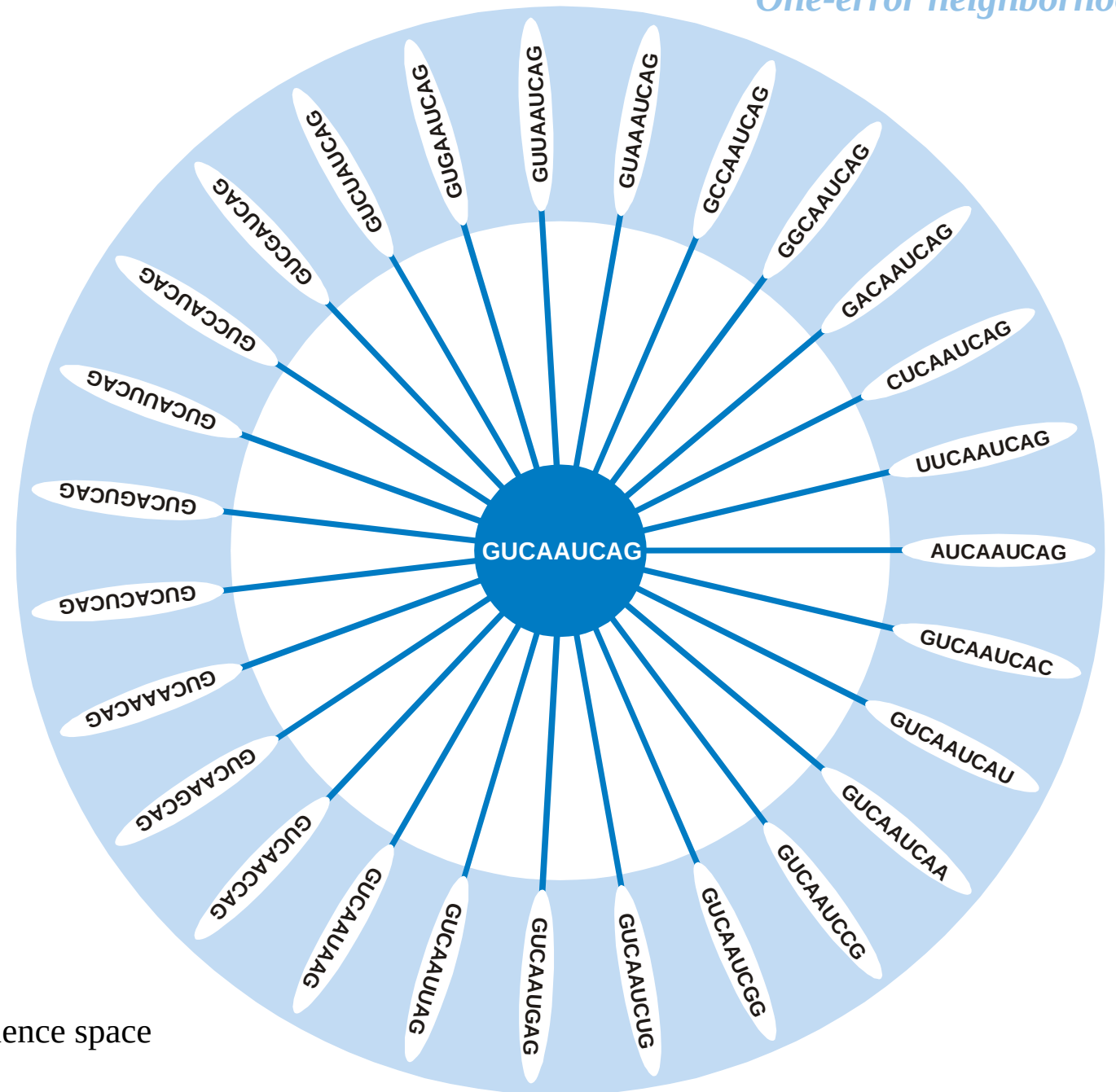
Mapping from sequence space into structure space


$S_k = \psi(I_.)$
Sequence space
Structure space

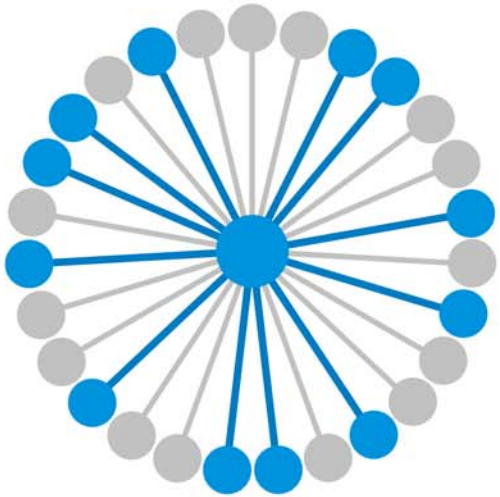


The pre-image of the structure S_k in sequence space is the **neutral network G_k**

One-error neighborhood



The surrounding of
GUCAAUCAG in sequence space



$$G_k = \psi^{-1}(S_k) \doteq \{ I_j \mid \psi(I_j) = S_k \}$$

$$\bar{\lambda}_k = \frac{\sum_{j \in |G_k|} \lambda_j(k)}{|G_k|}$$

$\lambda_j = 12 / 27 = 0.444$

Alphabet size κ :

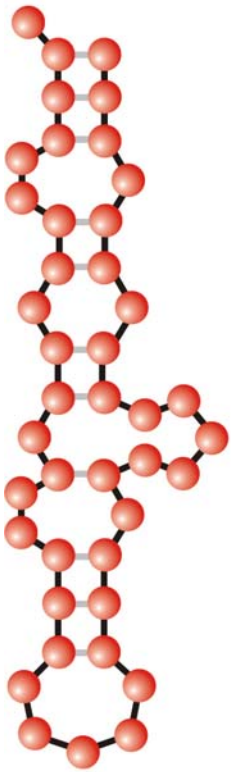
κ	λ_{cr}	
2	0.5	**AU,GC,DU**
3	0.423	**AUG , UGC**
4	0.370	**AUGC**

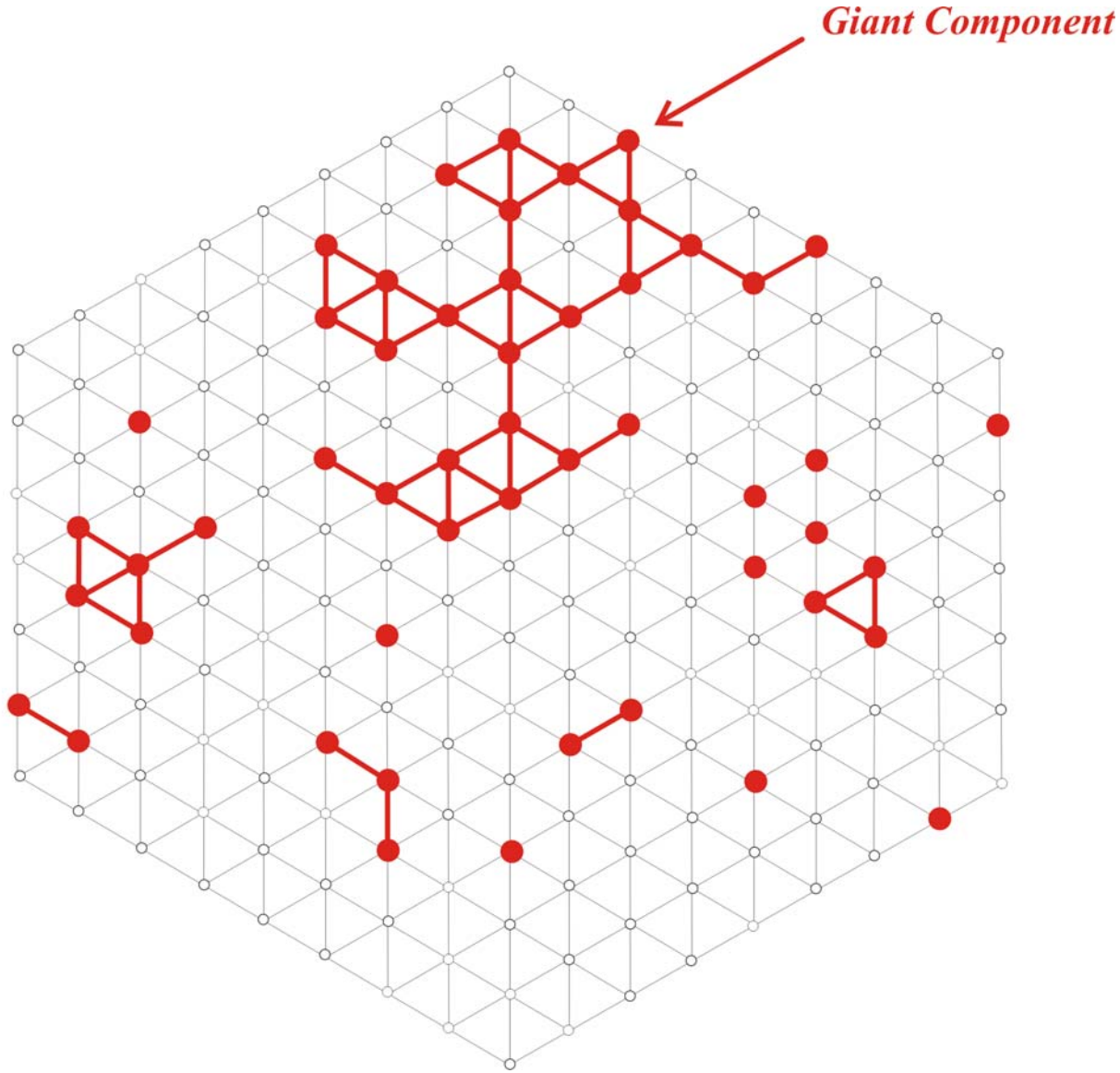
$\bar{\lambda}_k > \lambda_{cr}$ .... network G_k is connected

$\bar{\lambda}_k < \lambda_{cr}$ .... network G_k is **not** connected

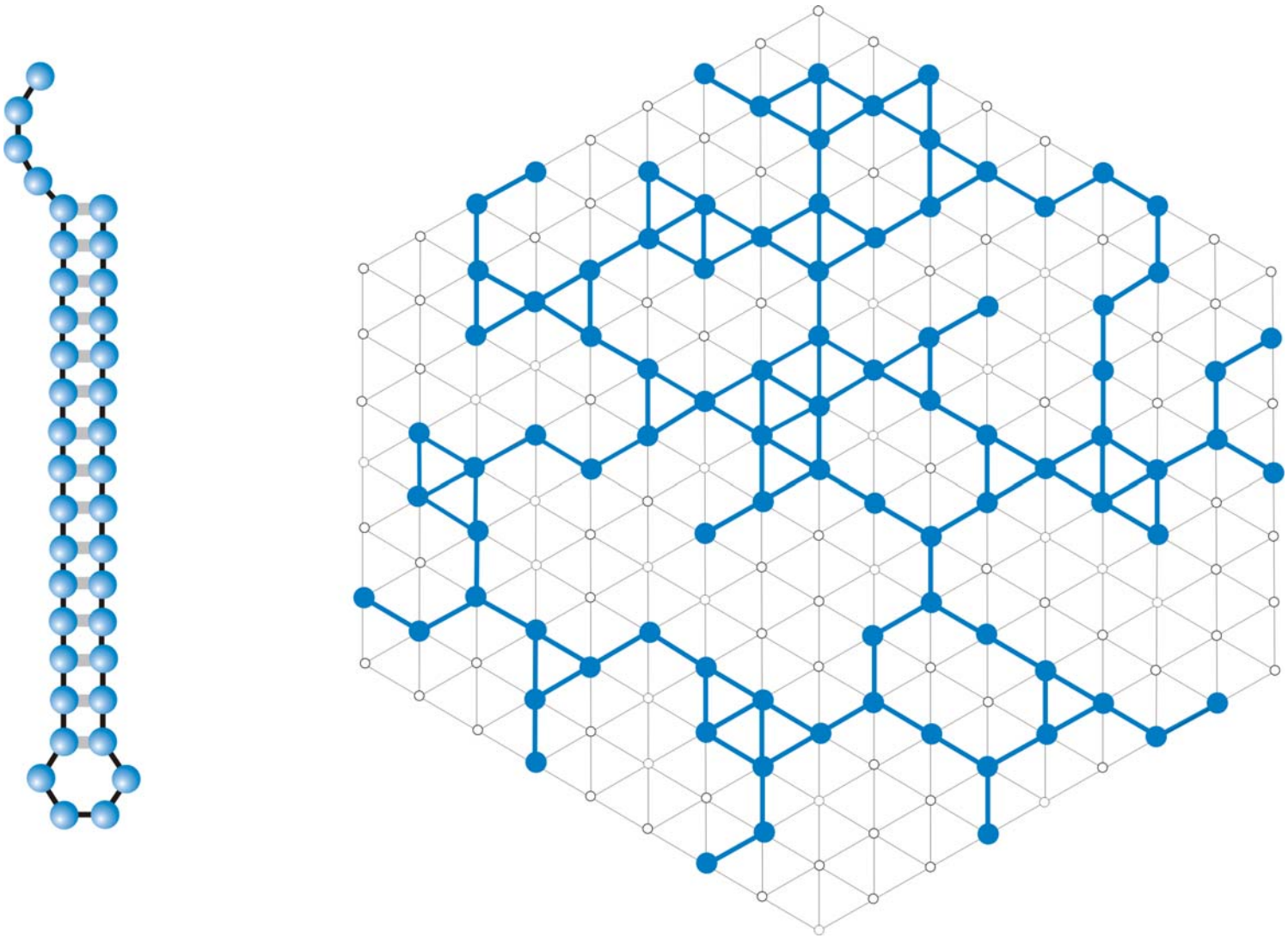
Connectivity threshold: $\lambda_{cr} = 1 - \kappa^{-1/(\kappa-1)}$

Degree of neutrality of neutral networks and the connectivity threshold





A multi-component neutral network formed by a rare structure: $\lambda < \lambda_{cr}$



A connected neutral network formed by a common structure: $\lambda > \lambda_{cr}$

1. Darwinian evolution in laboratory experiments
2. Modeling the evolution of molecules
3. From RNA sequences to structures and back
4. **Evolution on neutral networks**
5. Origins of complexity

Genotype = Genome

Mutation → GGCUAUCGUACGUUUACCCAAAAAGUCUACGUUGGACCCAGGCAUUGGAC.....G

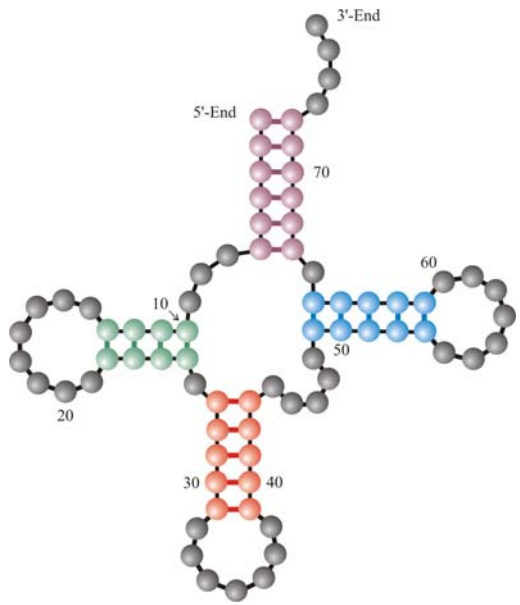
Unfolding of the genotype:

RNA structure formation

Fitness in reproduction:

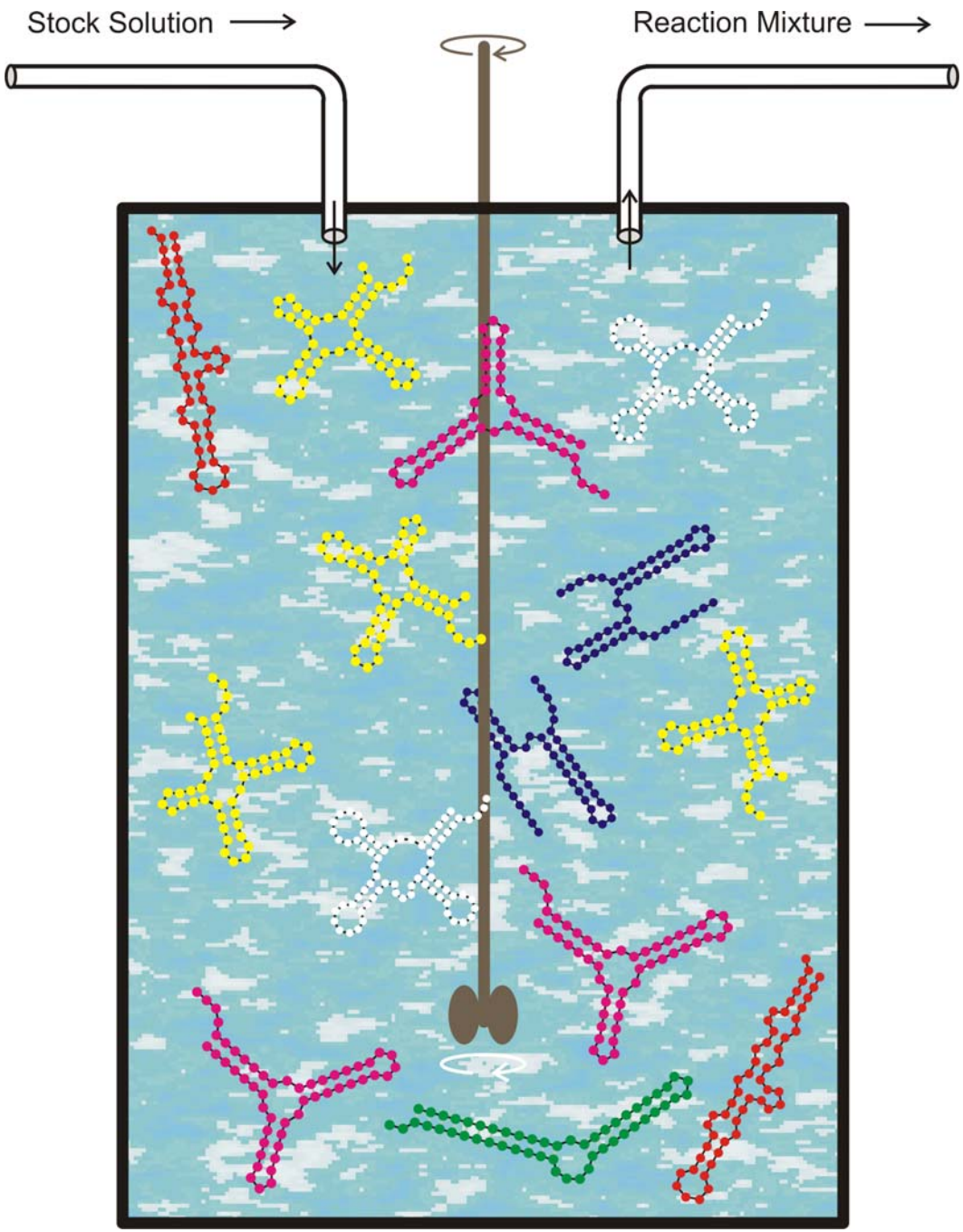
Number of genotypes in the next generation

Phenotype



Selection →

Evolution of phenotypes: RNA structures



Replication rate constant:

$$f_k = \gamma / [\alpha + \Delta d_S^{(k)}]$$

$$\Delta d_S^{(k)} = d_H(S_k, S_\tau)$$

Selection constraint:

Population size, N = # RNA molecules, is controlled by the flow

$$N(t) \approx \overline{N} \pm \sqrt{\overline{N}}$$

Mutation rate:

