Analysis of Free Energy Signals Arising from Nucleotide Hybridization between rRNA and mRNA Sequences during Translation in Eubacteria

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A decoding algorithm that mechanistically models the progressive alignments that arise as the mRNA moves past the rRNA tail during translation elongation is tested. Each of these alignments provides an opportunity for hybridization between the single-stranded, 3'-terminal nucleotides of the 16S rRNA and the spatially accessible window of mRNA sequence, from which a free energy value can be calculated. Using this algorithm, we show that a periodic energetic pattern of frequency 1/3 is revealed. This periodic signal exists in the majority of coding regions of eubacterial genes, but not in the noncoding regions encoding the 16S and 23S rRNAs. Signal analysis reveals that the population of coding regions of each bacterial species has a mean phase that is correlated in a statistically significant way with species (G+C) content. These results suggest that the periodic signal could function as a synchronization signal for the maintenance of reading frame and that codon usage provides a mechanism for manipulation of signal phase.

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1. INTRODUCTION

The complexity of living organisms makes them information-rich systems. As such, many processes are available for the application of signal processing analysis to reveal underlying mechanisms of information encoding and decoding. The mathematical methods of signal processing are well established and are used to extract encoded information from energetic patterns. These methods yield estimates of parameters that characterize the signal. Examples of the most basic parameters include frequency, phase, and magnitude. Through the study of system response to signal parameter change, the information content of signal parameters can be identified and the encoding and decoding rules can be defined. The application of signal processing analysis to a biological process requires the identification of a signal that could arise followed by characterization of signal parameters that correlate with process behavior.

It is well established that nucleic acid molecules, that is, DNA and RNA, encode information in their nucleotide sequences that is essential to a number of cellular processes.

Therefore, it is reasonable to use a signal processing approach to further our understanding of the rules and mechanisms of information encoding and decoding. The process of protein synthesis, or translation, is the most-studied biological process in which information encoded in the nucleotide sequence of mRNA is decoded into the correct sequence of amino acids in a polypeptide. Nucleic acids are long polymers of four nucleotide bases: adenine (A), guanine (G), cytosine (C), and thymidine (T, DNA) or uracil (U, mRNA). The chemical structure of the nucleotides provides for the formation of hydrogen bonds (hybridization) between pairs of nucleotide bases following specific rules. In Watson-Crick-type hybridization, the rules are that adenine forms two hydrogen bonds with either thymidine or uracil and guanosine forms three hydrogen bonds with cytosine. If two single-stranded nucleic acid sequences can spatially align such that the hybridization can occur, they will form a stable, double helical structure and are said to be complementary. Hybridization of two nucleic acid molecules results in a change in free energy that is proportional to the number of hydrogen bonds formed between the two molecules. Watson-Crick

hybridization can be thought of as a signal generating process in which the signal is the free energy change associated with nucleic acid alignment. Variation in the signal arises from the sequence variation which determines the degree to which the two sequences are complementary.

There are a number of biological processes that involve Watson-Crick hybridization and in which nucleic acids participate including tRNA hybridization to mRNA during translation, recognition of the correct site for Okazaki fragment polymerization by primase during DNA replication [1], snRNA hybridization to pre-mRNA sequences during intron splicing [2], and siRNA hybridization to mRNAs during gene silencing [3]. In translation, the precision of hybridization between the anticodon sequence of a tRNA molecule, carrying a specific amino acid, and the codon sequence of an mRNA molecule determines if that amino acid is polymerized into the polypeptide chain.

Two more examples of RNA-RNA hybridization encoding translation process information also exist. Shine and Dalgarno [4] observed sequence complementarity between the 3'-terminal single-stranded nucleotide sequence of the 16S rRNA (rRNA tail) and a window of mRNA sequence upstream of the start codon and they hypothesized that the resulting hybridization could stabilize the mRNA/30S ribosome subunit complex. This observation was confirmed experimentally [5, 6] and established 30S ribosome subunit recruitment as a role for the rRNA tail in translation initiation. More than a decade later, Weiss et al. [7, 8] showed that hybridization between the rRNA tail and the mRNA was a critical component regulating a shift of reading frame during bacterial translation of the mRNA encoding the RF2 protein in *E. coli*. This was the first direct evidence of a role for hybridization of the rRNA tail with the mRNA during translation elongation. The requirements for exact sequence and exact spacing of sequence lead the investigators to conclude that the rRNA tail "...scans the mRNA during elongation ..." [8].