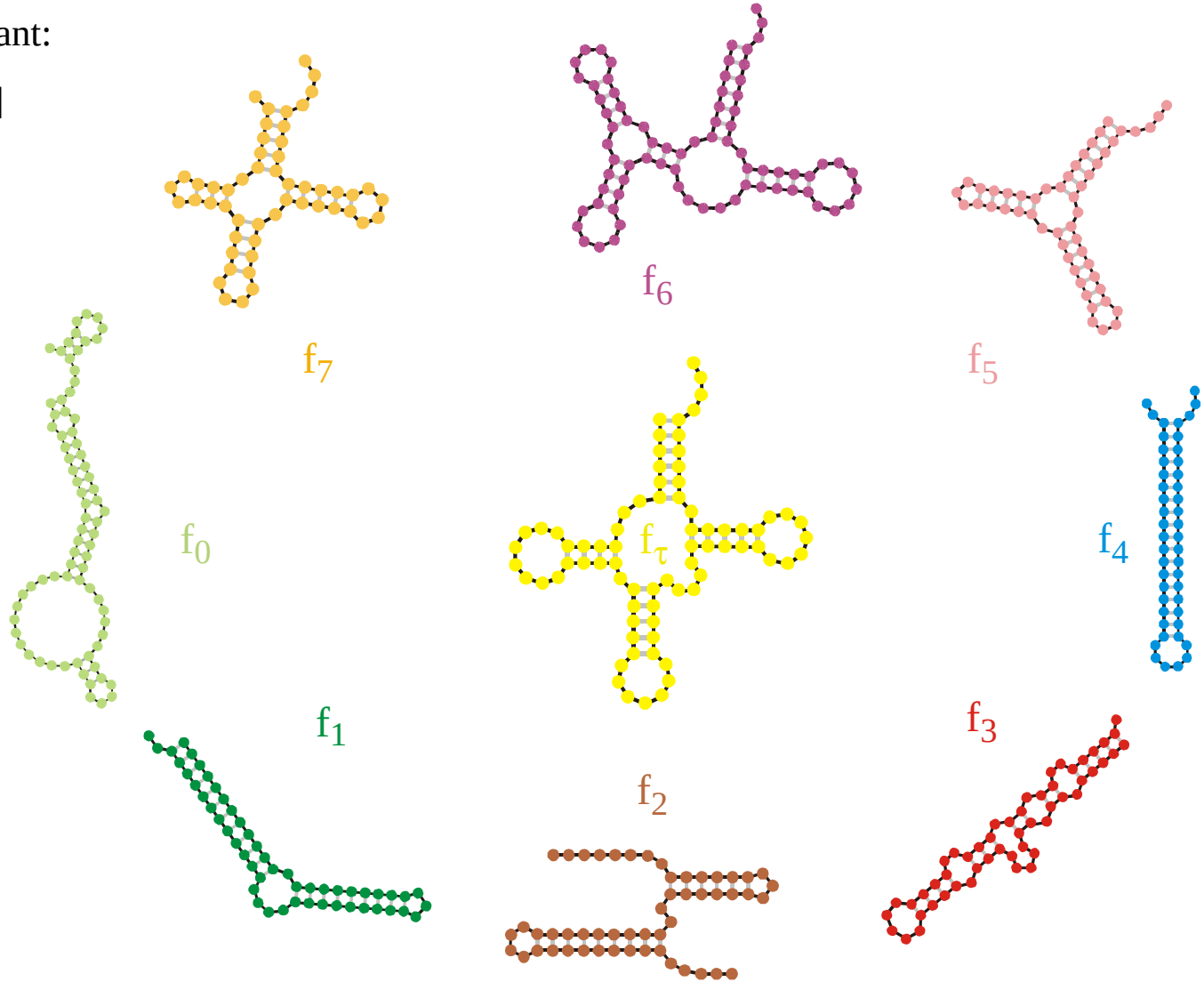
p = 0.001 / site × replication

The flowreactor as a device for **studies** of evolution ***in vitro*** and ***in silico***

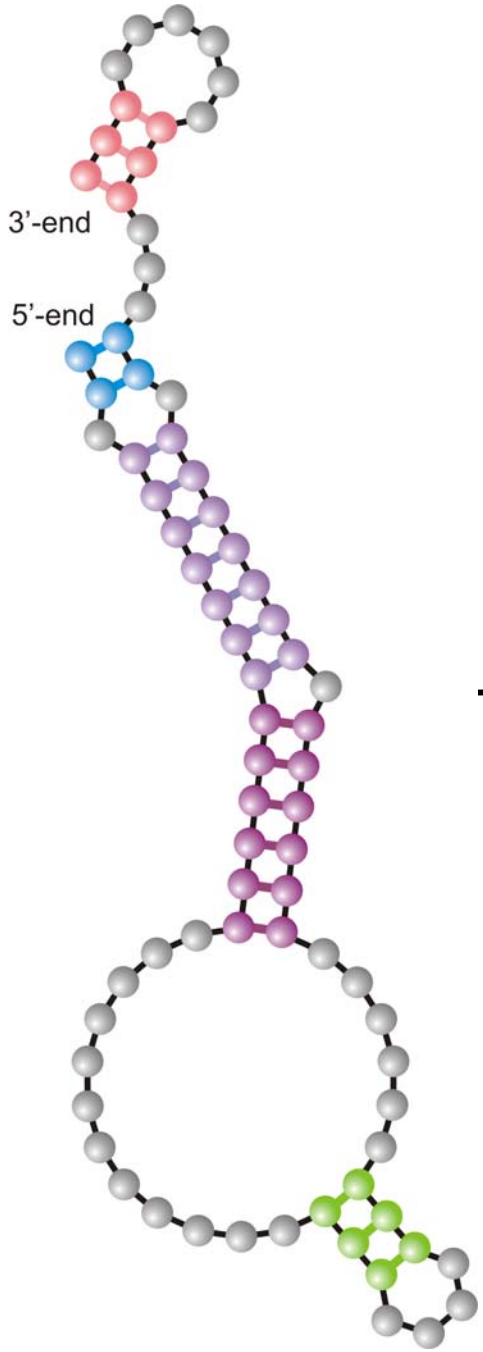
Replication rate constant:

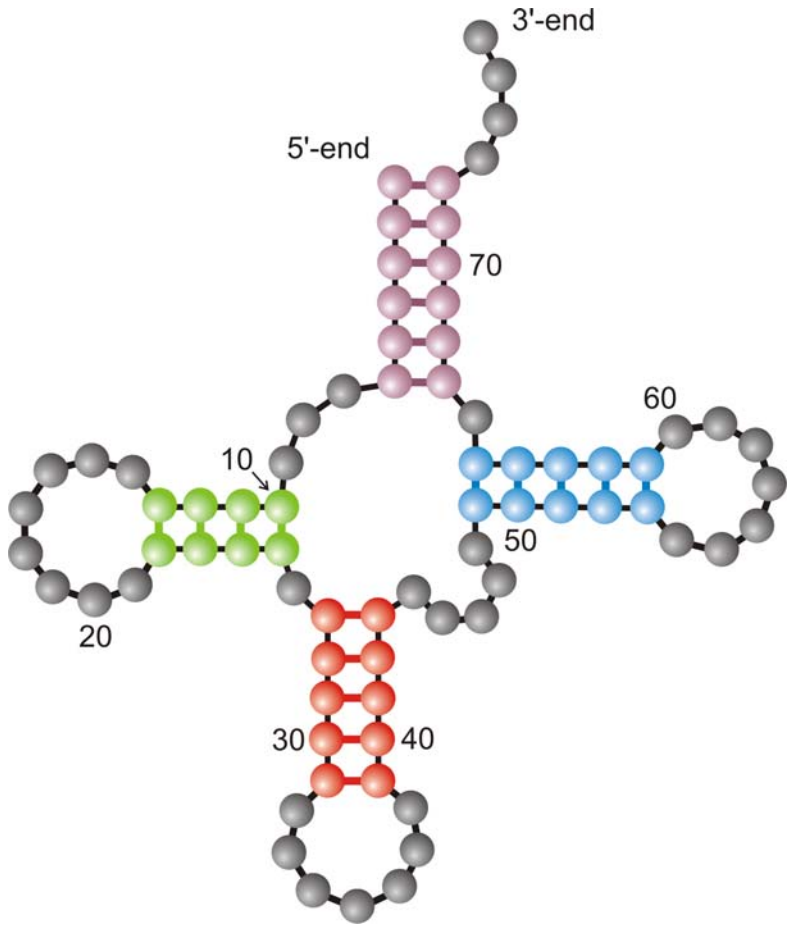
$$f_k = \gamma / [\alpha + \Delta d_S^{(k)}]$$

$$\Delta d_S^{(k)} = d_H(S_k, S_\tau)$$



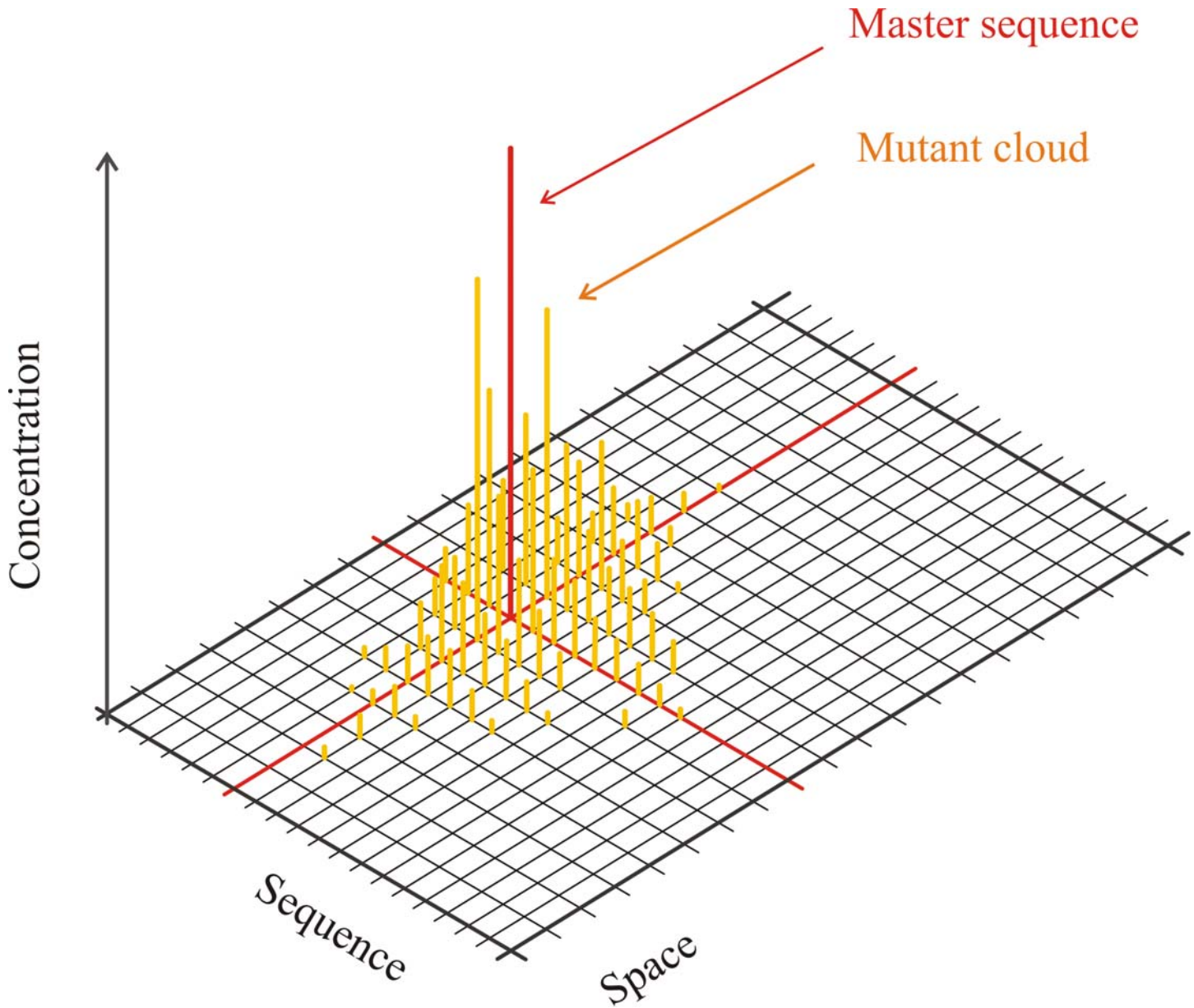
Evaluation of RNA secondary structures yields replication rate constants



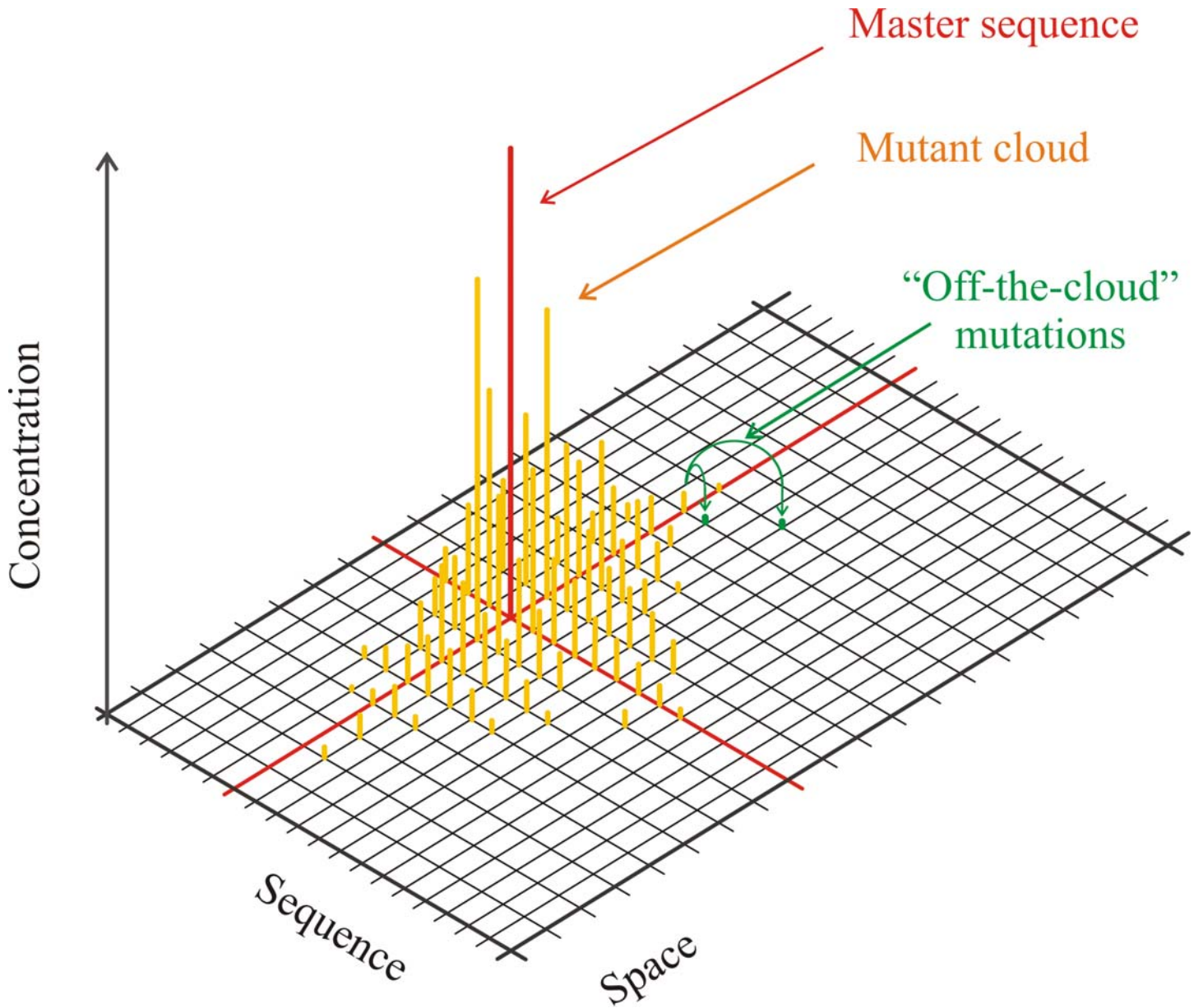


Randomly chosen initial structure

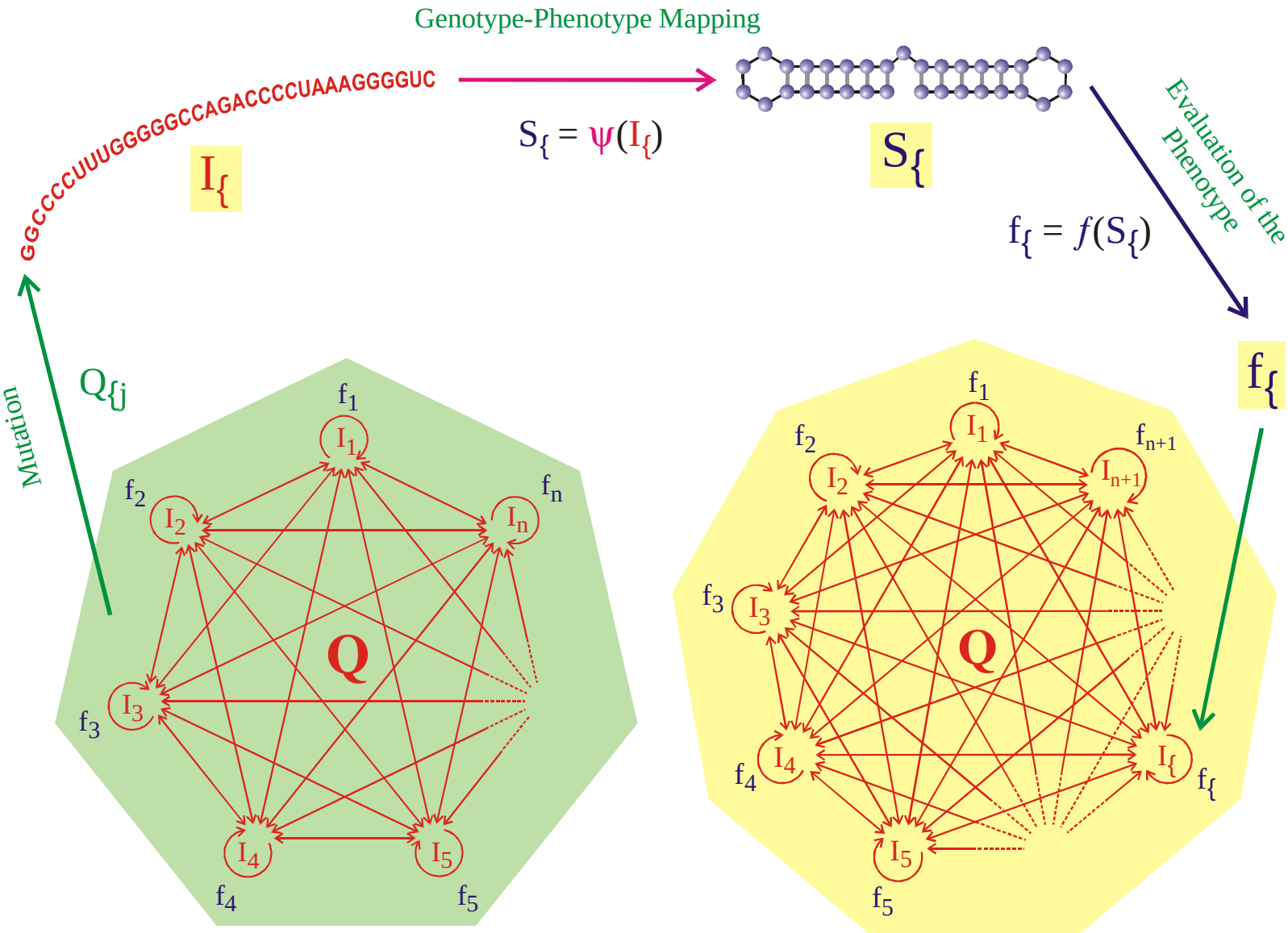
Phenylalanyl-tRNA as target structure



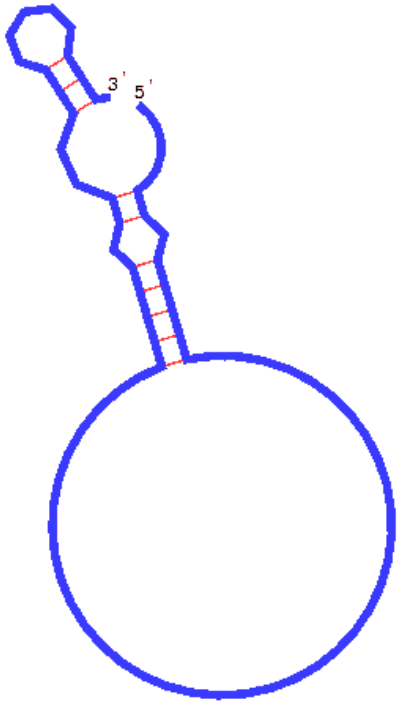
Formation of a quasispecies
in sequence space