The idea of one nucleic acid molecule, the rRNA tail, "scanning" a second nucleic acid molecule, the mRNA, suggested to us the structure of a decoding algorithm from which a signal could arise. Each scanning alignment step would produce a free energy of hybridization value whose magnitude would be proportional to the degree of sequence complementarity. The linear series of these free energy values could constitute a signal indexed by nucleotide position on the mRNA molecule. The work of Weiss et al. [8] suggested to us that such a signal could encode information that the translation process utilizes for the maintenance of reading frame.

In considering this hypothesis, two expectations seemed critical. If information for the maintenance of the reading frame exists in the rRNA tail signal, such an information signal would be expected to arise in the coding regions of a majority, if not all mRNA sequences. Additionally, if the signal did supply information for the maintenance of reading frame, it could exist across many species of bacteria if they employed the same mechanisms as *E. coli*. If the signal was found to exist across species, it would need to be maintained

Position 0.	Free energy value = 0.0
rRNA:	a u c c u c c a c u a g
mRNA:	G G U A A A A G A A U A A U G G C ...
Position 1.	Free energy value = 0.0
rRNA:	a u c c u c c a c u a g
mRNA:	... G G U A A A A G A A U A A U G G C ...
:	:
Position 63.	Free energy value = -1.7
rRNA:	a u c c u c c a c u a g
mRNA:	... U C A C C G A G A U C C U G G U C ...
:	:
Position N-2.	Free energy value = 0.0
rRNA:	a u c c u c c a c u a g
mRNA:	... G C C G U C U G G U G A U G U A A
Position N-1.	Free energy value = -0.7
rRNA:	a u c c u c c a c u a g
mRNA:	... G C C G U C U G G U G A U G U A A

FIGURE 1: Alignment of the 16S rRNA tail with the mRNA sequence of gene *aceF* in *E. coli*. Free energy values of 0 indicate unfavorable binding. The length of the gene is $N = 1893$ nucleotides.

regardless of (G+C) content, known to vary across bacterial species. The purpose of this study was to rigorously establish that a free energy signal can be decoded from mRNA sequences utilizing an algorithm that models the mechanical movement of the mRNA through the ribosome during translation. Our study then characterizes this signal in terms of frequency, phase, and magnitude. Our results indicate that coding regions of species tend to a mean species phase. Finally, we show that the signal phase is a function of sequence (G+C) content, an indirect measure of codon bias. This last finding suggests the possibility that regulation of translational efficiency through codon usage could be mediated by signal phase.

2. FREE ENERGY CALCULATIONS

A simple algorithm has been developed by Starmer et al. [9, 10] and utilized for this study which generates a free energy signal as a function of nucleotide position (the decoding algorithm). Briefly, the algorithm requires a short nucleic acid sequence as the "decoder" that is successively aligned with a longer "message" sequence in which information is encoded (Figure 1). At each alignment, the algorithm calculates a free energy of nucleotide hybridization, ΔG° , for the optimal helical structure between the "decoder," for this study the 3'-terminal, single-stranded, nucleotides of the 16S rRNAs of bacterial species (16S rRNA tails), and the "message," the mRNA sequence that would be aligned with the 16S rRNA tail as the mRNA moves through the ribosomal complex as it is translated. The actual free energy calculation utilizes dynamic programming extended to allow for internal loops, to identify the minimal free energy conformation and the Individual nearest-neighbor hydrogen bond model [11] to

TABLE 1: List of eubacteria used in our study.