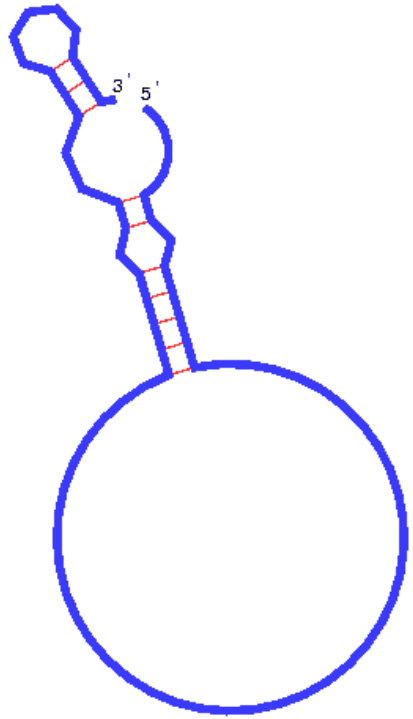


Migration of a quasispecies through sequence space



Evolutionary dynamics
including molecular phenotypes





AUGC alphabet

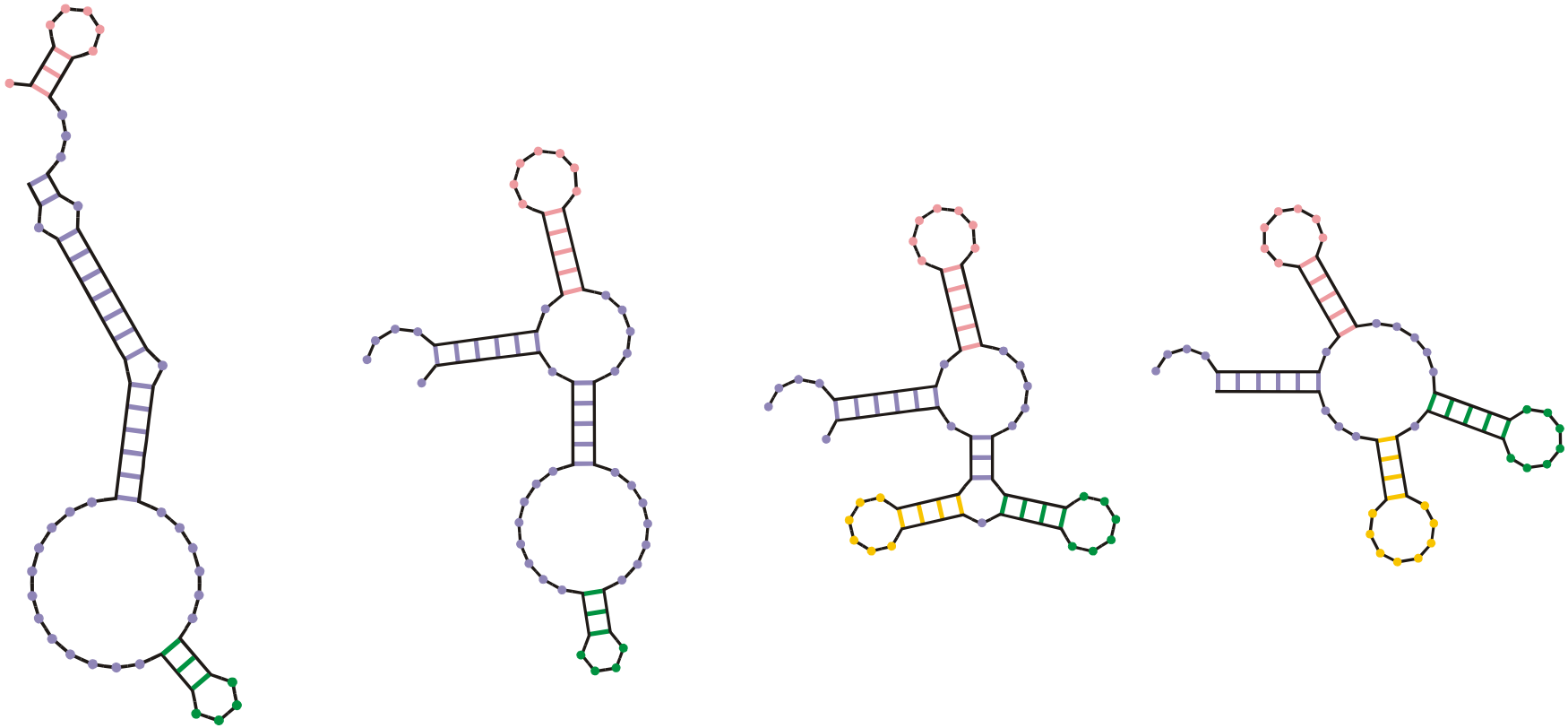
GC alphabet

connected

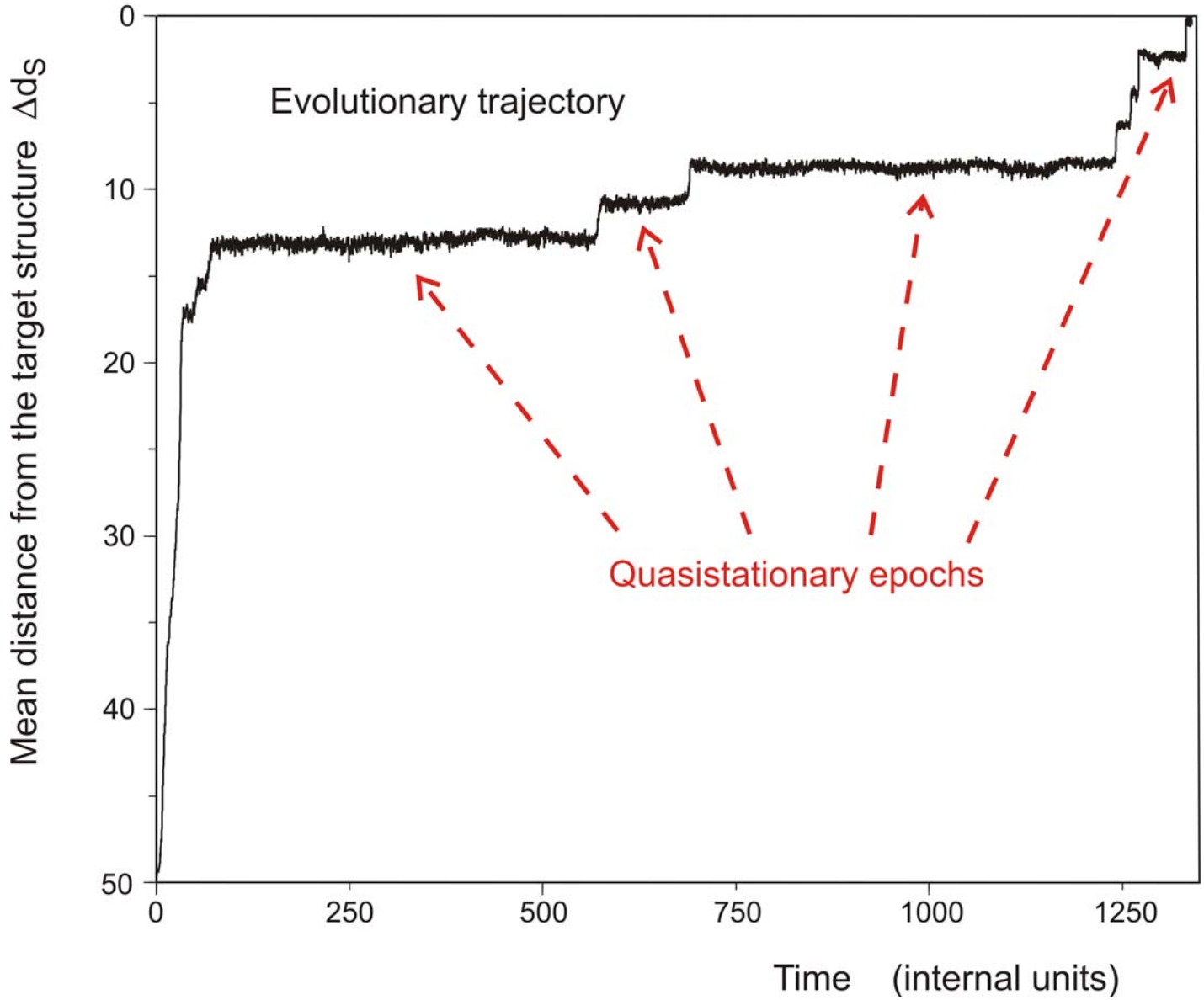
neutral network

disconnected

Evolutionary optimization of RNA structure

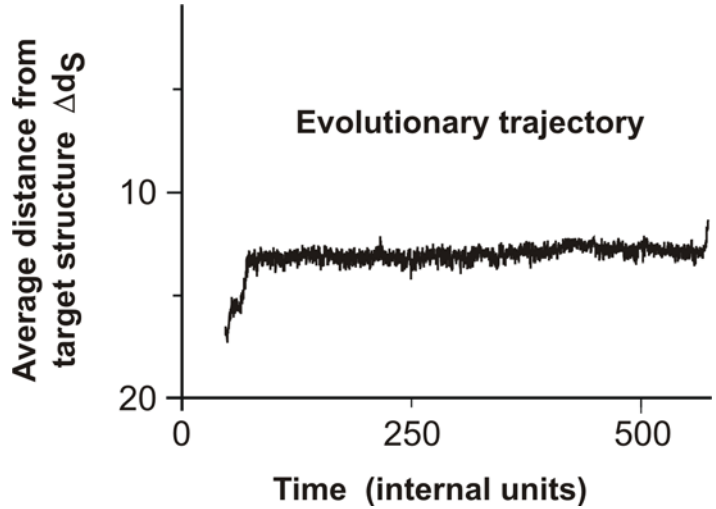


Three important steps in the formation of the tRNA clover leaf from a randomly chosen initial structure corresponding to three **main transitions**.



In silico optimization in the flow reactor: Evolutionary Trajectory

28 neutral point mutations during a long quasi-stationary epoch




```
entry   GGUAUGGGCGUUGAAUAGUAGGGUUUAAACCAAUCGGCCAACGAUCUCGUGUGCGCAUUUCAUAUCCCGUACAGAA
8       .((((((((((((.........(((....)))........)))))).....((((.........))))))))))....
exit    GGUAUGGGCGUUGAAUAAUAGGGUUUAAACCAAUCGGCCAACGAUCUCGUGUGCGCAUUUCAUAUCCCAUACAGAA
entry   GGUAUGGGCGUUGAAUAAUAGGGUUUAAACCAAUCGGCCAACGAUCUCGUGUGCGCAUUUCAUAUACCAUACAGAA
9       .((((((.((((.........(((....)))........)))))).....((((.........))))).))))))....
exit    UGGAUGGACGUUGAAUAACAAGGUAUCGACCAAACAACCAACGAGUAAGUGUGUACGCCCCACACACCGUCCCAAG
entry   UGGAUGGACGUUGAAUAACAAGGUAUCGACCAAACAACCAACGAGUAAGUGUGUACGCCCCACACAGCGUCCCAAG
10      .(((((..((((.........(((....)))........)))))).....((((.........))))).)))))....
exit    UGGAUGGACGUUGAAUAACAAGGUAUCGACCAAACAACCAACGAGUAAGUGUGUACGCCCCACACAGCGUCCCAAG
```

Transition inducing point mutations change the molecular structure

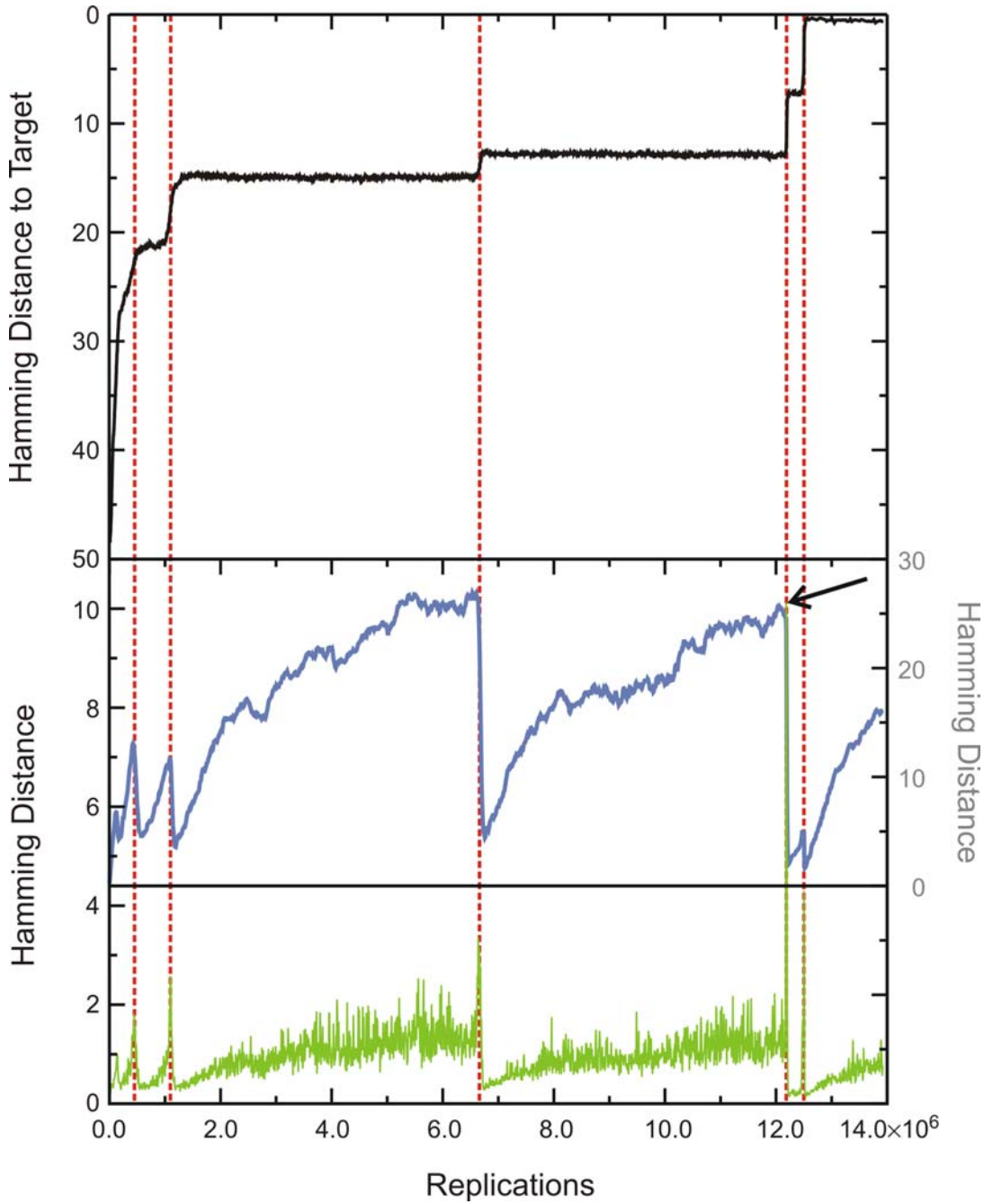
Neutral point mutations leave the molecular structure unchanged

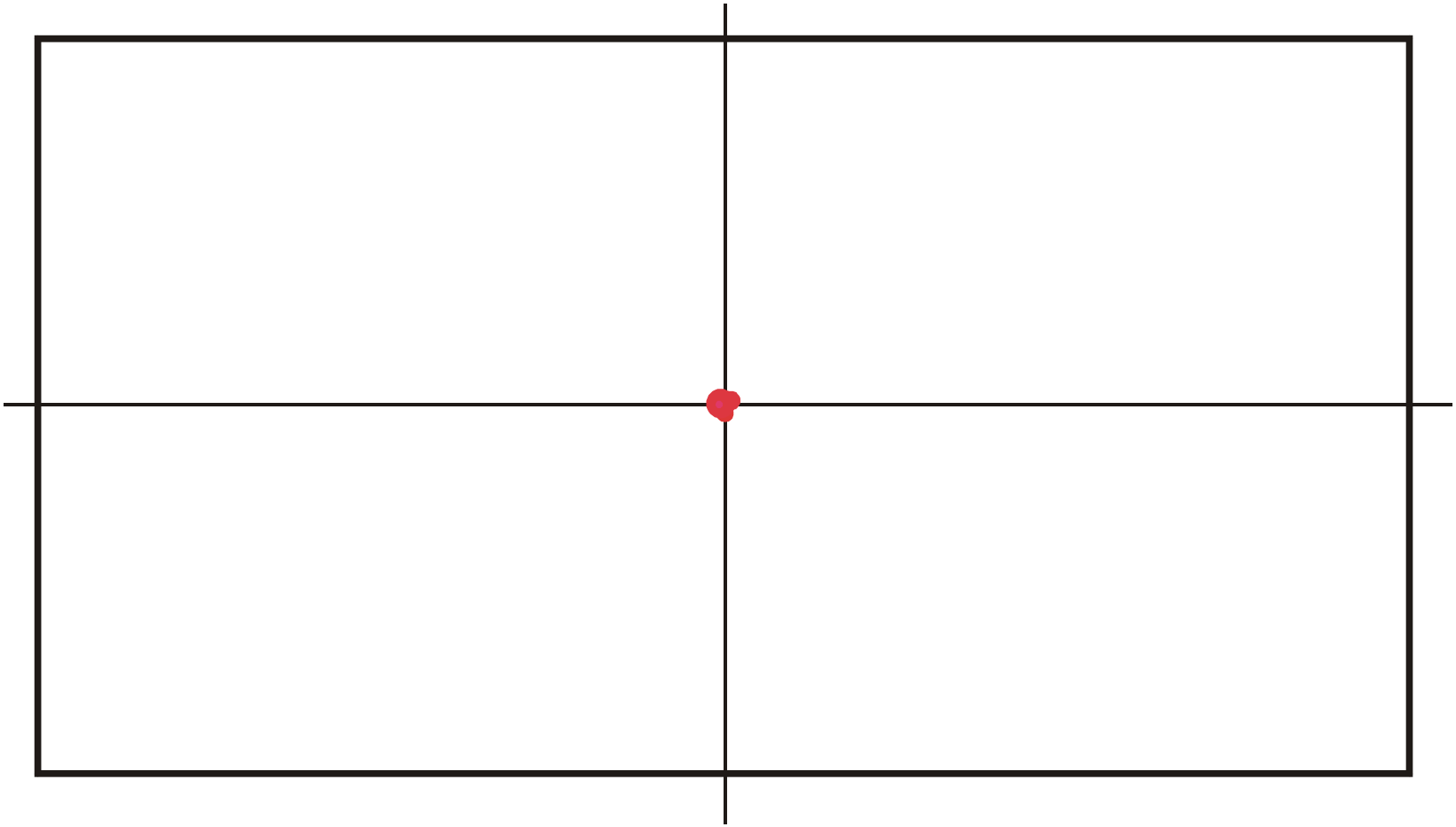
Neutral genotype evolution during phenotypic stasis

Evolutionary trajectory

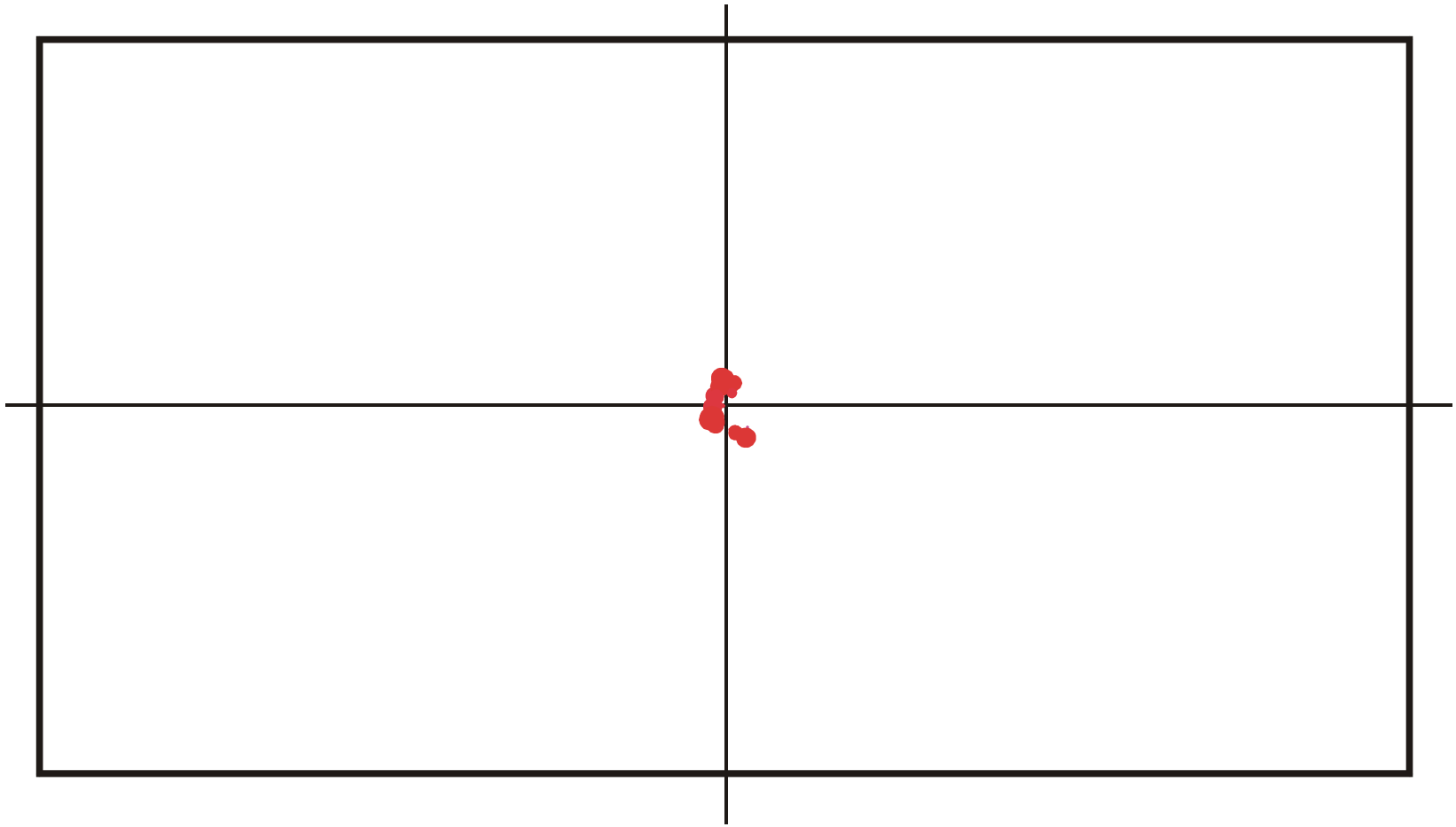
Spreading of the population on neutral networks

Drift of the population center in sequence space

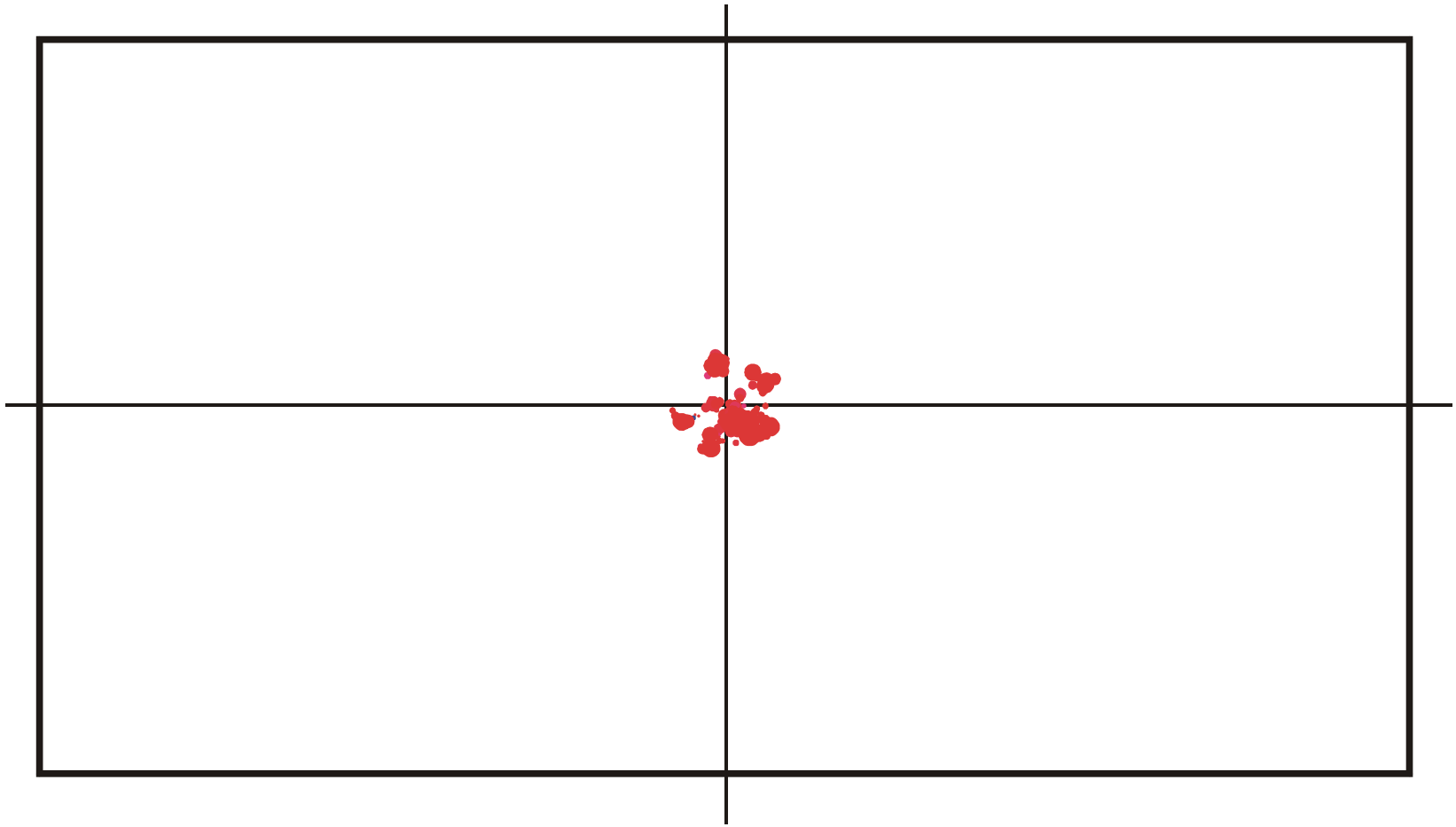




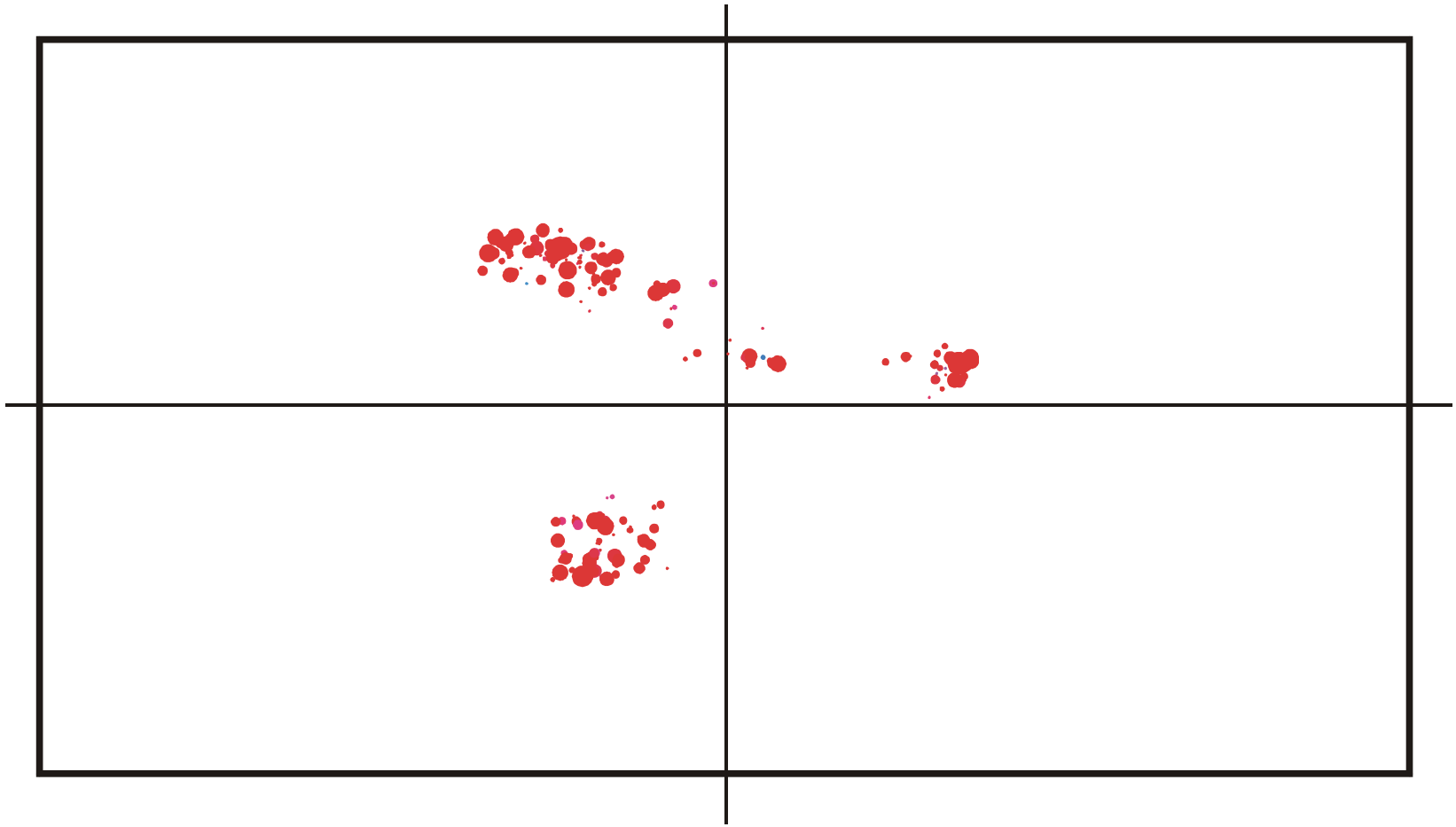
Spreading and evolution of a population on a neutral network: t = 150



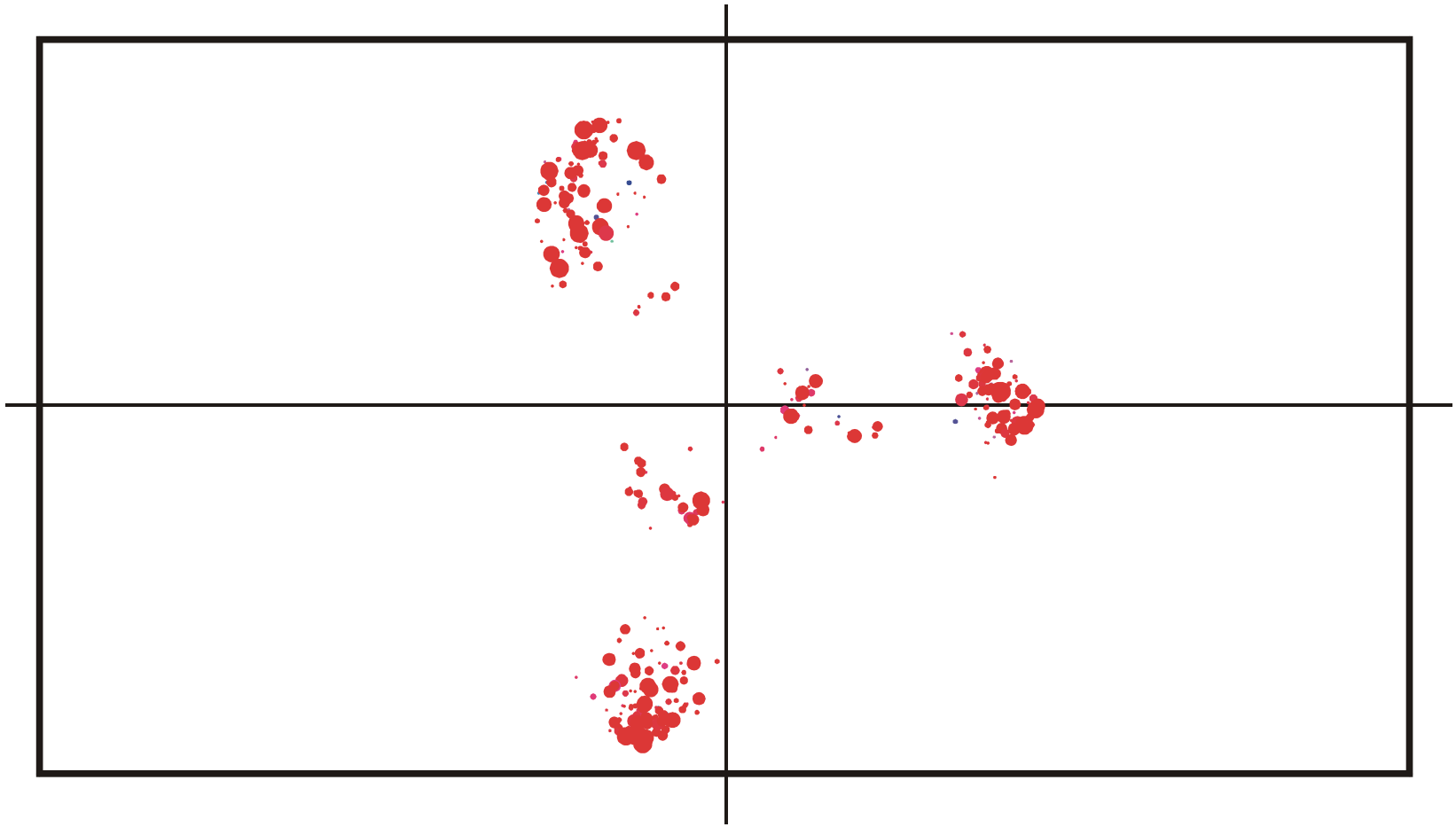
Spreading and evolution of a population on a neutral network : t = 170



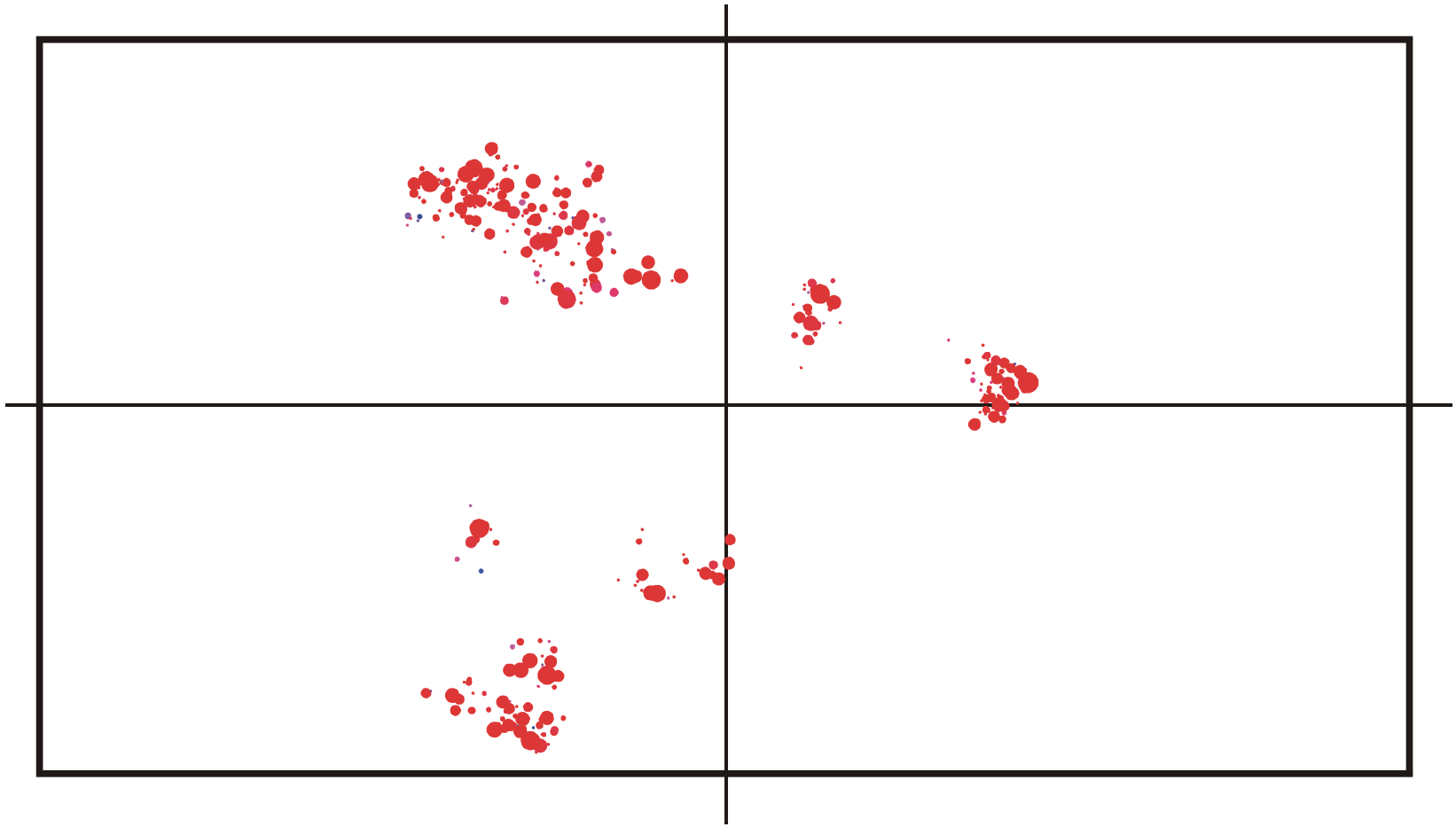
Spreading and evolution of a population on a neutral network : t = 200



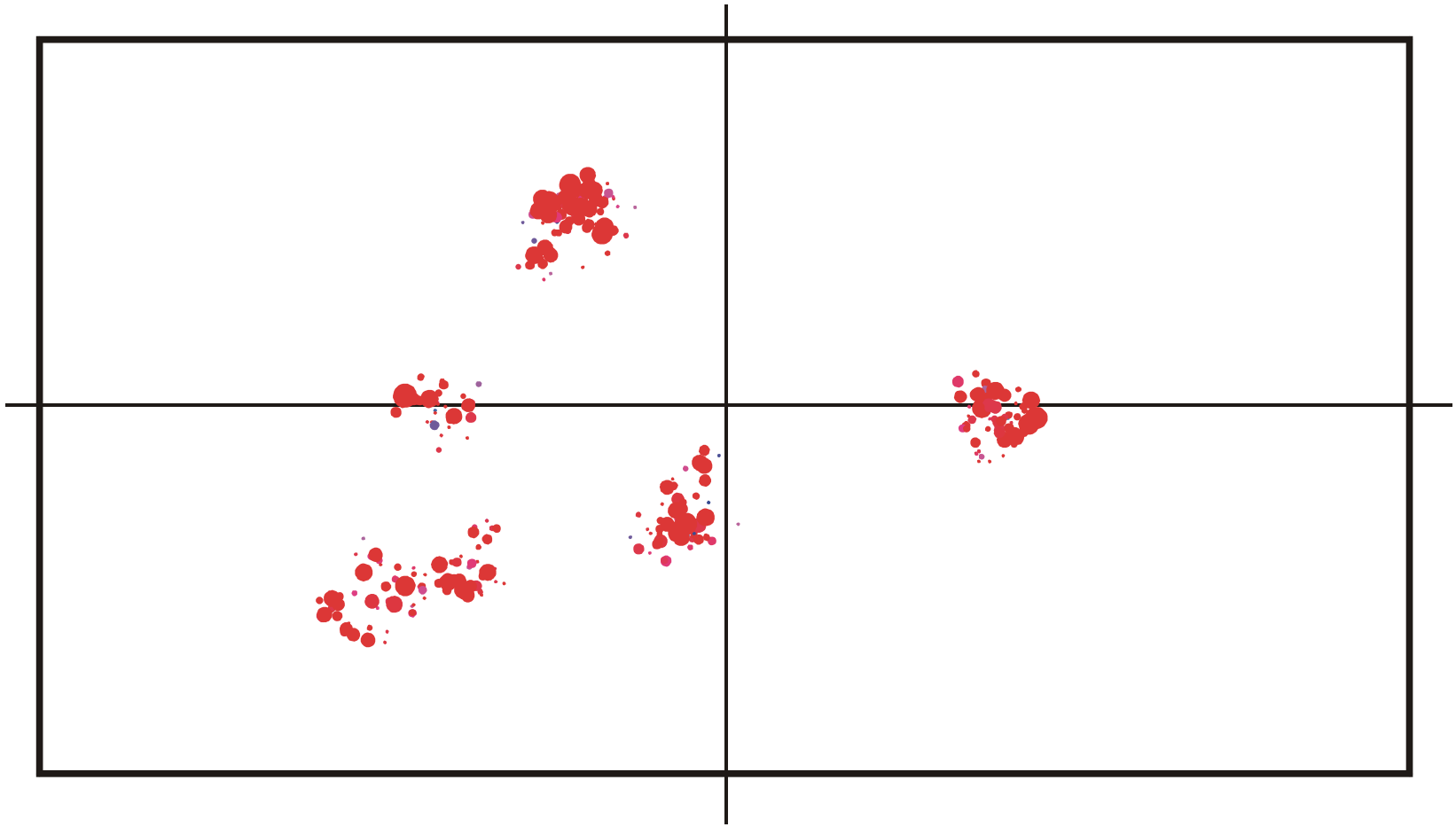
Spreading and evolution of a population on a neutral network : t = 350