Species name	GenBank accession number	16S tail	(G+C) percentage
<i>Buchnera aphidicola</i>	NC_004545	auuccuccacuag	26
<i>Borrelia burgdorferi</i>	NC_001318	uuuccuccacuag	28
<i>Bacillus licheniformis</i>	NC_006322	uuuccuccacuag	46.2
<i>Clostridium perfringens</i>	NC_003366	uuuccuccacuag	27
<i>Deinococcus radiodurans</i>	NC_001263	uuuccuccacuag	66.6
<i>Escherichia coli</i> K-12	NC_000913	auuccuccacuag	50
<i>Mycoplasma hyopneumoniae</i>	NC_006360	uuuccuccacuag	28.6
<i>Pseudomonas syringae</i>	NC_005773	auuccuccacuag	55.6
<i>Rhodobacter sphaeroides</i>	NC_007493	uuuccuccacuag	68.8
<i>Shigella boydii</i>	NC_007613	auuccuccacuag	47.4
<i>Salmonella enterica</i>	NC_006511	auuccuccacuag	52.2
<i>Thermus thermophilus</i>	NC_005835	uuuccuccacuag	69.4

estimate the associated free energy value for that conformation. Adjustments to the free energy values for loop penalties [12] and for G/U mismatches [13] are also incorporated. Bulges, more complex secondary structures involving only one of the two strands of RNA, are not considered in the calculation. This assumption was made based on structural models of the 70S ribosomal complex [14, 15] in which the estimated space of the mRNA channel is thought to be insufficient for bulges and secondary structures to exist. The algorithm assigns the free energy value to an mRNA nucleotide. The alignment is then shifted one nucleotide downstream (in the 3' direction along the mRNA) and the free energy value of the new alignment is calculated and assigned. This approach generates a set of free energy values for an entire mRNA sequence indexed by nucleotide position. Our analysis assumes that the linear array of free energy values constitutes a discrete signal. This signal was examined using methods of time-series analysis, with signal points indexed by nucleotide position, instead of time.

Sequence information and the genome databases used for this study are given in Table 1. Gene sequences for 12 eubacterial species, including *E. coli* K-12, were obtained from the NCBI GenBank database (<http://www.ncbi.nlm.nih.gov/>). Using GenBank annotation, the coding sequences were sorted into two categories: (1) verified sequences, that is, genes with a clearly annotated function and (2) hypothetical sequences, that is, genes listed as hypothetical or putative. For *E. coli*, sequences encoding the 16S and 23S rRNAs were also used, designated as "noncoding" sequences to indicate that they do not encode amino acid sequence information. The 3'-terminal nucleotide sequences of the 16S rRNA (16S rRNA tails) for each species are also presented in Table 1. When calculating the free energy signals from a species population of mRNAs, the species' own 16S rRNA tail was used. These tails are the 3'-single-stranded rRNA sequences that are potentially available for hybridization to the mRNA as it moves across the ribosome during translation.

FIGURE 2: Free energy signal for *aceF* (a) upstream region and (b) downstream region.

A sample free energy signal, computed using the gene *aceF* sequence in *E. coli*, is shown in Figure 2. The estimated free energy for the alignment of the 5'-terminal nucleotide of the tail with the first base of the start codon is plotted at position 0 on the horizontal axis. The free energy estimates calculated for downstream alignments are plotted at positive indices while negative indices on the horizontal axis indicate free energy estimates for upstream alignments.

Two features of this variable free energy pattern are of note. There is a trough of negative free energy at nucleotide position -6 . Earlier studies have identified the presence of an upstream free energy trough in genes of *E. coli* [16] and other bacteria [17]. This trough is interpreted as the signal feature

FIGURE 3: Periodogram for *aceF*.

for the Shine-Dalgarno region [16–22]. The other noteworthy feature is the pattern of negative free energy troughs that occur roughly every third nucleotide throughout the coding sequence. The suggestion of periodicity can be quantitatively confirmed using signal processing methodology.

3. SIGNAL ANALYSIS

The set of free energy estimates are assumed to be a discrete signal, denoted as

$$\mathbf{x} = [x_0, x_1, \dots, x_{N-1}]. \quad (1)$$

The periodogram is defined as [23]

$$I_k = \frac{1}{N} |X_k|^2, \quad k = 0 \dots (N-1), \quad (2)$$

where

$$X_k = \sum_{n=0}^{N-1} x_n e^{-j2\pi kn/N}, \quad k = 0 \dots (N-1). \quad (3)$$

The periodogram of the free energy signal for a sample gene *aceF* reveals a dominant frequency of 1/3 cycles/base (Figure 3). The absence of other strong periodic components suggests that this signal can be modeled as the sum of a sine wave of frequency $f = 1/3$ and noise. A model for the signal can be written as

$$x_n = \mu + A \sin(2\pi f n + \phi) + e_n, \quad (4)$$

where A is the amplitude, ϕ is the phase, $f = 1/3$ is the specified frequency, and e_n is Gaussian white noise with variance σ^2 . As per this model, if a periodic component of frequency $f = 1/3$ does not exist, the signal would be interpreted as

FIGURE 4: Power versus length at SNR = −18 dB.

TABLE 2: *E. coli* signal parameters.

Parameter	Mean	Std. dev.
Phase (degrees)	−14.53	23.26
SNR (dB)	−18.35	1.84

white noise. To test the hypothesis that a free energy signal can be modeled from the variable free energy pattern arising from hybridization of the rRNA tail with the mRNA, the assumption is made that such a signal exists in the majority of coding regions. However, coding regions vary in length and signal length will affect the power of the statistical test. To ensure that the statistical test has sufficient power, the relationship between signal length, defined as nucleotide sequence length, and power was determined for an SNR of −18 dB, the mean SNR for *E. coli* K-12 coding regions (Table 2). As shown in Figure 4, a power of 0.92 can be achieved using a signal length of greater than or equal to 900 nucleotides. Therefore, only coding regions of 900 nucleotides or greater were used to insure a robust statistical test.

The statistical test was performed with the null hypothesis that the free energy pattern contains only white noise, versus the alternate hypothesis that a signal does exist and it contains a dominant frequency component of $f = 1/3$ [24]. The signal model can be written in the equivalent form

$$x_n = \mu + C_1 \sin(2\pi f n) + C_2 \cos(2\pi f n) + e_n, \quad (5)$$

where $C_1 = A \cos(\phi)$ and $C_2 = A \sin(\phi)$ are nonrandom constants.

The signal sum-of-squares $|\mathbf{x}|^2$ can be partitioned by periodic components, allowing the construction of a test of hypothesis [24]. Our null hypothesis is

$$H_0 : C_1 = C_2 = 0 \quad (6)$$

TABLE 3: Detection results.

Species	Sequence type	Sample size	Passed
<i>Buchnera aphidicola</i>	Verified	206	197
	Hypothetical	34	32
<i>Borrelia burgdorferi</i>	Verified	265	242
	Hypothetical	140	99
<i>Bacillus licheniformis</i>	Verified	1318	1068
	Hypothetical	375	272
<i>Clostridium perfringens</i>	Verified	489	484
	Hypothetical	679	648
<i>Deinococcus radiodurans</i>	Verified	577	573
	Hypothetical	490	475
<i>Escherichia coli</i>	Verified	1193	1144
	Hypothetical	758	685
<i>Mycoplasma hyopneumoniae</i>	Verified	186	173
	Hypothetical	164	131
<i>Pseudomonas syringae</i>	Verified	1919	1888
	Hypothetical	472	440
<i>Rhodobacter sphaeroides</i>	Verified	977	972
	Hypothetical	359	357
<i>Shigella boydii</i>	Verified	875	838
	Hypothetical	715	653
<i>Salmonella enterica</i>	Verified	995	952
	Hypothetical	771	684
<i>Thermus thermophilus</i>	Verified	654	654
	Hypothetical	197	194

and our alternate hypothesis is

$$H_1 : C_1 \text{ and } C_2 \text{ are both not zero.} \quad (7)$$

From [24], we know that under H_0 ,

$$(2I_{N/3}) \sim \sigma^2 \chi^2(2) \quad (8)$$

and $I_{N/3}$ is independent of

$$\left(\sum_{i=0}^{N-1} x_i^2 - I_0 - 2I_{N/3} \right) \sim \sigma^2 \chi^2(N-3). \quad (9)$$

We may reject H_0 in favor of H_1 at level α if

$$\frac{(N-3)I_{N/3}}{\sum_{i=0}^{N-1} x_i^2 - I_0 - 2I_{N/3}} > F_{1-\alpha}(2, N-3). \quad (10)$$

The results of this test for the verified and hypothetical sequences greater than 900 nucleotides in various eubacteria are given in Table 3. The test is performed at level $\alpha = 0.05$. “Sample size” indicates the number of sequences in each category. “Passed” indicates the number of sequences whose

free energy signal shows only one periodic component of the assumed frequency for the hidden periodicity statistical test, that is, $f = 1/3$. We observe that 95.9% of the selected verified sequences and 90.4% of the chosen hypothetical sequences in *E. coli* demonstrate strong periodicity at $f = 1/3$ in their free energy signals. For the other bacterial species in our study, whose genomic (G+C) contents ranged from 26% to 69.4% (Table 1), the majority of their verified and hypothetical sequences were also found to demonstrate strong periodicity at $f = 1/3$.