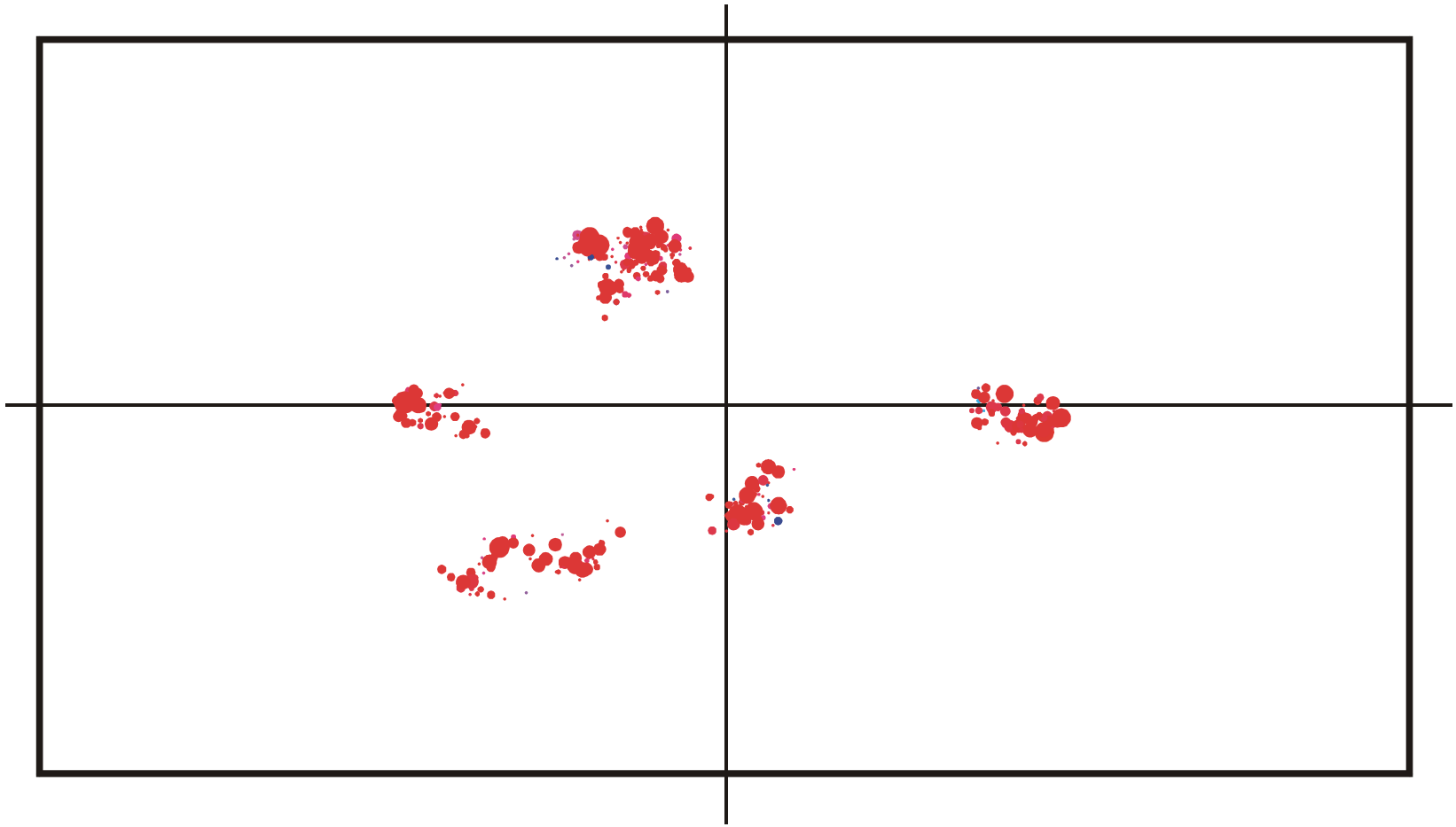
Spreading and evolution of a population on a neutral network : t = 500



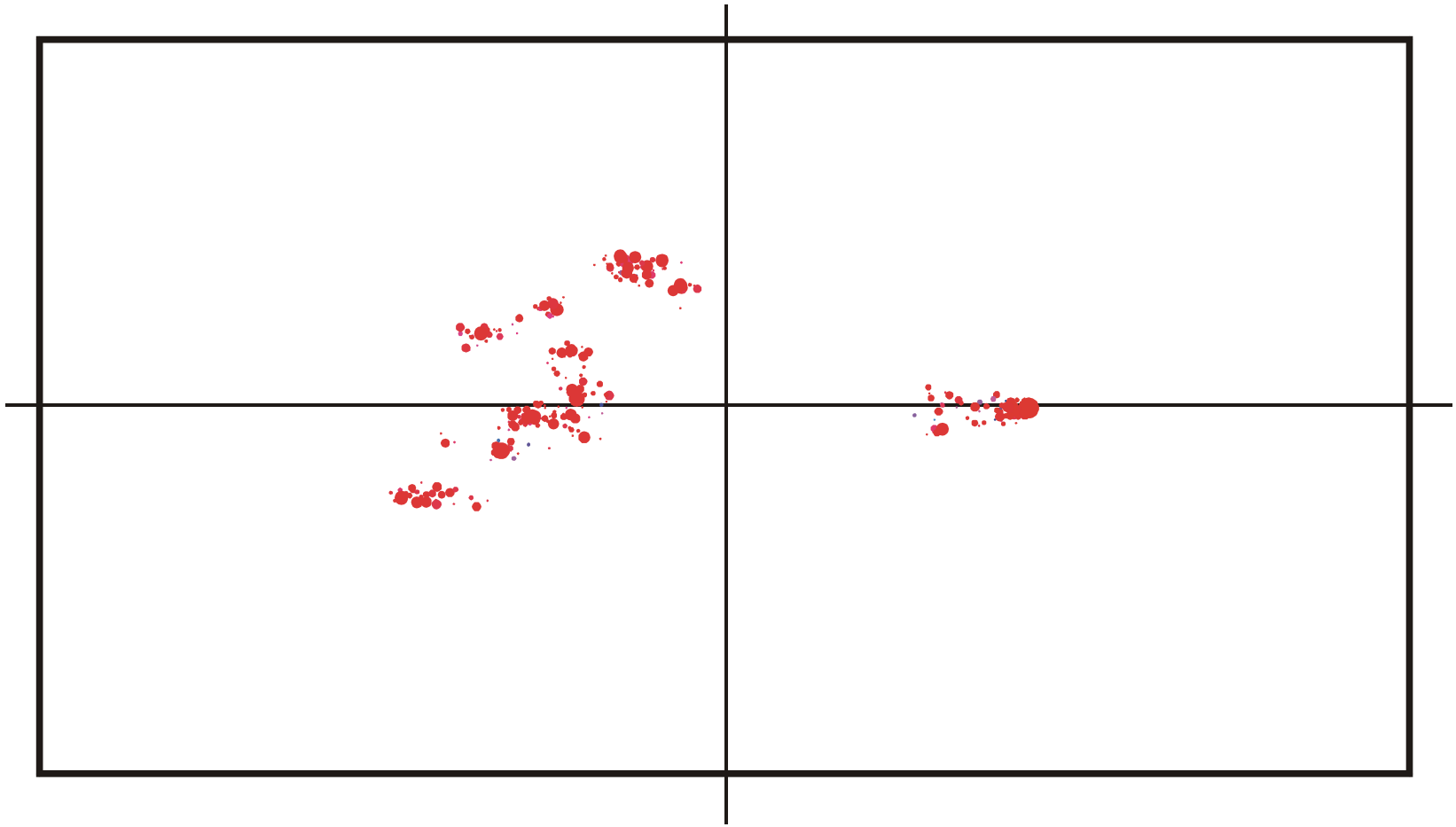
Spreading and evolution of a population on a neutral network : t = 650



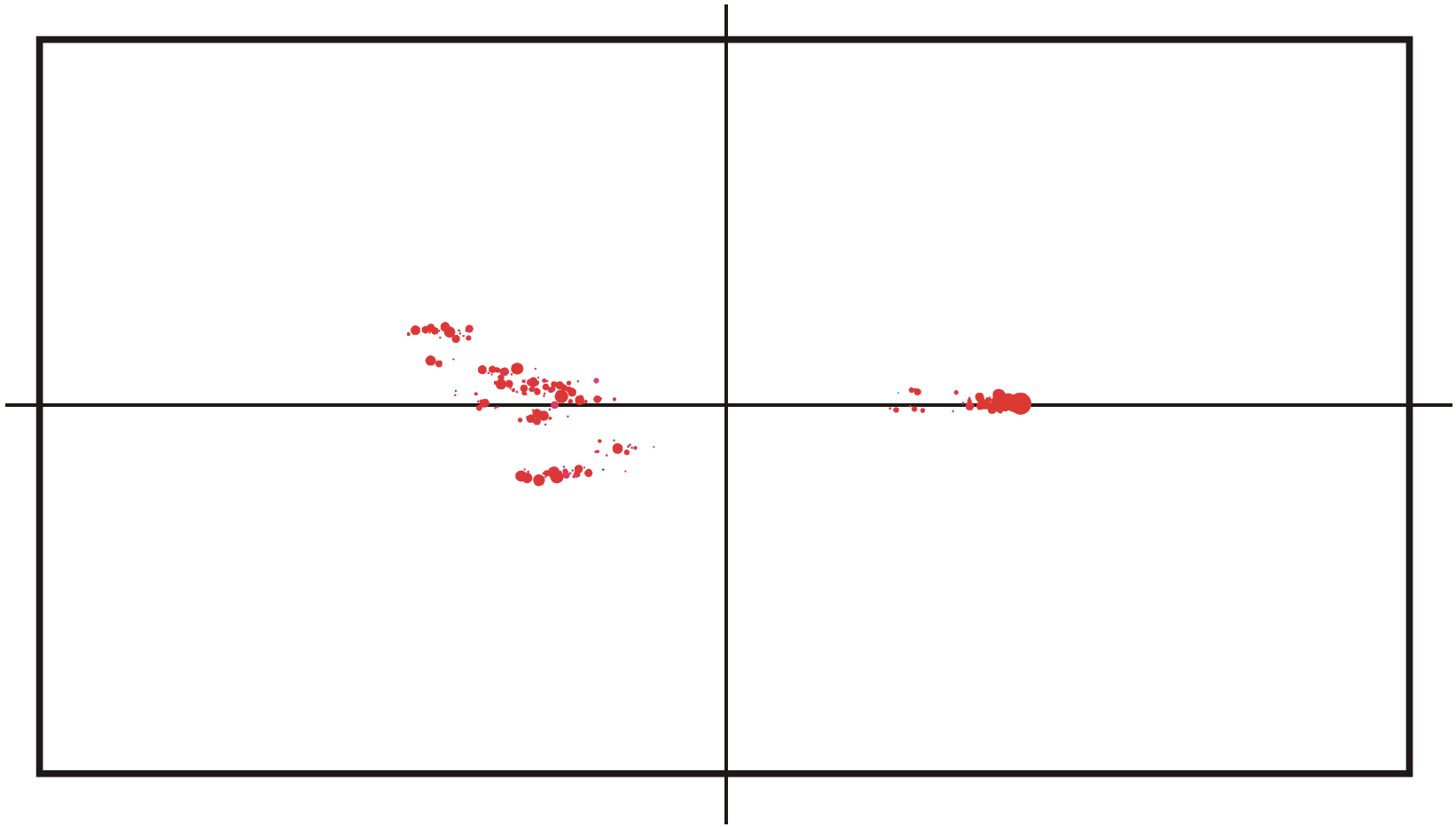
Spreading and evolution of a population on a neutral network : t = 820



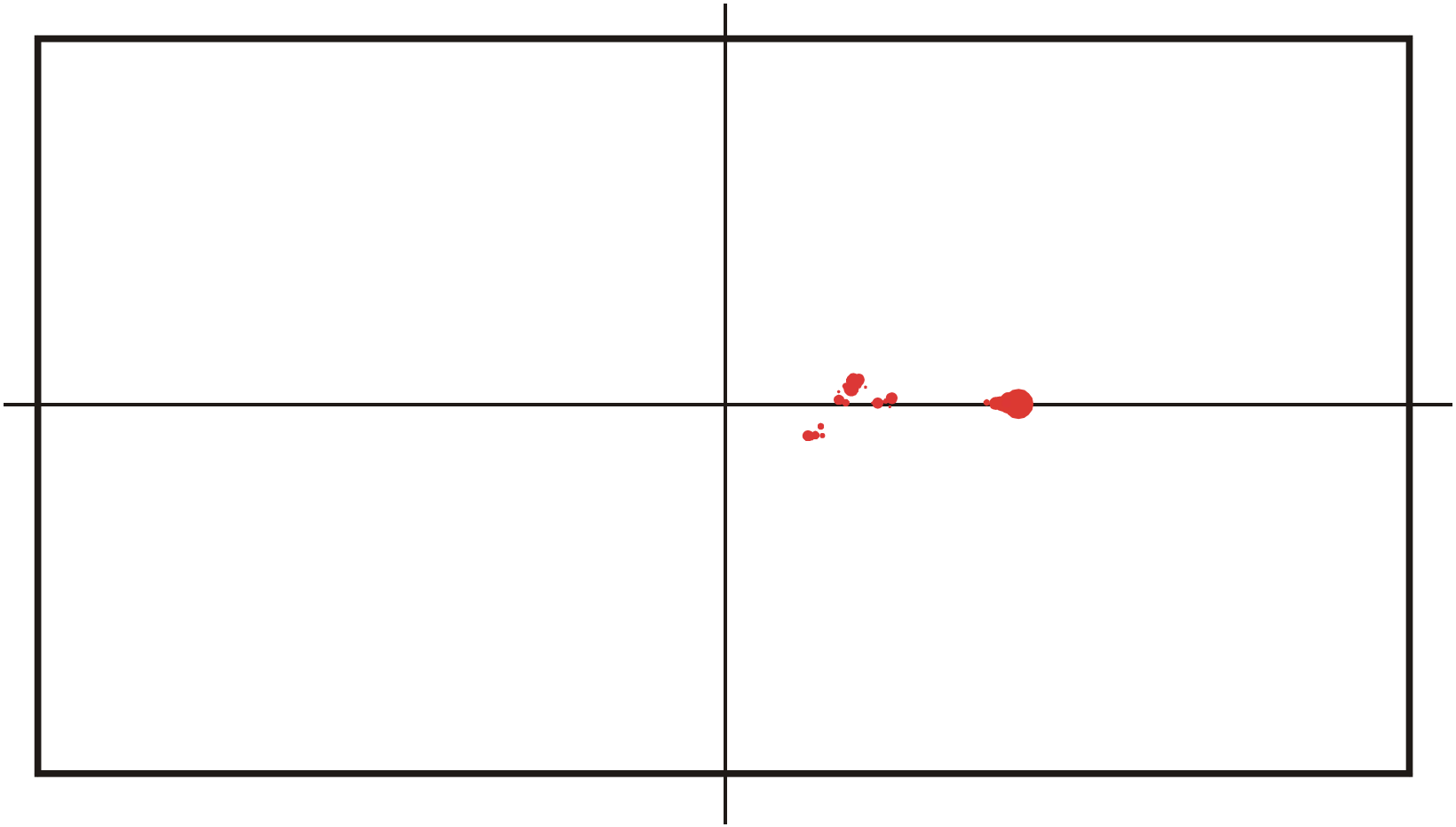
Spreading and evolution of a population on a neutral network : t = 825



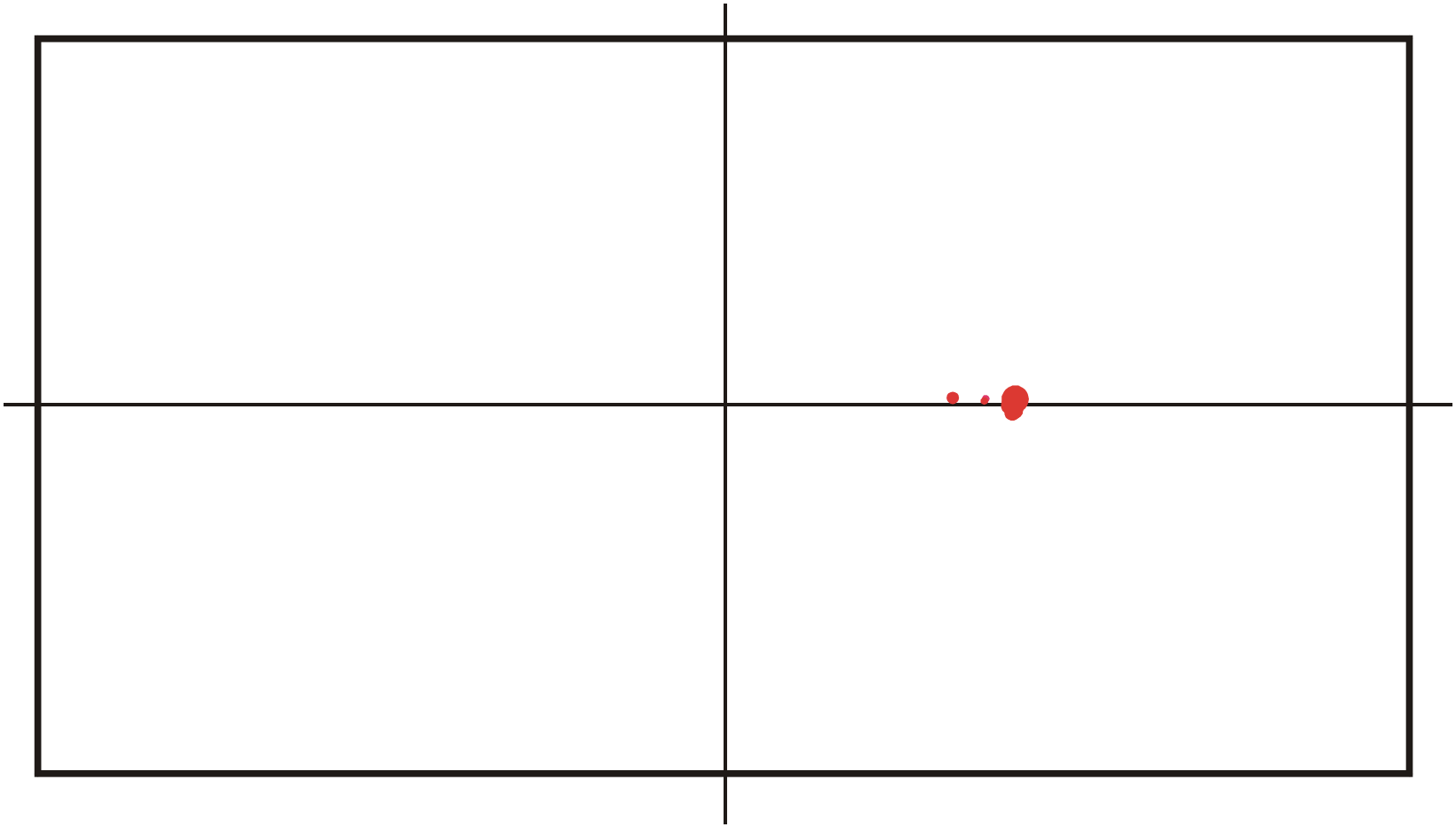
Spreading and evolution of a population on a neutral network : t = 830



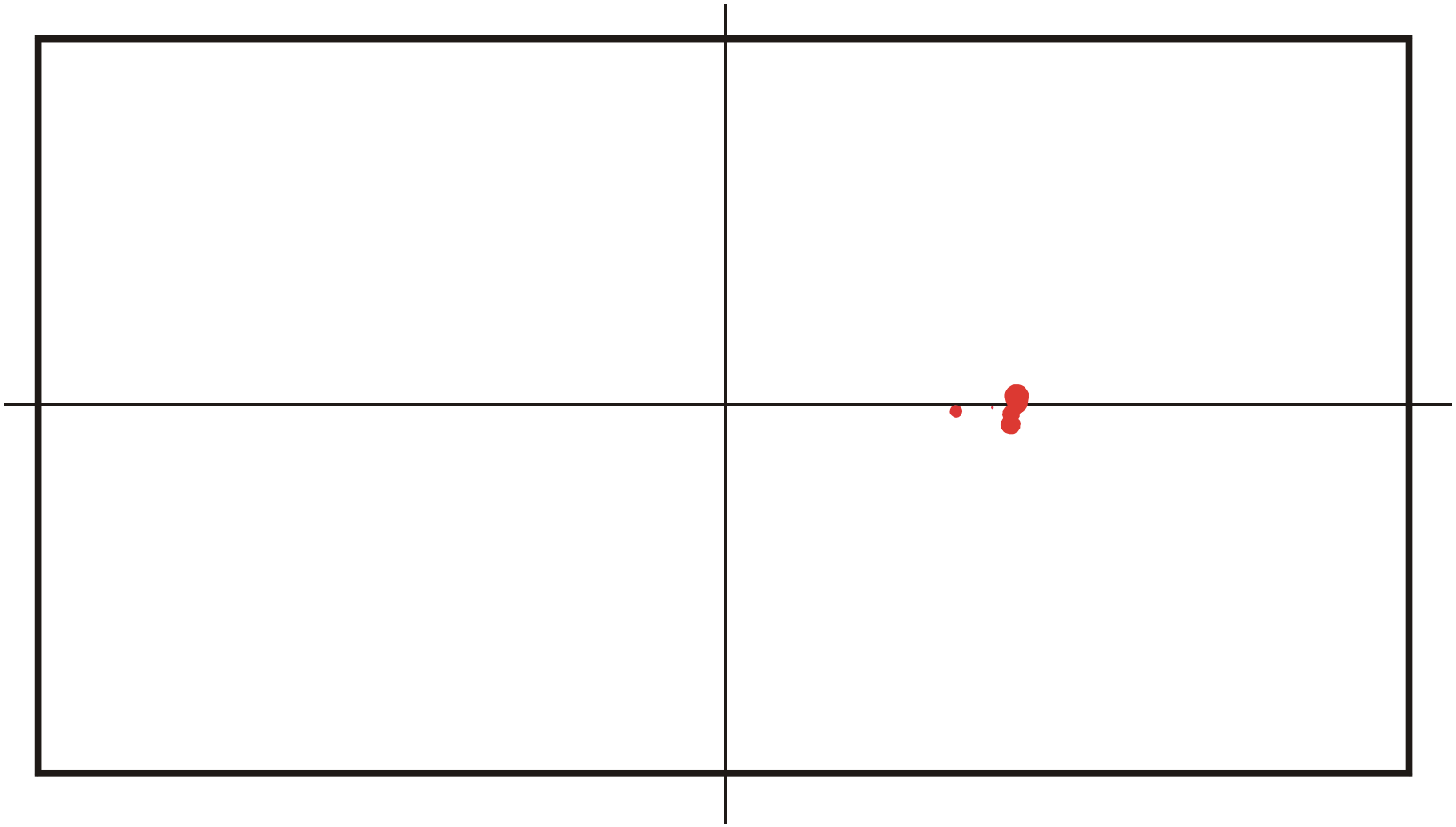
Spreading and evolution of a population on a neutral network : t = 835



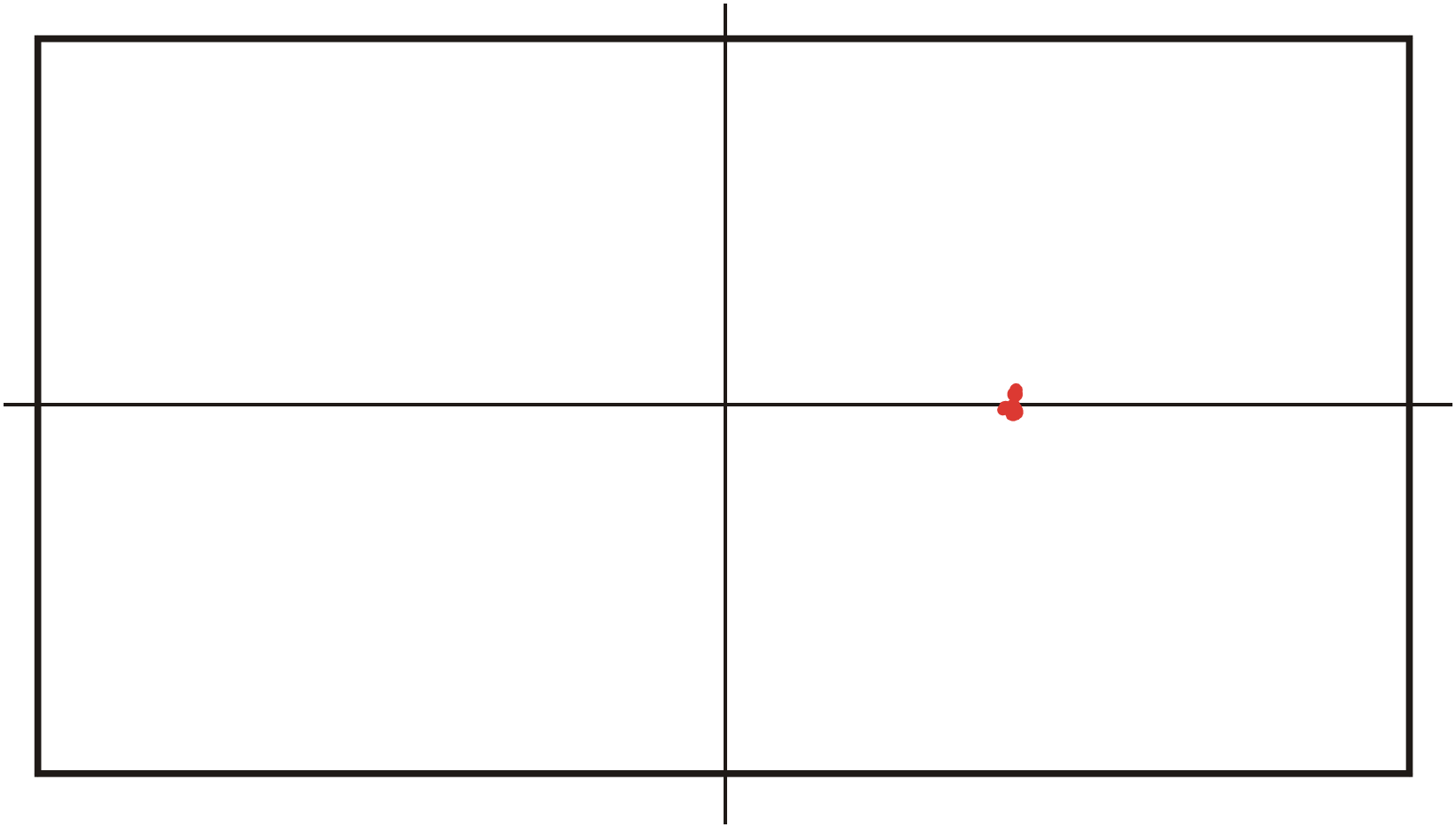
Spreading and evolution of a population on a neutral network : t = 840