If the information encoded by the periodic signal is relevant to translation, we might expect that it would only be present in the coding sequences and not in the sequences that are not translated. Testing this hypothesis would require applying our algorithm to noncoding sequences minimally 750 to 900 nucleotides in length, based on estimated relationship of statistical power and SNR, to have sufficient statistical power (Figure 4). In bacteria, the rRNA sequences are the only sequences that are sufficiently long to satisfy these considerations. Therefore, we used the 16S and 23S rRNA gene sequences, of which there are 7 each in *E. coli*, to test the hypothesis. The free energy patterns calculated using these sequences did not show periodicity at $f = 1/3$, consistent with the correlation between signal presence and periodicity and

FIGURE 5: Periodogram calculated using the free energy signal for a 23S rRNA sequence in *E. coli*.

sequences that are translated. Figure 5 shows an example of the periodogram of a noncoding sequence, 23S rRNA.

For those free energy signals for which our model (4) is valid, we can evaluate the power of the 1/3 harmonic and estimate the noise variance using trigonometric regression [25, 26]. The regression procedure performs a least-squares fit of the model described by (5) to the free energy signal $\mathbf{x}$.

The best-fit values of C_1 and C_2 , denoted by $\hat{C}_1$ and $\hat{C}_2$, respectively, can be used to estimate the magnitude and phase of the signal using (11) and (12). It can be shown that the regression procedure is equivalent to maximum-likelihood estimation, under the assumption that the i.i.d. noise, e_n , follows a normal distribution [25]:

$$\hat{A} = \sqrt{\hat{C}_1^2 + \hat{C}_2^2}, \quad (11)$$

$$\hat{\phi} = \arctan \frac{\hat{C}_2}{\hat{C}_1}. \quad (12)$$

The power of the sinusoidal component can be calculated using (13). The mean-squared error (MSE) from regression yields an estimate of the noise variance $\hat{\sigma}^2$. The power of the noise and the signal-to-noise ratio (SNR) are calculated using (14) and (15), respectively:

$$P_{\text{signal}} = 10 \log_{10} \left(\frac{\hat{A}^2}{2} \right) \text{ dB} \quad (13)$$

$$P_{\text{noise}} = 10 \log_{10} (\hat{\sigma}^2) \text{ dB}, \quad (14)$$

$$\text{SNR} = (P_{\text{signal}} - P_{\text{noise}}) \text{ dB}. \quad (15)$$

Histograms for signal phase and SNR for verified genes in *E. coli* are shown in Figures 6 and 7, respectively. The mean and standard deviation of the estimated parameter values are shown in Table 2. These values are calculated using verified genes in *E. coli* that pass our detection test (1144 in number).

FIGURE 6: Histogram of phase of verified sequences.

FIGURE 7: Histogram of SNR of verified sequences.

The revelation of a free energy signal embedded in coding regions provides the foundation for further studies to determine if the signal could provide information for the maintenance of reading frame. If this is its function, it would be reasonable to expect the signal to be present in coding regions of eubacterial species in general. To determine if this is true, we selected 12 eubacteria of varying (G+C) content, listed in Table 1. The verified genes that passed the detection test for each species were used for analysis. The free energy signals for each species were calculated using its specific 16S tail, shown in Table 1. We found that a periodic signal is present in the coding regions of genes in all the species tested and that the mean phase of these signals is roughly proportional to the (G+C) content (Figure 8). An ANOVA test indicated a significant effect of (G+C) content on the signal phase.

FIGURE 8: Phase as a function of (G+C) across eubacterial species.

4. DISCUSSION

Our algorithm models the movement of the ribosome relative to the mRNA during translation. This model assumes that a continual series of mRNA sequence windows is accessible for hydrogen bond formation to occur between the 16S rRNA tail and the mRNA as they move by each other during the translation process. The free energy associated with each of these windows is a function of the degree of complementarity between the 16S rRNA tail and the mRNA sequence window. Using this model, it is clear that a periodic signal is encoded in the free energy variation. Standard signal processing and statistical analyses show that this signal has a dominant frequency $1/3$ and that it is encoded in the majority of protein-encoding sequences of genes in a diverse group of eubacterial species, including *E. coli*. This periodic signal is not present in genomic sequences that encode rRNAs which do not participate in translation. Although this result is consistent with the signal being present only in sequences that are translated, the limited sample size (there are only 7 rRNA encoding genes in *E. coli*) prevents meaningful statistical confirmation of the hypothesis that the signal exists only in sequences encoding proteins. These results reveal a signal and provide a signal decoding mechanism, however they do not explain what parameters contribute to signal structure and what role it could play in translation.

In our model, the energetic variation of the signal arises from the variation in mRNA nucleotide sequence. That the signal has a frequency $1/3$ implies that the mRNA nucleotide sequence has a frequency $1/3$. Periodicity in the coding regions of genes has been observed prior to our results using statistical correlation analysis of coding regions. Lio et al. [27] have investigated prokaryotic and eukaryotic DNA sequences for the presence of subcodes following a periodicity rule based on the ideas of several investigators [28, 29]. The analysis of individual gene sequences from both prokaryotes and eukaryotes revealed period-three recurrence of (G+C) bases in the codon third position, coherent with

the reading frame for the gene ((G+C)⁻³ periodicity). This period-three recurrence was found in some translated sequences in both prokaryotes and eukaryotes but was not found in introns, repetitive DNA, or sequences encoding rRNAs or tRNAs [27]. These results are consistent with ours. The analysis of Lio et al. also identified translated sequences in which (G+C)⁻³ periodicity could not be resolved, however they did not exclude the possibility that a weaker period-three signal could be present. This result is consistent with a relatively low SNR for their signal, impairing resolution of all but the strongest signals.

The new observation of a mean phase for *E. coli* genes suggested the subsequent study to determine if the presence of coding region periodicity with constant phase is a feature peculiar to *E. coli* or that is a more general feature of prokaryotic genomes. Our results indicate that each bacterial genome does have a distribution of signal phase, however, the mean phase for each species is different. Knowing that the (G+C) content of genomes varies, and that this variation is a reflection of the species preference for certain codons (generally referred to as synonymous codon bias [30]), we hypothesized that the signal phase is a function of (G+C) content. Our regression results indicate that phase is a function of (G+C) content and that there is a significant difference in the signal phase of species that are widely distributed across (G+C) content. The functional relationship between phase and (G+C) content means that the signal phase can be manipulated through codon selection.

The role of Watson-Crick hybridization between 16S rRNA sequences, including the tail, and the mRNA during translation has long been the subject of investigation. Trifonov [31] suggested that this hybridization could play a role in maintenance of reading frame during translation. The elegant work of Weiss et al. [7, 8] using mutant analysis of both the mRNA and the 16S rRNA clearly showed that hybridization between these two molecules was critical in the shift of reading frame that regulates the production of RF2 protein in *E. coli*. Our results suggested that parameters of the energetic signal, that is, phase, could supply the translational process information for maintenance of reading frame.

Our findings are consistent with this hypothesis. To maintain the correct reading frame, the ribosome must translocate three nucleotides after each amino acid is incorporated into the polypeptide product of the translation process. Therefore, it would be expected that a signal encoding reading-frame information would have a dominant $1/3$ frequency, as our signal does. In addition, using a robust statistical test, we found the signal to be present in genomic sequences that encode proteins, again an expected result. Our results also imply that specific manipulation of codon usage, which would modify (G+C) content, could locally adjust phase and potentially impact reading frame fidelity.

The next step in establishing a role for our signal in maintenance of reading frame is a critical test of the hypothesis. Such a test is underway in our group, using the sequence encoding the RF2 protein, prfB, a sequence known to harbor a programmed +1 frameshift. If the free energy signal was supplying information that maintains or regulates the reading

frame of translation, we would expect that changes in reading frame during translation elongation would be accompanied by changes in the phase of the free energy signal. Preliminary results [32] indicate that an abrupt phase shift occurs in the prfB sequence at the location of the programmed frameshift. This result has encouraged us to refine and further develop our model of reading frame maintenance, confirming the value and utility of the signal processing approach.

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