Spreading and evolution of a population on a neutral network : t = 845



Spreading and evolution of a population on a neutral network : t = 850

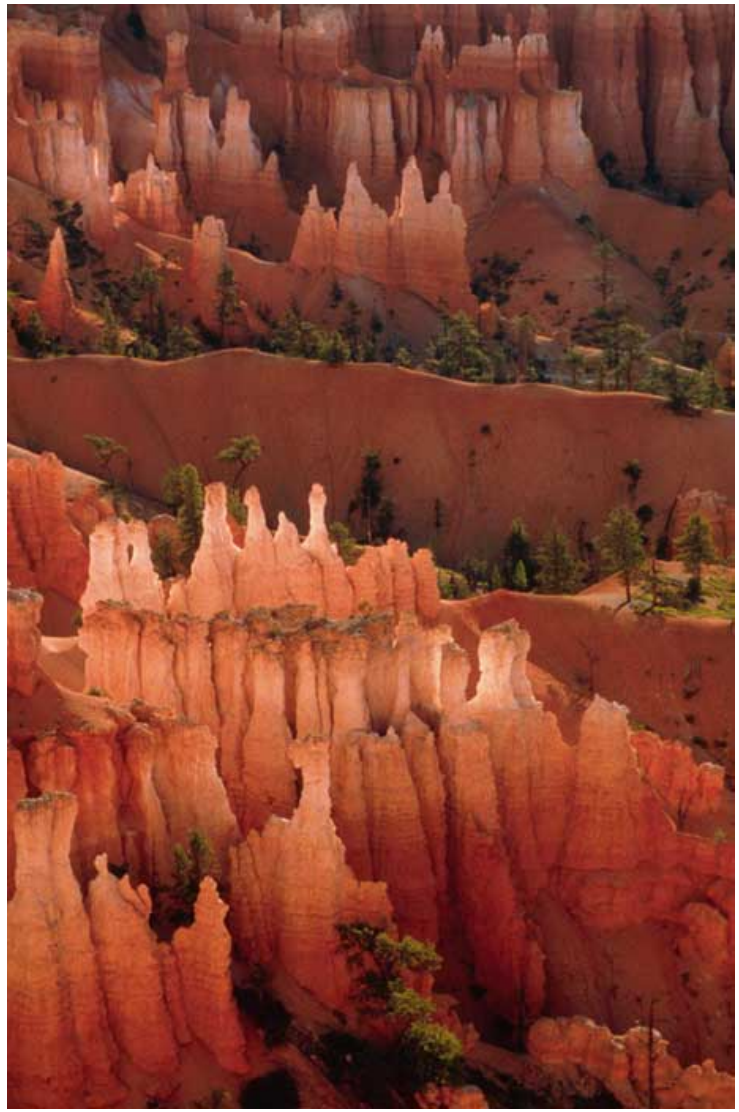


Spreading and evolution of a population on a neutral network : t = 855

Mount Fuji

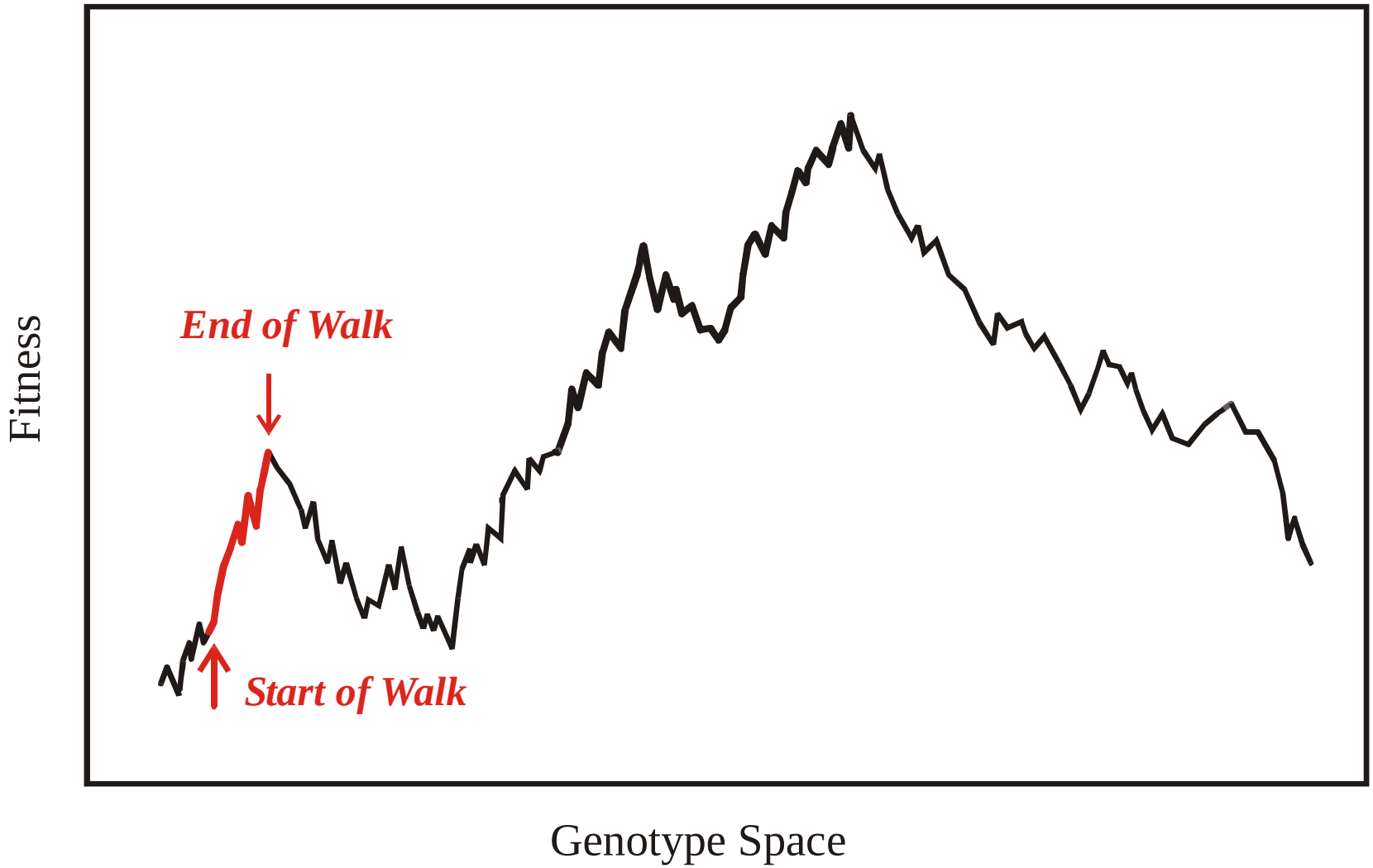
Example of a smooth landscape on Earth

Dolomites

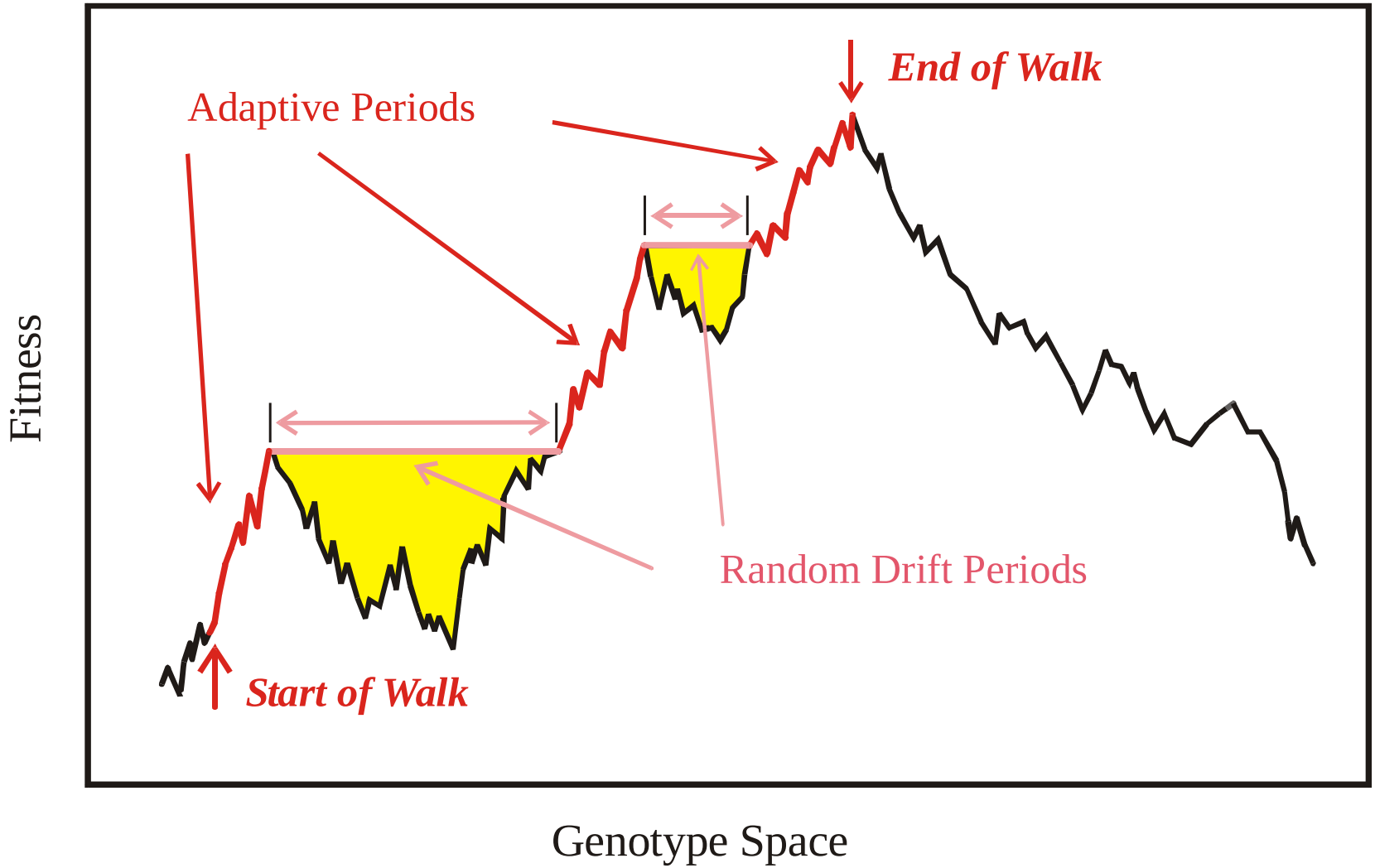


Bryce Canyon

Examples of rugged landscapes on Earth



Evolutionary optimization in absence of neutral paths in sequence space



Evolutionary optimization including neutral paths in sequence space

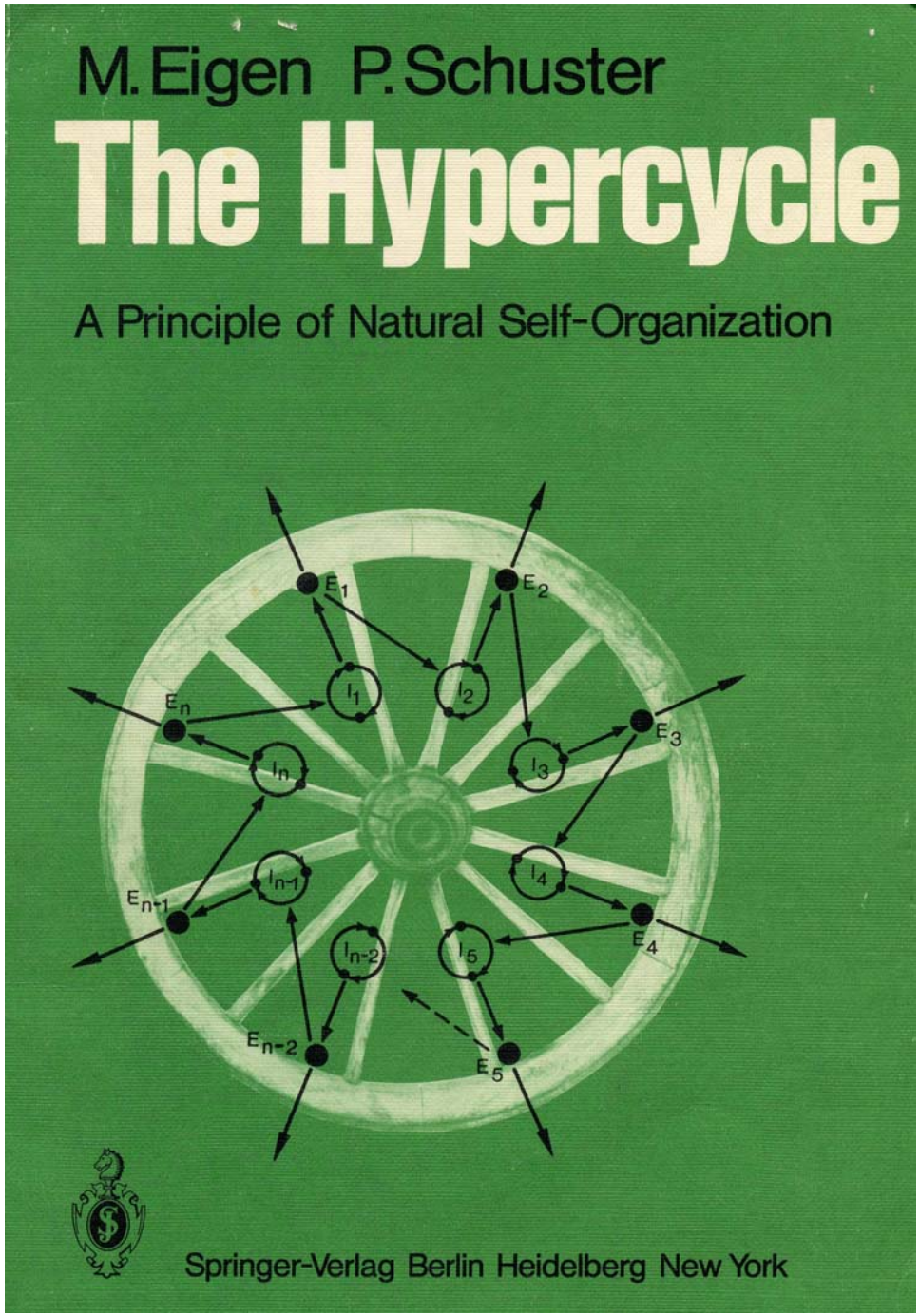
Grand Canyon

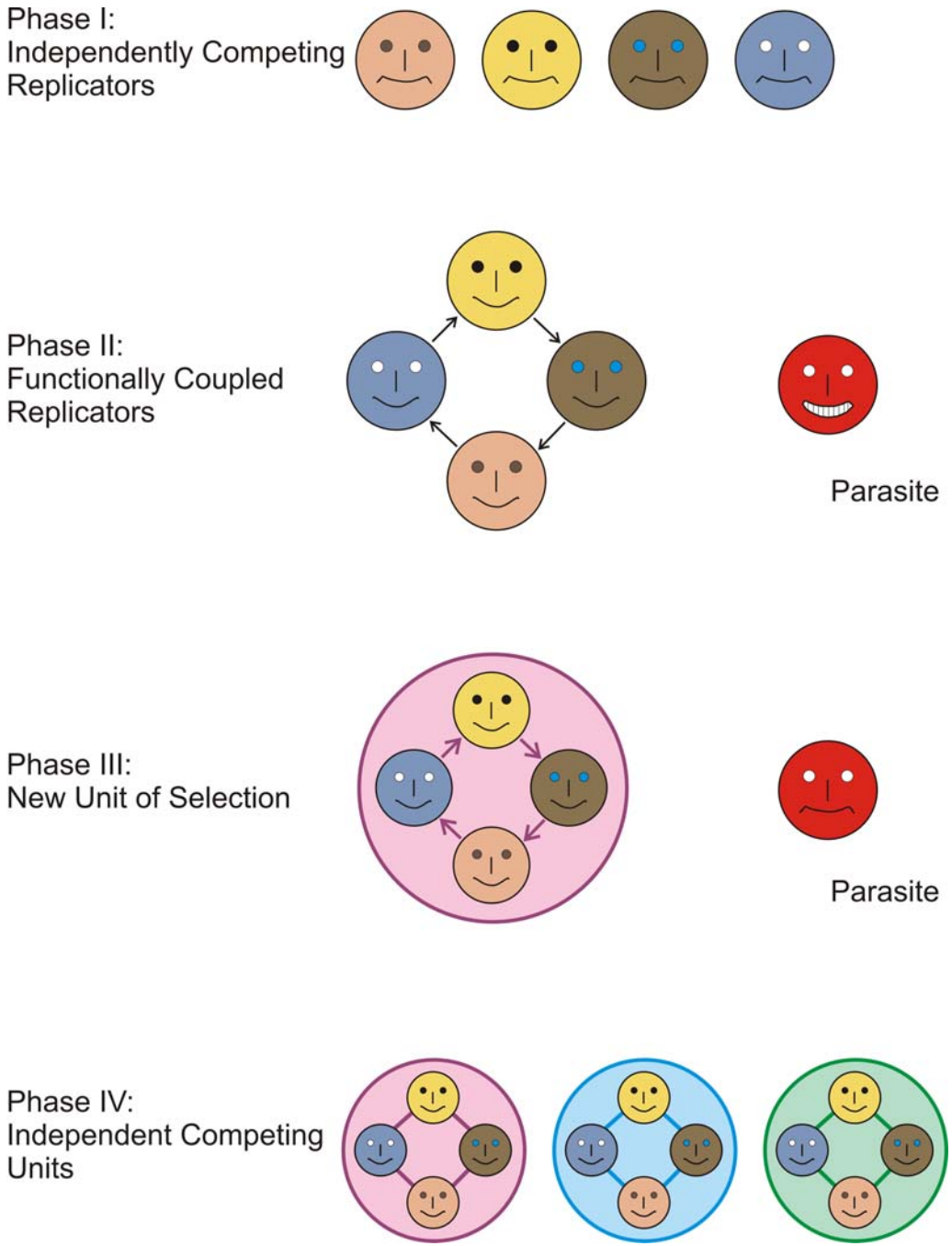
Example of a landscape on Earth with ‘neutral’ ridges and plateaus

1. Darwinian evolution in laboratory experiments
2. Modeling the evolution of molecules
3. From RNA sequences to structures and back
4. Evolution on neutral networks
5. **Origins of complexity**

Chemical kinetics of molecular evolution

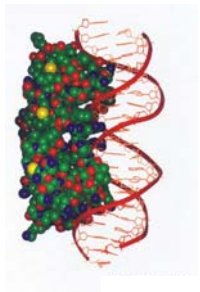
M. Eigen, P. Schuster, `The Hypercycle´, Springer-Verlag, Berlin 1979

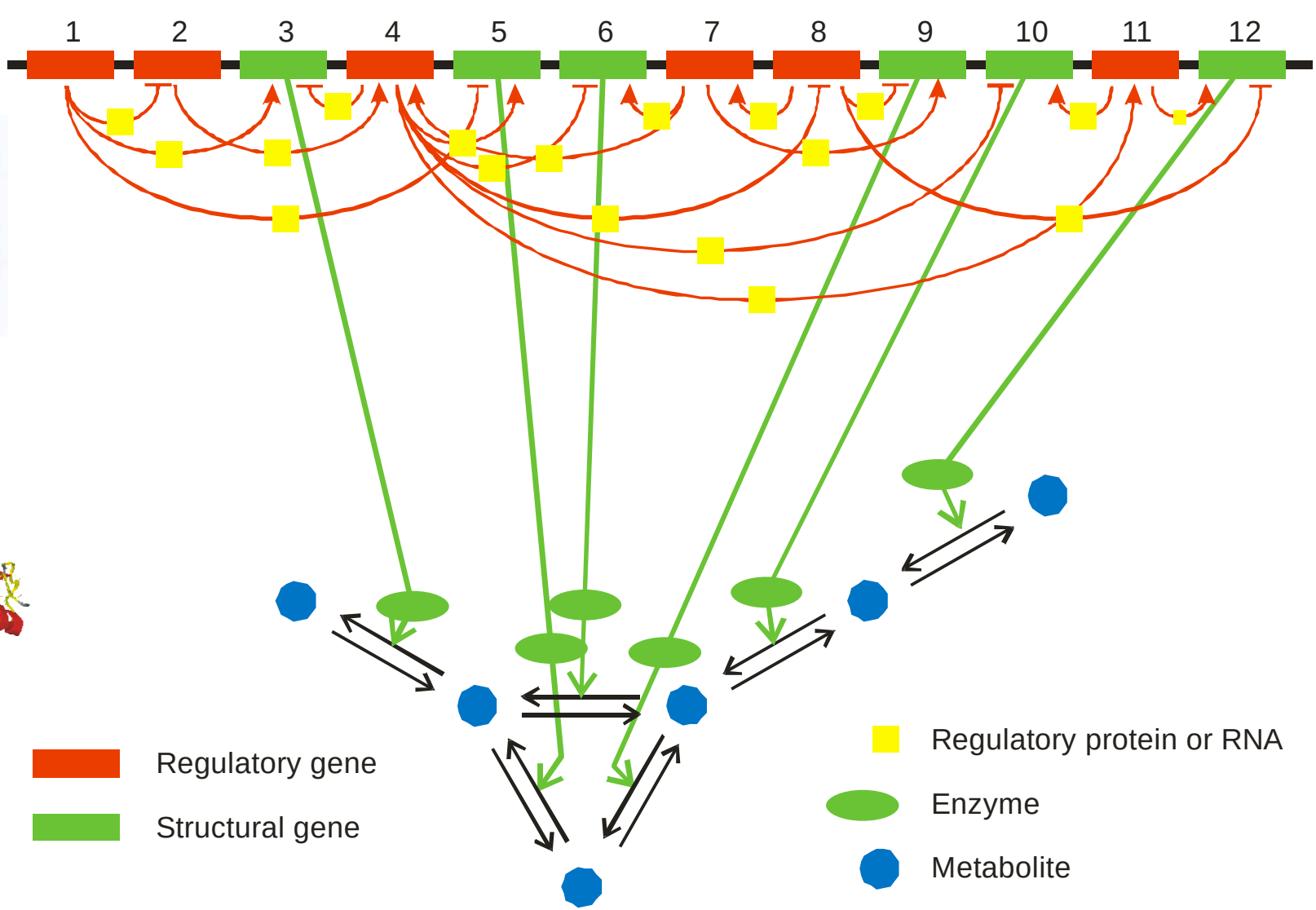




Four phases of major transitions leading to radical innovations in evolution

M.Eigen, P.Schuster: 1978
J.Maynard Smith, E. Szathmáry: 1995





Sketch of a genetic and metabolic network

All higher forms of life share the almost same sets genes.

Differences come about through different expression of genes and multiple usage of gene products.

Are there molecules with multiple functions ?

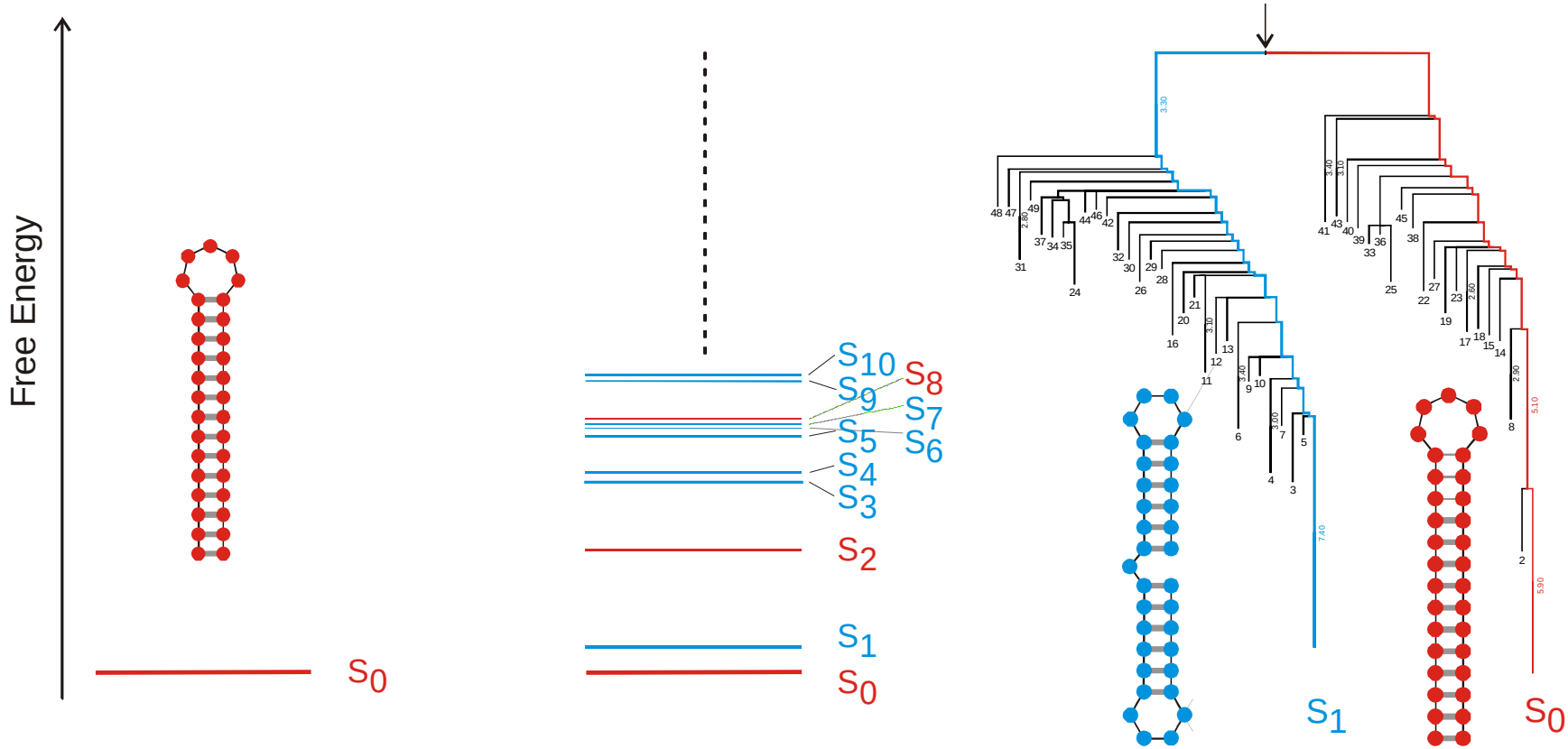
How do they look like?

RNA switches as an example

One sequence - one structure

Many suboptimal structures
Partition function

Metastable structures
Conformational switches

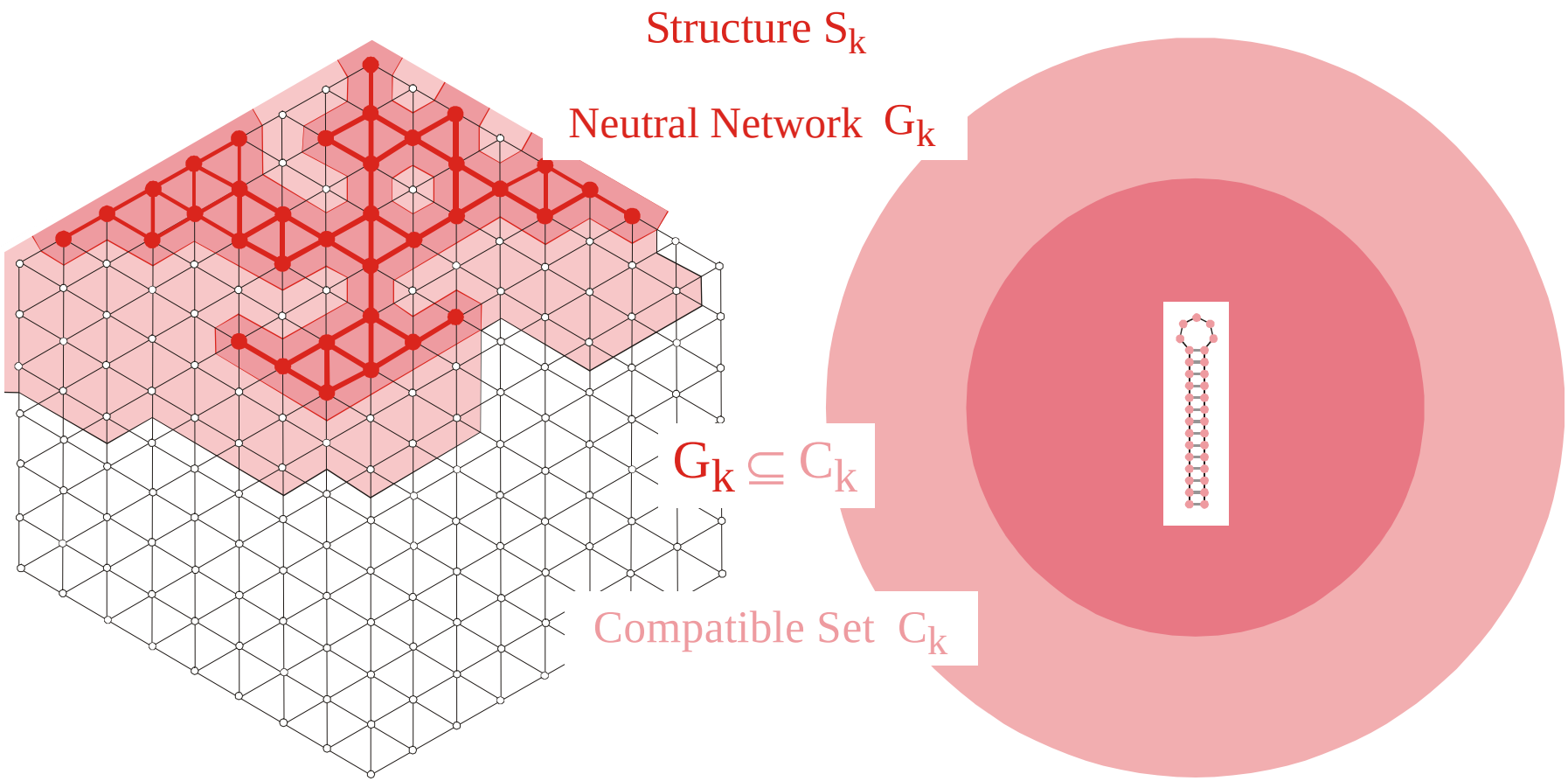


Minimum free energy structure

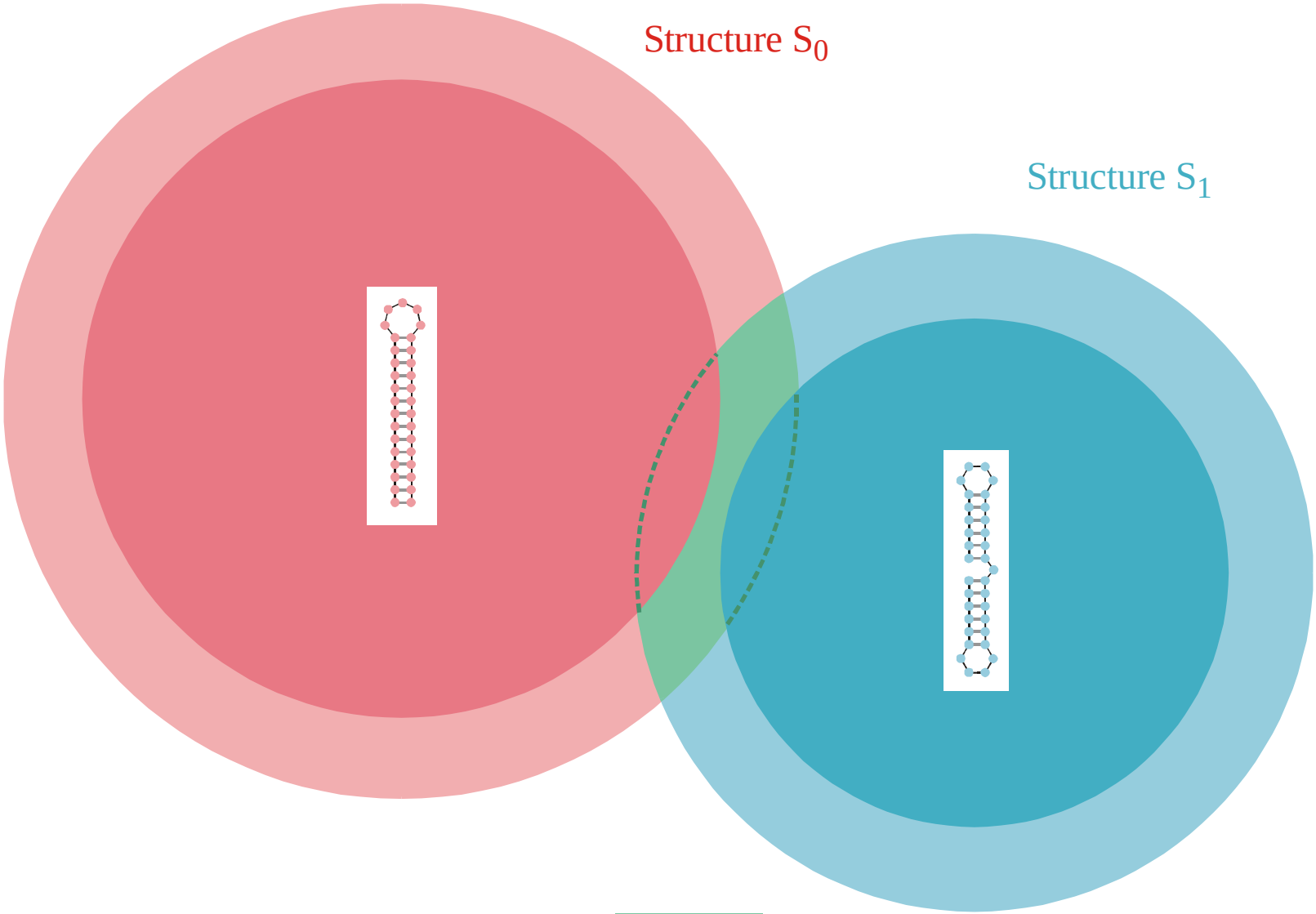
Suboptimal structures

Kinetic structures

RNA secondary structures derived from a single sequence



The **compatible set C_k** of a structure **S_k** consists of all sequences which form **S_k** as its minimum free energy structure (the neutral network **G_k**) or one of its suboptimal structures.



Intersection of two compatible sets: $C_0 \cap C_1$

The intersection of two compatible sets is always non empty: $C_0 \cap C_1 \notin \varnothing$

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GENERIC PROPERTIES OF COMBINATORY MAPS: NEUTRAL NETWORKS OF RNA SECONDARY STRUCTURES[1]

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Random graph theory is used to model and analyse the relationships between sequences and secondary structures of RNA molecules, which are understood as mappings from sequence space into shape space. These maps are non-invertible since there are always many orders of magnitude more sequences than structures. Sequences folding into identical structures form *neutral networks*. A neutral network is embedded in the set of sequences that are *compatible* with the given structure. Networks are modeled as graphs and constructed by random choice of vertices from the space of compatible sequences. The theory characterizes neutral networks by the mean fraction of neutral neighbors (λ). The networks are connected and percolate sequence space if the fraction of neutral nearest neighbors exceeds a threshold value ($\lambda > \lambda^*$). Below threshold ($\lambda < \lambda^*$), the networks are partitioned into a largest "giant" component and several smaller components. Structures are classified as "common" or "rare" according to the sizes of their pre-images, i.e. according to the fractions of sequences folding into them. The neutral networks of any pair of two different common structures almost touch each other, and, as expressed by the conjecture of *shape space covering* sequences folding into almost all common structures, can be found in a small ball of an arbitrary location in sequence space. The results from random graph theory are compared to data obtained by folding large samples of RNA sequences. Differences are explained in terms of specific features of RNA molecular structures. © *1997 Society for Mathematical Biology*

THEOREM 5. INTERSECTION-THEOREM. *Let* s *and* s′ *be arbitrary secondary structures and* **C**[s], **C**[s′] *their corresponding compatible sequences. Then,*

$$\mathbf{C}[s] \cap \mathbf{C}[s'] \neq \varnothing.$$

Proof. Suppose that the alphabet admits only the complementary base pair $[XY]$ and we ask for a sequence x compatible to both s and s'. Then $\gamma(s, s') \cong D_m$ operates on the set of all positions $\{x_1, \ldots, x_n\}$. Since we have the operation of a dihedral group, the orbits are either cycles or chains and the cycles have even order. A constraint for the sequence compatible to both structures appears only in the cycles where the choice of bases is not independent. It remains to be shown that there is a valid choice of bases for each cycle, which is obvious since these have even order. Therefore, it suffices to choose an alternating sequence of the pairing partners X and Y. Thus, there are at least two different choices for the first base in the orbit. ■

Remark. A generalization of the statement of theorem 5 to three different structures is false.

Reference for the definition of the intersection and the proof of the **intersection theorem**

A ribozyme switch

E.A.Schultes, D.B.Bartel, Science **289** (2000), 448-452

minus the background levels observed in the HSP in the control (Sar1-GDP–containing) incubation that prevents COPII vesicle formation. In the microsome control, the level of p115-SNARE associations was less than 0.1%.
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50. GST-SNARE proteins were expressed in bacteria and purified on glutathione-Sepharose beads using standard methods. Immobilized GST-SNARE protein (0.5 μM) was incubated with rat liver cytosol (20 mg) or purified recombinant p115 (0.5 μM) in 1 ml of NS buffer containing 1% BSA for 2 hours at 4°C with rotation. Beads were briefly spun (3000 rpm for 10 s) and sequentially washed three times with NS buffer and three times with NS buffer supplemented with 150 mM NaCl. Bound proteins were eluted three times in 50 μl of 50 mM tris-HCl (pH 8.5), 50 mM reduced glutathione, 150 mM NaCl, and 0.1% Triton X-100 for 15 min at 4°C with intermittent mixing, and elutes were pooled. Proteins were precipitated by MeOH/CH_3Cl and separated by SDS–polyacrylamide gel electrophoresis (PAGE) followed by immunoblotting using p115 mAb 13F12.
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69. We thank G. Waters for p115 cDNA and p115 mAbs; G. Warren for p97 and p47 antibodies; R. Scheller for rbet1, membrin, and sec22 cDNAs; H. Plutner for excellent technical assistance; and P. Tan for help during the initial phase of this work. Supported by NIH grants GM 33301 and GM42336 and National Cancer Institute grant CA58689 (W.E.B.), a NIH National Research Service Award (B.D.M.), and a Wellcome Trust International Traveling Fellowship (B.B.A.).

20 March 2000; accepted 22 May 2000

One Sequence, Two Ribozymes: Implications for the Emergence of New Ribozyme Folds

Erik A. Schultes and David P. Bartel*

We describe a single RNA sequence that can assume either of two ribozyme folds and catalyze the two respective reactions. The two ribozyme folds share no evolutionary history and are completely different, with no base pairs (and probably no hydrogen bonds) in common. Minor variants of this sequence are highly active for one or the other reaction, and can be accessed from prototype ribozymes through a series of neutral mutations. Thus, in the course of evolution, new RNA folds could arise from preexisting folds, without the need to carry inactive intermediate sequences. This raises the possibility that biological RNAs having no structural or functional similarity might share a common ancestry. Furthermore, functional and structural divergence might, in some cases, precede rather than follow gene duplication.

Related protein or RNA sequences with the same folded conformation can often perform very different biochemical functions, indicating that new biochemical functions can arise from preexisting folds. But what evolutionary mechanisms give rise to sequences with new macromolecular folds? When considering the origin of new folds, it is useful to picture, among all sequence possibilities, the distribution of sequences with a particular fold and function. This distribution can range very far in sequence space (*1*). For example, only seven nucleotides are strictly conserved among the group I self-splicing introns, yet secondary (and presumably tertiary) structure within the core of the ribozyme is preserved (*2*). Because these disparate isolates have the same fold and function, it is thought that they descended from a common ancestor through a series of mutational variants that were each functional. Hence, sequence heterogeneity among divergent isolates implies the existence of paths through sequence space that have allowed neutral drift from the ancestral sequence to each isolate. The set of all possible neutral paths composes a "neutral network," connecting in sequence space those widely dispersed sequences sharing a particular fold and activity, such that any sequence on the network can potentially access very distant sequences by neutral mutations (*3–5*).

Theoretical analyses using algorithms for predicting RNA secondary structure have suggested that different neutral networks are interwoven and can approach each other very closely (*3*, *5–8*). Of particular interest is whether ribozyme neutral networks approach each other so closely that they intersect. If so, a single sequence would be capable of folding into two different conformations, would have two different catalytic activities, and could access by neutral drift every sequence on both networks. With intersecting networks, RNAs with novel structures and activities could arise from previously existing ribozymes, without the need to carry nonfunctional sequences as evolutionary intermediates. Here, we explore the proximity of neutral networks experimentally, at the level of RNA function. We describe a close apposition of the neutral networks for the hepatitis delta virus (HDV) self-cleaving ribozyme and the class III self-ligating ribozyme.

In choosing the two ribozymes for this investigation, an important criterion was that they share no evolutionary history that might confound the evolutionary interpretations of our results. Choosing at least one artificial ribozyme ensured independent evolutionary histories. The class III ligase is a synthetic ribozyme isolated previously from a pool of random RNA sequences (*9*). It joins an oligonucleotide substrate to its 5′ terminus. The prototype ligase sequence (Fig. 1A) is a shortened version of the most active class III variant isolated after 10 cycles of in vitro selection and evolution. This minimal construct retains the activity of the full-length isolate (*10*). The HDV ribozyme carries out the site-specific self-cleavage reactions needed during the life cycle of HDV, a satellite virus of hepatitis B with a circular, single-stranded RNA genome (*11*). The prototype HDV construct for our study (Fig. 1B) is a shortened version of the antigenomic HDV ribozyme (*12*), which undergoes self-cleavage at a rate similar to that reported for other antigenomic constructs (*13*, *14*).

The prototype class III and HDV ribozymes have no more than the 25% sequence identity expected by chance and no fortuitous structural similarities that might favor an intersection of their two neutral networks. Nevertheless, sequences can be designed that simultaneously satisfy the base-pairing requirements

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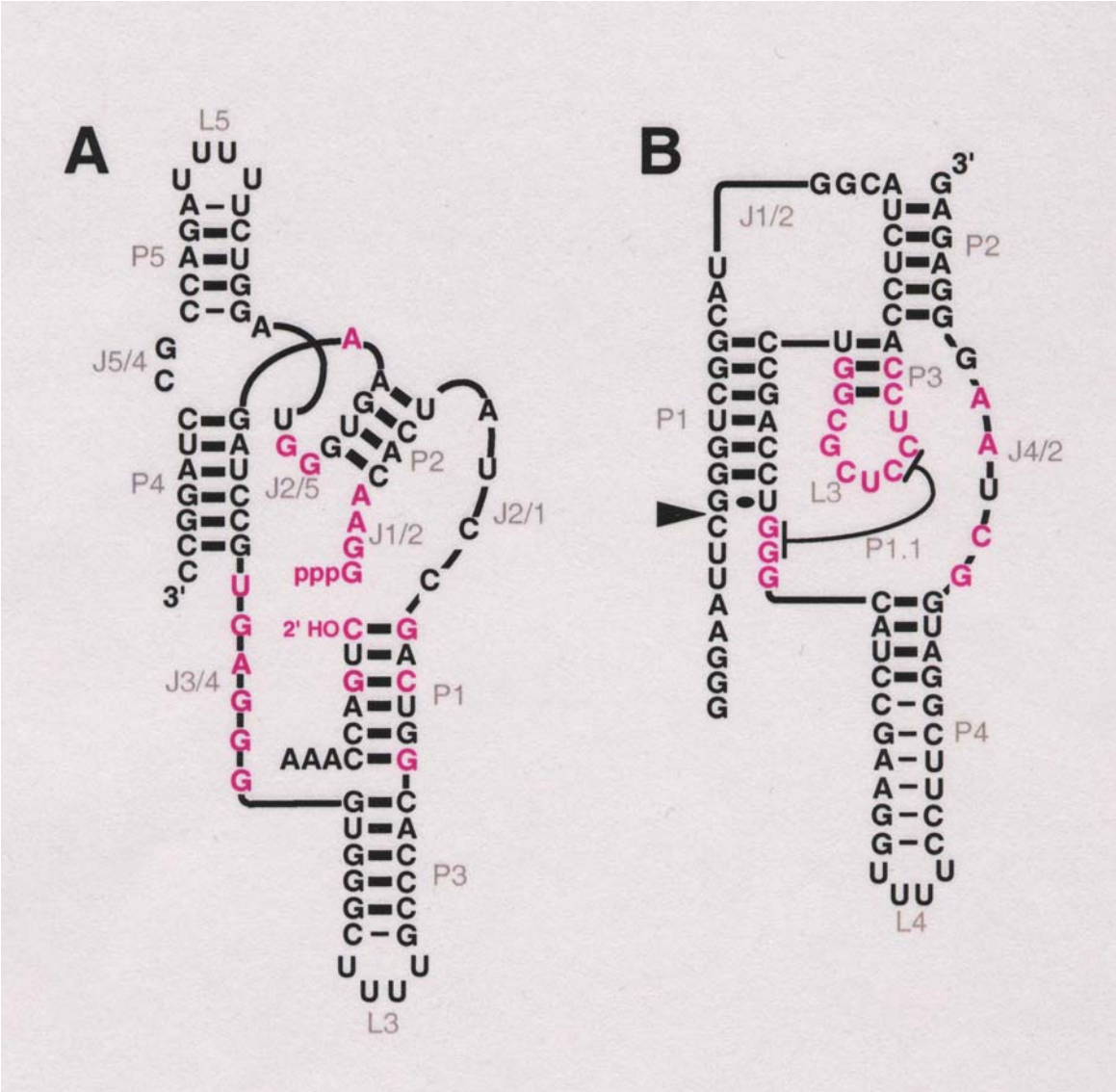
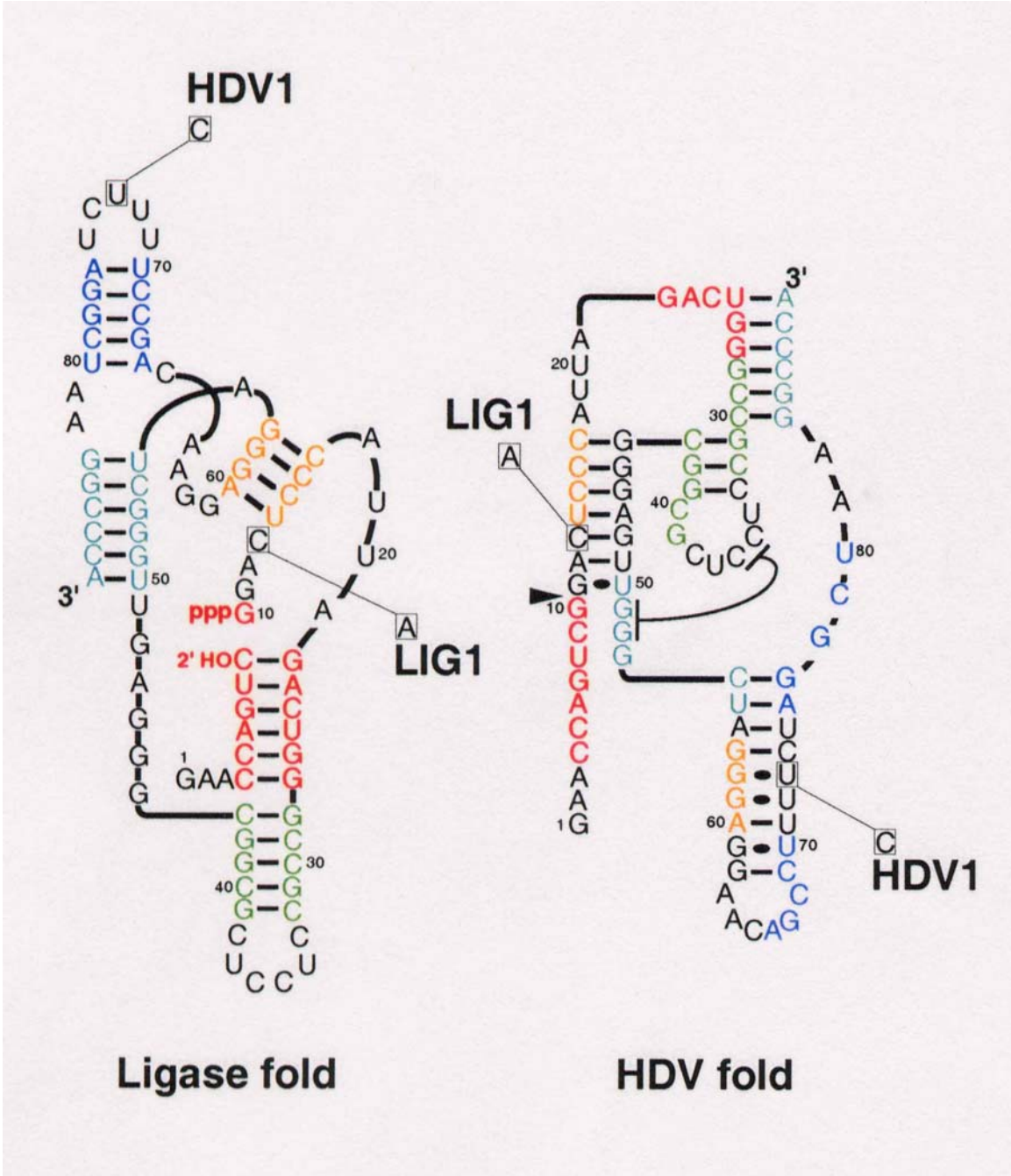
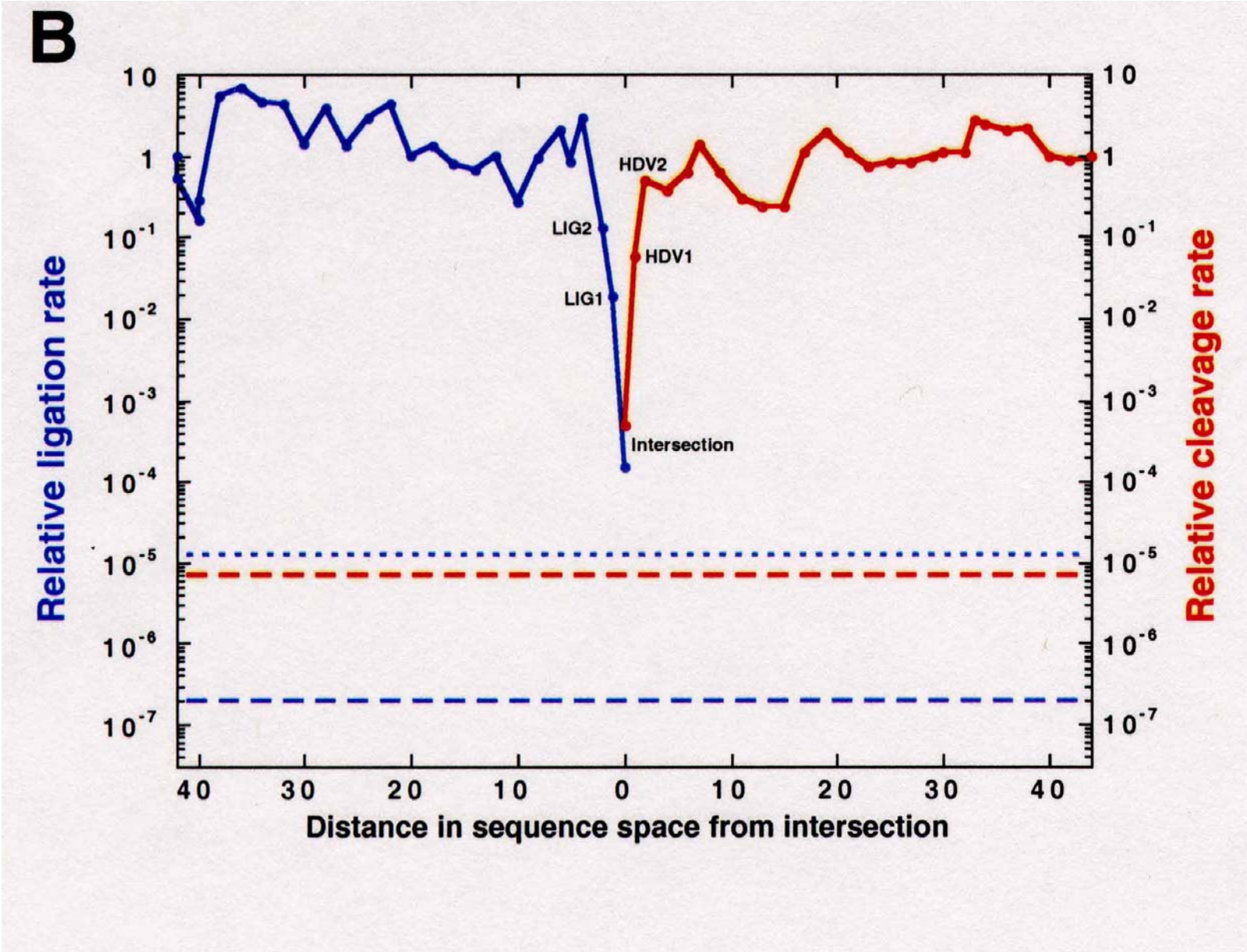


Two ribozymes of chain lengths n = 88 nucleotides: An artificial ligase (**A**) and a natural cleavage ribozyme of hepatitis-δ-virus (**B**)

The sequence at the ***intersection***:

An RNA molecules which is 88 nucleotides long and can form both structures





Two neutral walks through sequence space with conservation of structure and catalytic activity

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